Identification and functional analysis of diet-responsive genes in Spodoptera litura (Fabricius)

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Abstract

Spodoptera litura is one of the most devastating agricultural pests with a wide range of host plants. To study larval performance on different diets and midgut adaptation at transcriptional levels, feeding assay and RNA-Seq experiments were conducted. RNA interference technology was used to explore the detoxification and metabolism of two cytochrome P450 genes.

The bioassay data showed that S. litura larvae developed more quickly when fed on cabbage than when fed on soybean, corn and cotton, tannin can inhibit the growth of S. litura . The result of RNA-Seq indicated that S. litura midgut modified gene expression levels to accommodate different diets, and the most differentially expressed genes were detoxification-related and digestion-related genes . Further analysis showed that the glutathione metabolism pathway was the common detoxification pathway in S. litura. The expression of cytochrome P450 genes showed a clear response to different plant hosts, and these differences may play key functions in primary detoxification of secondary metabolites from host plants. Meanwhile, the digestive enzymes of proteinases, lipases, and carbohydrases in midgut showed special responses to different plant hosts. After injection of dsRNA of CYP321A19 and CYP6AB60 , the expression level of target gene were decreased, and the sensitivity of insect to plant allelochemicals increased and the weight increase significantly slowed.

In this study, genes involved in detoxification were identified, and the results demonstrate the genes and pathways S. litura utlize to detoxify specific plant-host allelochemicals. These results may also provide a theoretical basis for S. litura management.

Background

Plants face a large number of biotic and abiotic stresses in nature, among them, herbivorous insects are one of the important threats to plant survival [1]. After a long period of evolution, plants biosynthesize a broad range of secondary metabolites to resist the attack of herbivorous insects, and these metabolites can influence the growth, survival, and reproduction of herbivorous insects [2, 3]. In cotton plants, terpenoid gossypol [4, 5], tannins [6] and flavonoids [7] are the major classes of secondary metabolites. Glucosinolates are particularly abundant and they are important plant secondary metabolites in the Brassicaceae plant of Chinese cabbage [8, 8, 10, 11]. Fabaceae plants, including soybean (*Glycine max* (L.) Merr.), are rich in isoflavones. [12, 13]. Derivatives of 1, 4-benzoxazin-3-one (BX) are the most common secondary metabolites in the plants of Poaceae, including maize (*Zea mays* L.) [14, 15].

Conversely, herbivorous insects have developed various defense systems to detoxify secondary compounds found in host plants [16, 17]. A series of detoxification-related enzymes, including cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GST) and UDP-glycosyltransferases (UGT), and ATP binding cassette (ABC) transporters, participate in the detoxification and metabolism of plant secondary metabolites [18]. P450s are an important group of enzymes involved in detoxification of host secondary metabolites in insects [19]. For polyphagous insects, their P450

detoxification enzymes need to process more plant toxic secondary metabolites. For example, *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) is a typical polyphagous pest. The insect has a wide host range and can feed on soybean, cotton, corn, tobacco, flax, tea, and some common vegetables [20, 21].

It is generally known that the insect *P450* gene are expressed in many tissues. Not surprisingly, the midgut is a rich source of P450-dependent metabolism of model substrates. Krieger et al [22] proposed that the midgut of Lepidoptera larvae is an important tissue for detoxification and metabolism, and the detoxification enzymes in the midgut evolved in response to plant defense chemical reactions [23]. Studies have shown that herbivorous insects feeding on different host plants exhibit significantly different levels of *P450* genes [24]. Liu et al confirmed that cytochrome P450 of *H. armigera* played an important role in the metabolism of plant secondary substances [25]. Studies have shown that *CYP6AE14* in the midgut of *H. armigera* was highly expressed when gossypol was added in the diet [26]. Gene *slgste1* was up-regulated in the midgut of *S. litura* at the transcriptional and protein levels when the insect fed on Brassica juncea or diet containing phytochemicals [27].

The objective of the current study was to determine global changes in the midgut gene expression of *S. litura* larvae fed on different diets, as well as to compare the performance of *S. litura* larvae when fed on these diets. To investigate these transcriptional responses, we used a replicated RNA-Seq approach. In our analyses of the transcriptional responses of *S. litura* larval midgut, we focused mainly on the digestion-related and detoxification-related genes, with a particular emphasis on cytochrome *P450* genes. In addition, two *P450* genes were selected in the transcriptome, and the function was verified by RNA interference technology.

