Transcriptome analysis of Spodoptera litura reveals the molecular mechanism to pyrethroids resistance

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Abstract

Background

Spodoptera litura is a destructive agricultural pest and has 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 comparing to pyrethroid-susceptible population (GX), with increased resistant ratio ranging from 40.8 to 1764.0-fold. To characterize pyrethroid resistance mechanism, the transcriptomes between pyrethroid-resistant (LF and NJ) and pyrethroid-susceptible (GX) populations were compared by RNA-sequencing. Results showed that multiple differentially 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 UGT, 4 COEs and 1ABC, were verified in the additional pyrethroid resistant populations CZ and JD. The expression levels of CYP3 and GST3 , which were homologous to CYP321A8 and GST1 , respectively, showed positive correlation with their pyrethroid resistance levels within CZ, LF, NJ and JD populations. While the expression levels of CYP12 , CYP14 , COE4 and ABC5 showed good correlation with their pyrethroid resistance levels in at least three populations. UGT5 had the highest expression level among the tested UGT genes in the four pyrethroid resistant populations.

Conclusion

CYP3 , CYP12 , CYP14 , GST3 , COE4 , UGT5 and ABC5 play important roles in pyrethroid resistance among the four field-collected populations. Our work provides a valuable clue for further study of pyrethroid resistance mechanisms in *S. litura* .

Background

The common cutworm, *Spodoptera litura* (Fabricius), is a polyphagous agricultural pest worldwide and causes enormous losses to many economical crops. Insecticide resistance has developed rapidly in the past decades due to its extensively usage. At present, *S. litura* has evolved high resistance to multiple conventional and new insecticides, resulting in both the failure of pest control and the destruction of natural enemies [1-4].

Insecticide 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 [5]. TSR is endowed by gene mutations in target enzymes, which can be easily detected by sequencing. Multiple mutations in sodium channel, such as L1014F, L1014S, M918V, L925I, E435K, C785R, P1999L, F1538I, A1410V, M1524I, D1549V, T929I and M918T, had been identified

conferring resistance to pyrethroid insecticides in insect species [6-7]. NTSR is achieved by mechanisms reducing insecticide concentration reaching the target-site, including metabolism-based resistance, which were participated by cytochrome P450 monooxygenase (P450), glutathione S-transferase (GST), carboxylesterases (COE), UDP-glycosyltransferase (UGT) and ATP-binding cassette transporters (ABC).

In *S. litura*, several genes had been reported to be related with insecticides resistance. *CYP4M14*, *CYP4S9*, *Slest2* and *Slest3* were suggested to be overexpressed in the resistant strain and deduced to be related with deltamethrin resistance [8]. The expression of *CYP9A40* could be induced by deltamethrin and methoxyfenozide, and RNAi mediated silencing of *CYP9A40* increased the mortality to them [9]. While the expression of *CYP321B1* could be induced by chlorpyrifos, β-cypermethrin and methomyl, and RNAi of *CYP321B1* increased the mortality to chlorpyrifos and β-cypermethrin [10]. Western blot indicated that *SlGSTE1* could be induced by chlorpyrifos, and the recombinant SlGSTE1 possessed high binding activities to chlorpyrifos, deltamethrin, malathion, phoxim and DDT [11]. The researches above indicated that these genes are involved in metabolism-based resistance, but fails to comprehensively identify the individual genes responsible for insecticide resistance.

RNA-Sequencing (RNA-Seq) is a powerful tool for the comprehensive analysis of pest transcriptome without reference genome in greater depth than ever before [12]. Wang et al [13] identified four P450 genes involved in chlorantraniliprole resistance by comparing the transcriptome of resistant and susceptible *Spodoptera exigua* strains using RNA-Seq. In four *Plutella xylostella* populations, the expression of 19 P450 unigenes and 3 GST unigenes were identified to be gradient up-regulated with the increasing resistance to chlorantraniliprole by RNA-Seq [14]. When compared with the susceptible strain, highly expressed CYP genes in the resistant *Spodoptera frugiperda* strain were concluded to be the main reason for high resistance to lufenuron by RNA-Seq [15].