Results

Growth rate on different diets

The larvae grew differently on the different diets. Two days later (T_1), the weight of *S. litura* fed on cabbage was 61.02 mg, which was significantly heavier than those fed on other diets, and especially for Tannin1 (29.91mg).

At the last time point (T_3), the average weight of *S. litura* fed on cabbage (520.37 mg) was heaviest. Those larvae fed on soybean (365.38 mg), cotton (272.09 mg), and artificial diet (193.25 mg) had a middle range weight, and those fed on corn (91.01 mg), Tannin2 (49.24 mg), and Tannin1 (36.98 mg) were significantly lighter than the others (Figure 1). Larvae fed on cabbage showed higher growth rates at time points 1 and 2 than the others, and the lowest growth rates were found for the larvae fed on the artificial diet with 15 g of tannin at any time point (Table 1).

S. litura larvae had a higher growth rate and a higher final weight when fed on cabbage. *S. litura* larvae fed on Tannin1 had growth rates and final weights that were significantly lower.

Table 1 Growth rate of *S. litura* larvae fed on different diets.

Treatment	T_1	T_2	T_3
Cotton	0.35 ± 0.01 de	0.23 ± 0.00 a	0.16 ± 0.00 a
Cabbage	0.40 ± 0.00 a	0.24 ± 0.00 a	0.16 ± 0.00 a
Corn	0.37 ± 0.01 cd	0.21 ± 0.00 b	0.15 ± 0.00 a
Soybean	0.39 ± 0.01 ab	0.23 ± 0.00 a	0.16 ± 0.00 a
AD	0.38 ± 0.00 bc	0.23 ± 0.00 a	0.16 ± 0.00 a
Tannin1	0.33 ± 0.01 f	0.17 ± 0.01 d	0.12 ± 0.00 a
Tannin2	0.35 ± 0.02 e	0.19 ± 0.01 c	0.13 ± 0.00 a

Those in the same column (mean ± SD) followed by different letters show significant difference at the $P < 0.05$ level by Duncan's multiple range test. AD: artificial diet. T: timepoint.

Illumina sequencing, sequence assembly, and gene identification

Transcriptome analysis of 21 samples was completed, and clean reads were obtained. The percentage of Q30 bases was not less than 91.22%. Clean reads of each sample were sequenced with the designated reference genome of *S. litura* [28], and the alignment efficiency ranged from 83.84% to 89.66%. A total of 570 new genes were identified by filtering out sequences that were either too short (less than less than 150 nucleotide ORFs) or contained only a single exon and 349 of them got functional annotation. Sequence alignment of the new genes was conducted using BLAST software with the databases of NR, swiss-prot, GO, COG, KOG, Pfam, and KEGG. KEGG Orthology results of the new genes were obtained using KOBAS2.0 [29]. After the prediction of amino acid sequences of the new genes, the HMMER [30] software was used to compare our results with the database of Pfam to obtain the annotation information of the new genes (Table 2). The transcriptome sequences had submitted to NCBI database and the SRA accession number was PRJNA528696

Table2 Statistic results of new gene function annotations

Annotated databases	New Gene Number
COG	27
GO	91
KEGG	69
KOG	100
Pfam	136
Swiss-Prot	87
eggNOG	223
nr	345
All	349

Differentially expressed genes between treatments

Gene expression was analyzed based on the comparison of results. Differentially expressed genes were identified according to their expression levels in different samples, and functional annotation and enrichment analyses were performed. *S. litura* midgut transcriptomes were studied by RNA-seq to determine changes in expression level in response to feeding from different diets. Gene expression levels for each replicate were assessed using principal component analysis (PCA), and the results revealed obvious differences from different diets. The gene expression in larvae fed on artificial diet that contained 15 g/L and 7.5 g/L tannin clustered together and were far from the other treatments based on sample scores for the first (PC1) principal components. These results indicated that *S. litura* differential gene expression in midgut levels in response to different diets (Supplement figure 1A).

The purpose of this study was to identify the key genes of *S. litura* that enable the insect to adapt to plant secondary metabolite, In order to investigate this, we carried out a pair-wise comparison of *S. litura* fed on other diets against artificial diets was carried out. Samples fed on cabbage, corn, cotton, soybean, and artificial diet (including tannin) exhibited 1,912 (964 up and 948 down), 1,395 (769 up and 626 down), 2,069 (1025 up and 1044 down), 2,998 (1516 up and 1482 down), and 1533 (838 up and 695 down) differentially expressed genes, respectively. larvae grown on artificial diet contain 15 g/L and 7.5 g/L tannin displayed similar gene expression, so those samples were combined as one sample set (Supplement figure 1B).

We next used a venn diagram to analyze the distribution of differentially expressed genes. There were only 45 up-regulated (Supplement figure 1E) and 64 down-regulated (Supplement figure 1F) differentially expressed genes when all treatments were compared together, and there were 238 up-regulated (Supplement figure 1C) and 244 down-regulated (Supplement figure 1D) differentially expressed genes when tannin treatments were excluded from venn diagram analysis. The unique differentially expressed gene numbers

were higher in cotton and soybean treatment samples, both in up-regulated and down-regulated genes analysis.

Identification of putative pathway related to diet adaption

The differentially expressed genes in pair-wise comparison of *S. litura* fed on various plant diets against artificial diets were subjected to KEGG pathway database analysis to discovery any significant changes to metabolic pathways genes. In top 5 up-regulated pathways, three enriched pathways (Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450, and Pentose and glucuronate interconversions) were present across four treatments. Two enriched pathways (Glutathione metabolism and Ascorbate and aldarate metabolism) were present across three treatments. In the top 5 down-regulated pathways, one enriched pathway (Neuroactive ligand-receptor interaction) was shared across four treatments, and 5 enriched pathways (Endocytosis, Phosphatidylinositol signaling system, N-Glycan biosynthesis, Alanine, aspartate and glutamate metabolism and other glycan degradation) were shared across the treatments (Supplement figure 2).

The three KEGG pathways of metabolism of xenobiotics by cytochrome P450, drug metabolism cytochrome P450, and glutathione metabolism were mostly up-regulated in cabbage, corn, cotton, and soybean feed samples as compared to artificial diets, but were down-regulated in the artificial diet with tannin feed samples compared to the artificial diet alone.

The character of genes involved in enriched pathway

In up-regulated pathway, there were many similar genes involved in different pathways. Glutathione S-transferase and UDP-glucuronosyltransferase were two important genes present in up-regulated pathway. Both of these genes are important secondary metabolism detoxification enzymes in insects. Glutathione S-transferase was present in three pathways out of the top 5 pathways determined in our analysis. We observed metabolism of xenobiotics by cytochrome P450, Drug metabolism-cytochrome P450 and Glutathione metabolism, and because metabolism of xenobiotics by cytochrome P450 and drug metabolism - cytochrome P450 were common in vertebrate animals and few reports in insect, and no cytochrome P450 genes were involved in those pathways, it indicated that glutathione metabolism was the main pathway, in which Glutathione S-transferase was involved.

A total of 17 glutathione S-transferase genes, 1 gamma-glutamyltransferase (GGT), 3 glutathione peroxidases (GPx), and 1 isocitrate dehydrogenase (IDH) gene were involved in the glutathione metabolism pathway. Meanwhile, elevated expression of genes involved in glutathione disulfide produced in feed on cabbage and cotton samples was observed, and gamma-glutamyltransferase genes were elevated when insects were fed on cotton (Supplement figure 3).

Identification of cytochrome P450s related to detoxification

As our focus was primarily on the response of detoxification-related genes of *S. litura* fed on various diets, we paid special attention to the cytochrome *P450* gene family, which is involved in primary detoxification metabolism. The main cytochrome P450 gene involved in detoxification metabolism in insects is typically the special cytochrome P450. However, in the enriched up-regulated pathways from our analysis, we cannot find common up-regulated P450 genes in all treatments.

A total of 24 cytochrome *P450* genes of which FPKM >100 were chosen in *S. litura* midgut fed on plant hosts. Of these, 19 out of 24 cytochrome *P450* genes belonged to the CYP6 family. Considering the cytochrome *P450* genes found in the CYP family: 2 belonged to CYP4, 2 belonged to CYP9, and 1 belonged to CYP12. Unlike in Glutathione metabolism pathway, the expression of cytochrome *P450* genes showed a clear response to different plant hosts. There were more induced cytochrome *P450* genes when *S. litura* fed on cabbage and cotton than on other diets (12 genes associated with cabbage and 9 genes associated with cotton). Only 3 cytochrome *P450* genes were higher expressed when fed on artificial diet, 2 genes when fed on soybean, and 1 gene when fed on corn.