Pyrethroid insecticides were widely applied to control *S. litura* and other agricultural pests, for its high efficiency and low toxicity to mammal. Yet excessive usage has imposed pyrethroid resistance the most severe situation [16]. To minimize the evolution of resistance and prolong the life time of insecticide, the molecular mechanisms for pyrethroid resistance in this pest should be addressed. In this study, RNA-Seq was performed by Illumina HiSeq™ 2000 sequencing platform and the transcriptomes of pyrethroid-resistant and pyrethroid-susceptible populations were compared. These analyses will provide new insights into mechanism of metabolism-based resistance to pyrethroids in *S. litura*.

Results

Toxicities of insecticides to *S. litura*

Eleven commonly used insecticides were applied to the larvae of GX, LF and NJ populations to test their toxicities. When compared with the insecticide-susceptible population, named as GX, higher resistant level to fenvalerate, beta-cypermethrin and cyhalothrin were observed, which were 809.5-, 141.3-, 1764.0-fold in LF population and 322.0-, 40.8-, 951.0-fold in NJ population, respectively (Table 1). 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, cyantraniliprole, imidacloprid and methomyl, with RR ranging from 4.0 to 19.8-fold. The results above indicated that field-collect populations of *S. litura* had high levels of pyrethroid resistance, which was urgent to be managed.

To determine the involvement of detoxification enzymes for pyrethroid resistance in *S. litura*, PBO, DEF and DEM, which were the synergists of P450, COE and GST, respectively, were used before pyrethroids treatment. Results showed that pyrethroid toxicities were distinctly increased in both LF and NJ populations by PBO. Fenvalerate and cyhalothrin toxicities were slightly increased by DEF and DEM, while beta-cypermethrin toxicity was not influenced by DEF and DEM (Figure 1).

Illumina sequencing and reads assembly

RNA-Seq was conducted with the cDNA libraries of GX, LF and NJ populations. 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 (Additional file 1). More than 96.5% raw reads had Phred-like quality score at Q20 level (an error probability of 1%). Clean reads were used to assemble 140003 transcripts and 82014 unigenes ranging from 201 to 29587 bp (Additional file 2). Pearson correlation coefficients of RNA samples in the same population ranged from 0.868 to 0.916, which was higher than that in different populations (Additional file 3).

Functional annotation of unigenes

Seven databases, NCBI non-redundant protein sequences database (Nr), NCBI non-redundant nucleotide sequences data-base (Nt), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), Swiss-port, KyotoEncyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) database were used for unigenes functional annotation. There are 47185 (57.53%) unigenes annotated in at least one database, 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 (Additional file 4). More than 90% annotated unigenes had nucleotides similarity above 60% (Additional file 5). 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 *P. xylostella*, 1.9 % to *Helicoverpa armigera* and 1.6% to *Papilio xuthus* (Additional file 6).

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. For MF, binding and catalytic activity formed the largest two subgroups (Additional file 7).

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, including cellular

processes (A), environmental information processing (B), genetic information processing (C), metabolism (D), and organism systems (E). 3002 unigenes (14.89%) were involved in A; 3023 unigenes (14.99%) were involved in B; 2954 unigenes (14.65%) were involved in C; 5563 unigenes (27.59%) were involved in D; 5622 unigenes (27.88%) were involved in E (Additional file 8).

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 $|\log_2$ (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 11162 DEGs were found in both LFvsGX and NJvsGX groups, including 5720 up-regulated and 5442 down-regulated genes (Additional file 9).

GO and KEGG enrichment of the DEGs

To further analyze the function of DEGs, GO and KEGG enrichment analyses were conducted. Based on 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 2A, B).

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 2C, D).

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 3). The results of GO and KEGG enrichment suggested that metabolism was the main enriched terms of DEGs and should be a main reason for pyrethroid resistance in *S. litura.*

Validation of the candidate DEGs

Based on high reading count and significant high expression levels in RNA-Seq, 14 P450, 5 COE, 5 GST, 5 UGT and 6 ABC up-regulated unigenes were screened out and validated by quantitative RT-PCR (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 2). *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.

The expression of candidate DEGs in additional two field populations

CZ and JD are another two field collected 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, while JD showed 9123.5-fold resistance to fenvalerate, 642.0-fold resistance to beta cypermethrin and 6986.0-fold resistance to cyhalothrin (Table 3).

Based on the pyrethroid RR, resistance level of JD>LF>NJ>CZ>GX could be concluded. To further validate the role of candidate DEGs in pyrethroid resistance, 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 pyrethroid 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 pyrethroid resistant level in at least three field-collected populations (Table 4).

Discussion

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 pest control. In the present study, the transcriptome of GX, NJ and LF populations *S. litura* were sequenced by Illumina platform with high criteria of data quality (Additional file 1), enabling assembling of 140003 transcripts and 82014 unigenes (Additional file 2, 4). GO and KEGG enrichment analysis of DEGs indicated that LF and NJ populations have relative higher ability in oxidation-reduction activity, hydrolase activity and P450 involved metabolic process than that in GX population (Figure 2, 3). Cheng et al. [17] conducted the genome sequencing of *S. litura*, and 138 P450, 47 GST, 110 COE and 54 ABC genes were annotated, which was similar with our transcriptome results (Table 5). In addition, the validation of genes expression level showed that RNA-Seq results were well coincident with that of qRT-PCR (Table 4). Therefore, the RNA-Seq results were reliable and this information will facilitate further researches on pyrethroid resistance and other physiological processed in *S. litura*.

Bioassay result in LF and NJ populations of *S. litura* showed high level resistance to fenvalerate, beta cypermethrin and cyhalothrin (Table 1). No cross-resistance between organophosphates and pyrethroids was found in LF or NJ populations, which is similar with the result of Huang et al [8]. Multiple studies had revealed a direct link between elevated P450 activities and organophosphate and pyrethroid resistances in insects [18]. 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 [5]. For example, chlorpyrifos can be converted to chlorpyrifos-oxon by P450 and chlorpyrifos-oxon is 100-fold toxicity than chlorpyrifos [19-20]. This may be the reason for high pyrethroid resistance and low organophosphates resistance in LF and NJ populations.

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 2A, B). P450s involved metabolism of xenobiotics and drug metabolism pathways were also enriched in KEGG enrichment analysis (Figure 3). Besides, P450s inhibitor (PBO) significantly increased the toxicity to pyrethroids in LF and NJ populations (Figure 1). Therefore, up-regulated P450s should be the main reason for pyrethroid 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 4). The expression level of *CYP3*, *CYP12* and *CYP14*, which were annotated as *CYP321A8*, *CYP6AE43* and *CYP6AB31*, respectively, showed good correlation with pyrethroid resistance level among GX, CZ, NJ, LF and JD populations. *CYP321A8* and *CYP6AB31* in *S. exigua* could be induced by lambda-cyhalothrin [21]. The expression of *CYP321A8* in *S. frugiperda* could be detected in the midgut, fatbody and malpighian tubules, while *CYP6AE43* could only be detected in the midgut and malpighian tubules [22]. Building evidence had indicated that midgut, malpighian tubules and fat body are the key tissues for the metabolism of most compounds [23]. P450 genes with high 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 high 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 [24]. For instance, the over-expression of carboxylesterase *E4* in *Myaus persicae* enhanced degradation and sequestration of pyrethroids, organophosphates and carbamates [25]. According to Go enrichment analysis of up-regulated DEGs, hydrolase activity was enriched in LF and NJ populations (Figure 2A, B). Validation in qRT-PCR revealed that the 4 selected COEs were higher expressed in resistance populations, especially for *COE4* (Table 4). Although only slight synergism to fenvalerate and cyhalothrin by DEF was observed (Figure 1), COE may also play an important role in pyrethroid resistance in consideration of the enrichment analysis and high expression level in the four resistance populations.

Insect GSTs are a superfamily of detoxification enzymes and had been reported to be associated with resistance to pyrethroids, organophosphorus and organochlorine insecticides [26]. 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 [27-28]. 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 positive correlation with the resistance level among the four resistant populations, was reported to possess high binding activities to deltamethrin and high peroxidase activity [11]. *SlNrf2* was reported to bind with the promoter of *GST1* and regulate the peroxidase activity in response to the stress induced by deltamethrin and other xenobiotics [29]. The studies above indicated the involvement of *GST1* in pyrethroid 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. Li et al. found that *UGT2B17* was overexpressed in four chlorantraniliprole-resistant *P. xylostella* populations and RNAi of *UGT2B17* increasde the toxicity to chlorantraniliprole [30]. In *S. exigu*, the expression of *UGT42B5*, *UGT40D5*, *UGT33J3* and *UGT33T3* were reported to be induced by lambda-cyhalothrin [31]. *UGT5*, annotated as *UGT33T2*, was significantly over expressed and had the highest expression level among the tested UGT genes in the four pyrethroid resistant populations. The involvement of UGT5 in pyrethroid resistance needs to be further studied.

Several researches had indicated that ABC genes were involved in the resistance to pyrethroid, organophosphorus, emamectin benzoate and Bt [32-36]. Pyrethroid resistance were mainly resulted from genes of ABCB subfamily. High expression of *P-gp* (*ABCB1*) in resistant *H. armigera* strain resulted in pyrethroid resistance [32]. Among the 6 selected ABC unigenes, only *ABC5* (annotated as *ABCB3*) was significantly up-regulated in the four pyrethroid resistant populations, which indicated a possible role of *ABC5* in pyrethroid resistance.

Decreased penetration rate was well documented to be involved in insecticides resistance [37]. 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 constitutent of cuticle term (Figure 2C, D, Additional file 10).

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 were the major reason for pyrethroid 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 pyrethroid resistance for their higher expression level in several resistant populations. Besides, different insecticide penetration may also account for pyrethroid 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 pyrethroid resistant level. Our study provides a comprehensive understanding that CYPs, COEs, GSTs, UGTs, and ABC transporters may associate with pyrethroid resistance in *S. litura*, which will greatly extend our understanding of

metabolism-based insecticide resistance mechanism, and facilitate the further identification of pyrethroid 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. LF, NJ, CZ and JD populations were collected from Langfang, Nanjing, Changzhou, and Shanghai, respectively. They had been reared on artificial diet in the laboratory without exposure to any insecticide for 3 years. The rear condition was 26±1℃ and 60-70% relative humidity with a 14:10 h of light:dark photoperiod. Insecticides of pyrethroid (fenvalerate, beta cypermethrin and cyhalothrin), organophosphorus (phoxim, profenofos and chlorpyrifos), carbamates (methomyl), diamide (chlorantraniliprole and cyantraniliprole), chloronicotinyl (imidacloprid) and emamectin benzoate were used for bioassay. Each insecticide was dissolved and diluted to 7 concentrations in acetone. 36 larvae were included in each concentration. One microlitre of serial dilution insecticide was applied on the thoracic dorsum of the third instars larvae by Hamilton syringe. For synergism experiments, PBO, DEF, and DEM were dissolved in acetone and applied 1 h before insecticide treatment at 10 μg larva^{-1} (synergist at this dosage had no effects on larval mortalities), respectively, and pyrethroid insecticides were applied at the tenth of LD_{50}. Control groups were applied with acetone or synergist alone. Larvae were held individually in a 12-well tissue culture plates with artificial diet. Mortality was checked after exposure to fenvalerate, beta cypermethrin, cyhalothrin, phoxim, profenofos, chlorpyrifos, methomyl, imidacloprid and emamectin benzoate for 48 h, and to chlorantraniliprole and cyantraniliprole for 72 h [38].

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 ploy-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 [39] 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 $E\text{-}value \le 10^{-5}$. The GO terms for functional categorization were analyzed using Blast2go software with the $E\text{-}value$ threshold $\le 10^{-6}$. The pathway assignments were carried out by sequence searches against the KEGG database, with the $E\text{-}value$ threshold $\le 10^{-10}$.

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-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 [40]. KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathways [41].

Validation of the RNA-Seq DEGs

To validate the transcriptional results from RNA-Seq and computational analysis, 35 unigenes were selected qRT-PCR. *EF1a* and *RPL10* were used as housekeeping genes to normalize the expression of each gene [42]. 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 11. SuperReal PreMix Plus (SYBR Green, TIANGEN, Beijing, China) was used in a 20 μl system with 2×Super real SYBR Mix (10 μl), cDNA (1 μl), 50×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 $2^{-\triangle\triangle Ct}$ method. Student t-test was performed to analyze the statistical difference between means. A *p*-value<0.05 was considered statistically significant.

Additional Files

Additional file 1: The sequencing data quality of nine *S. litura* samples. (DOCX 13 kb)

Additional file 2: Summary of assembly quality of *S. litura* RNA-seq. (DOCX 13 kb)

Additional file 3: Pearson correlation between susceptible (GX) and resistant populations (LF and NJ). (PDF 132 kb)

Additional file 4: Gene annotation by BLAST-searching against public databases. (DOCX 12 kb)

Additional file 5: Similarity distribution of sequence comparison by BLAST search. (PDF 68 kb)

Additional file 6: Species that *Spodoptera litura* unigenes were annotated by BLAST search in Nr database. (PDF 73 kb)

Additional file 7: Gene ontology (GO) classification of unigenes function. The unigenes were summarized in subgroups of biological process, cellular component and molecular function. (PDF 116 kb)

Additional file 8: 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. (PDF 140 kb)

Additional file 9: 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 72 kb)

Additional file 10: 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 144 kb)

Additional file 11: Primers used for qRT-PCR validation. (DOCX 16 kb)

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 Biotecnology 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-sequencing; SRA, sequence read archive; TSR: target-based resistance; UGT, UDP-glycosyltransferase.

Declarations

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.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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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 RQL. XLC and CJW provided helpful suggestion in data analysis and manuscript revision. All authors read and approved the final manuscript.

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Tables

Table 1. Toxicity of insecticides to different *S. litura* populations

Insecticides	Population	Slope (SE)	LD$_{50}$ (95% FI) (µg/larva)	R^2	RR
Fenvalerate	GX	2.592 (0.289)	0.004 (0.003-0.005)	0.951	1.0
	LF	1.312 (0.302)	3.238 (1.933-4.804)	0.925	809.5
	NJ	1.330 (0.339)	1.288 (0.379-2.172)	0.981	322.0
Beta cypermethrin	GX	3.238 (0.364)	0.004 (0.002-0.011)	0.833	1.0
	LF	1.962 (0.290)	0.565 (0.410-0.753)	0.929	141.3
	NJ	1.679 (0.351)	0.163 (0.073-0.249)	0.901	40.8
Cyhalothrin	GX	2.346 (0.274)	0.002 (0.001-0.002)	0.957	1.0
	LF	1.063 (0.223)	3.528 (2.129-5.941)	0.971	1764.0
	NJ	1.347 (0.264)	1.902 (1.176-2.927)	0.990	951.0
Phoxim	GX	2.238 (0.334)	0.219 (0.122-0.540)	0.879	1.0
	LF	1.752 (0.298)	0.092 (0.058-0.128)	0.978	0.4
	NJ	2.911 (0.410)	0.121 (0.095-0.151)	0.986	0.6
Profenofos	GX	3.226 (0.508)	0.065 (0.052-0.080)	0.979	1.0
	LF	4.018 (0.659)	0.118 (0.096-0.143)	0.999	1.8
	NJ	2.120 (0.451)	0.253 (0.172-0.343)	0.969	3.9
Chlorpyrifos	GX	1.617 (0.286)	0.243 (0.175-0.349)	0.948	1.0
	LF	1.879 (0.375)	0.086 (0.039-0.129)	0.933	0.4
	NJ	2.125 (0.297)	0.095 (0.072-0.126)	0.957	0.4
Chlorantraniliprole	GX	3.848 (0.586)	0.001 (0.001-0.002)	0.970	1.0
	LF	1.777 (0.267)	0.004 (0.003-0.005)	0.960	4.0
	NJ	1.662 (0.254)	0.005 (0.003-0.007)	0.978	5.0
Bromine cyantraniliprole	GX	1.064 (0.157)	0.002 (0.001-0.003)	0.938	1.0
	LF	1.583 (0.261)	0.011 (0.008-0.017)	0.997	5.5
	NJ	1.329 (0.236)	0.017 (0.011-0.027)	0.898	8.5
Imidacloprid	GX	1.882 (0.273)	0.573 (0.425-0.784)	0.965	1.0
	LF	3.120 (0.487)	3.298 (2.604-4.126)	0.991	5.8
	NJ	1.210 (0.462)	11.33 (5.887-66.473)	0.920	19.8
Methomyl	GX	1.743 (0.367)	0.010 (0.004-0.015)	0.853	1.0
	LF	1.250 (0.178)	0.048 (0.008-0.168)	0.830	4.8

Emamectin benzoate	NJ	1.985 (0.326)	0.077 (0.057-0.115)	0.962	7.7
	GX	1.524 (0.440)	0.011 (0.004-0.016)	0.798	1.0
	LF	1.090 (0.253)	0.028 (0.008-0.052)	0.981	2.6
	NJ	1.952 (0.293)	0.030 (0.021-0.041)	0.976	2.7

Resistance ratio (RR) = LD_{50} value of field population/ LD_{50} value of GX population

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

Table 3. Toxicity of pyrethroids insecticide to CZ and JD population

Insecticides	Population	Slope (SE)	LD$_{50}$ (95% FI) (µg/larva)	R^2	RR
Fenvalerate	CZ	1.119 (0.266)	0.338 (0.101-0.609)	0.952	84.5
	JD	1.603(0.253)	36.496 (25.858-51.746)	0.956	9123.5
Beta cypermethrin	CZ	1.462 (0.333)	0.046 (0.014-0.083)	0.991	11.5
	JD	1.745(0.265)	2.598 (1.898-3.702)	0.982	642.0
Cyhalothrin	CZ	1.006 (0.254)	1.357 (0.619-2.581)	0.966	678.5
	JD	1.213(0.239)	13.972 (7.025-21.737)	0.951	6986.0

Table 4. 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 5. The number and expression level of metabolism related genes among GX, LF and NJ populations

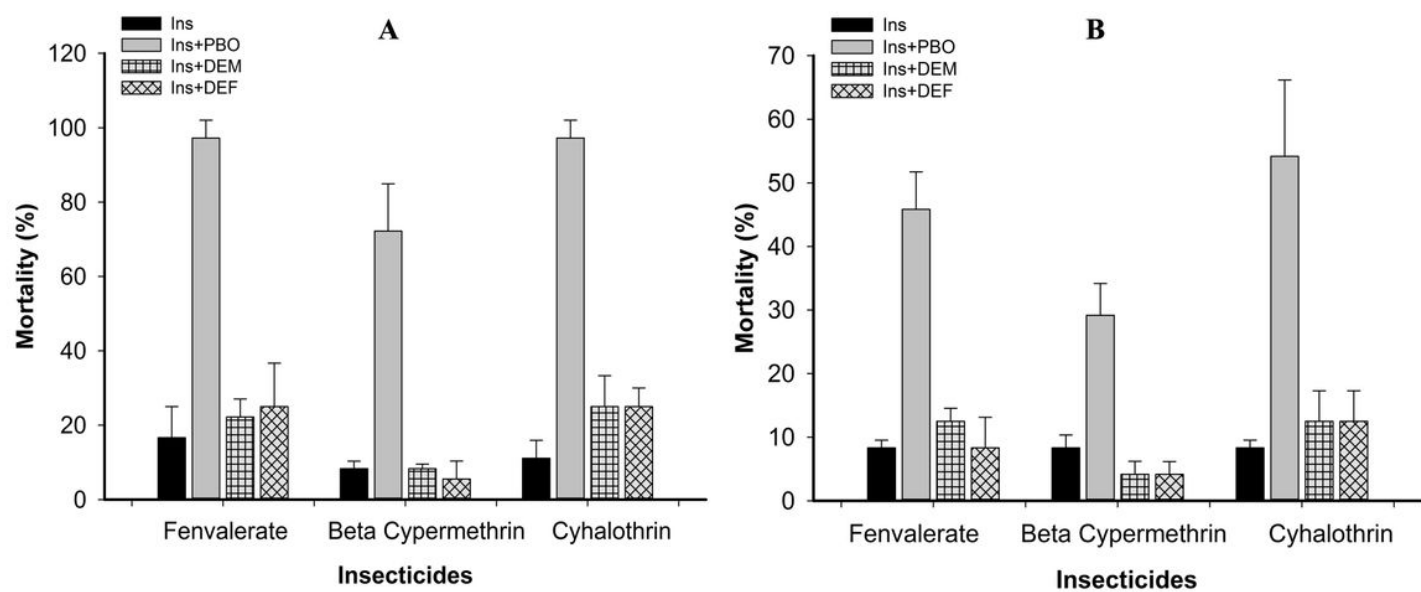| Classes of Enzyme	Total Number	Expression level of LF/GX ≥1	Expression level of NJ/GX ≥1	Expressed higher both in LF and NJ
P450	139	101	97	65
GST	32	27	29	23
COE	77	50	46	35
UGT	38	32	33	25
ABC	60	47	45	35

Figures

Figure 1

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

Figure 2

GO enrichment of up-regulated and down-regulated unigenes in LFvsGX (A, C) and NJvsGX (B, D) groups. A is unigenes up-regulated in LFvsGX. B is unigenes up-regulated in NJvsGX. C is unigenes down-regulated in LFvsGX. D is unigenes down-regulated in NJvsGX.
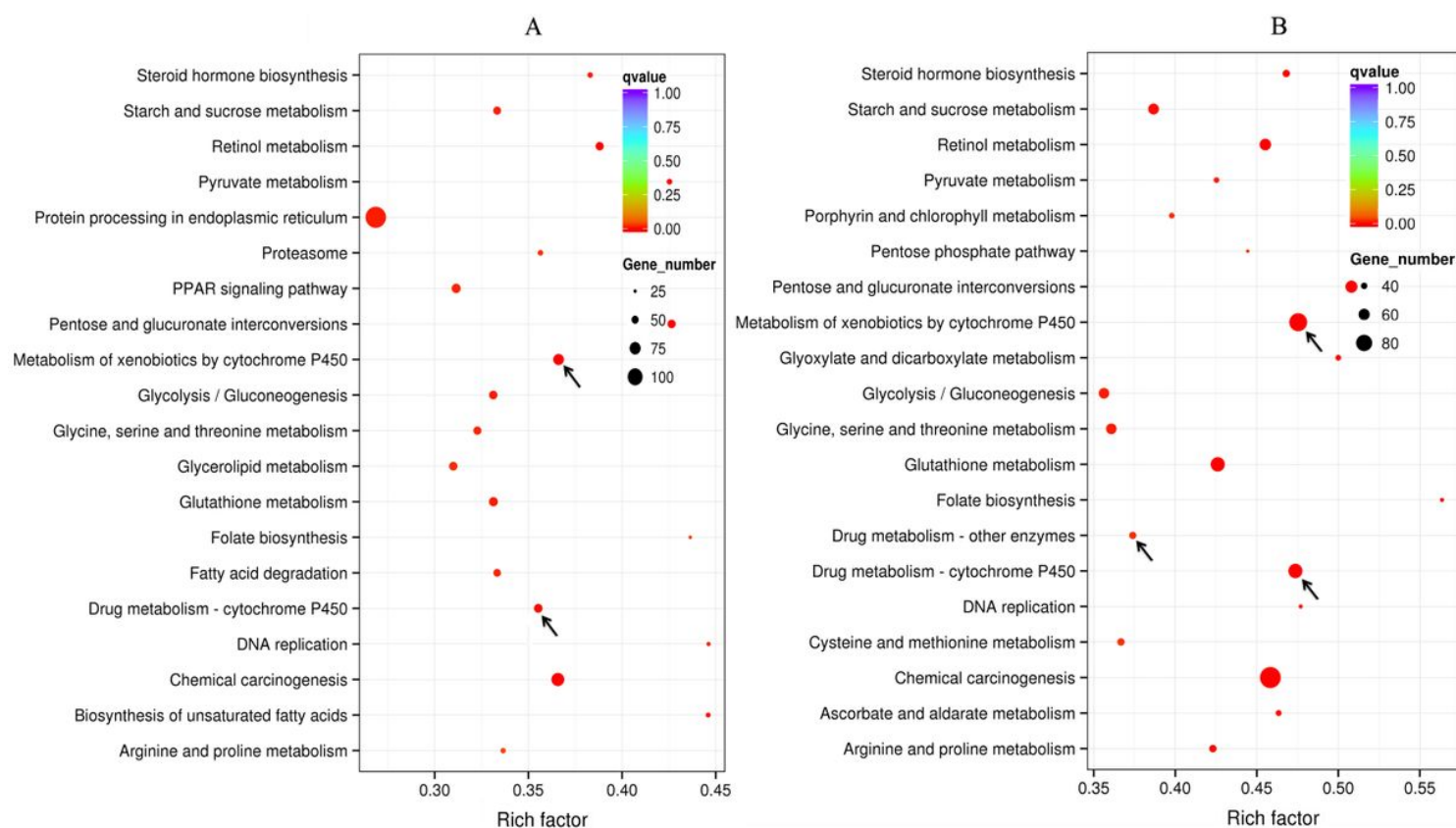
Figure 3

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

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