Artificial diets containing tannin induced 2 cytochrome *P450* genes to be expressed in insects, but suppressed the expression of 2 cytochrome *P450* genes which had higher expression when feed on artificial diets alone (Supplement figure 4).

Expression pattern of *CYP321A19* and *CYP6AB60* in different developmental stages and tissues

In order to obtain the expression profile of *CYP321A19* and *CYP6AB60,* RT-qPCR analysis showed that *CYP321A19* and *CYP6AB60* transcript was detected in all tissues and age. For *CYP6AB60* gene, it is highly expressed at the 4th and 6th instar larva, and the expression level is lower at the 1st instar larva and pupa (Figure 2A), and the expression levels were significantly higher in the midgut and fat body (Figure 2B). Similarly,

CYP321A19 was also highly expressed in 4th instar larvae (Figure 2C), with the highest expression in fat body and midgut (Figure 2D).

Expression of *CYP321A19* and *CYP6AB60* was induced by plant allelochemicals

The expression of *CYP321A19* in the midgut and fat body of the larvae was increased and showed a significant difference with control artificial diet, when fed with an artificial diet containing quercetin (Figure 3A). Similarly, when coumarin or soy isoflavones were added to the artificial diet, the expression levels of *CYP6AB60* increased significantly compared with the control group (Figure 3B, C).

Functional analysis of *CYP321A19* and *CYP6AB60* by RNAi

To evaluate the role of *CYP321A19* and *CYP6AB60* in the detoxification of plant allelochemicals, *CYP321A19* and *CYP6AB60* were silenced by RNAi technique in 4 instar larvae. Compared to the control group (injection of ds*GFP*), transcriptional levels of *CYP321A19* were significantly decreased by 83.9% and 66.2% in the midgut and fat body at 72h (Figure 4A, B). Similarly, in both midgut and fat body tissues, *CYP6AB60* transcript levels were significantly reduced following dsRNA injection (Figure 4C-F).

When the larvae were exposed to the plant allelochemicals, the net weight gain on day 5 was lower in the treatment group than in the control group (*CYP321A19*: 0.57 g vs. 0.70 g) (Figure 5A). Daily weight gain was lower in the treated larvae than in the control larvae (Figure 5B).Thus, *CYP321A19* silenced larvae showed both net weight gain and daily growth significantly lower than the control group. In addition, larvae injected with ds*CYP6AB60* and fed with coumarin and soy isoflavones, exhibited significantly lower weight gains than ds*GFP*-injected controls exposed to the same allelochemicals (Figure 5C-F).

Identification of digestive enzymes related to diet adaption

When *S. litura* fed on different diets, it faced different secondary metabolism stresses when deal with different nutrients. Proteinases, lipases, and carbohydrases make up the main digestive enzymes of insects [31]. In our transcriptome data, digestive enzymes in

midgut were identified, and included proteinases (trypsin and chymotrypsin), lipases, and carbohydrases (alpha-amylase and glucosidase).

A total of 34 trypsin genes and 4 chymotrypsin genes were found to be more highly expressed in *S. litura* midgut (FPKM >100). We found more than 10 induced trypsin genes when *S. litura* fed on cotton, soybean, and artificial diet, but few trypsin genes were induced when fed on cabbage and corn. Most of the high expression trypsin genes were uniquely induced by diets. We found 8, 11, and 6 unique high expression genes when fed on artificial diet, soybean, and cotton, respectively, and 5 higher expression trypsin genes were induced when fed on cotton and soybean. The highest expression of chymotrypsin genes were detected in samples of larvae fed on artificial diets, cotton, and soybean.

Considering the lipid digestion and absorption process in the midgut, 13 higher exressing triacylglycerol lipase genes were (FPKM >100) were found. The triacylglycerol lipase genes were induced when insects were feed on corn, cotton, and soybean, and had the highest induced gene numbers when fed on soybean (triacylglycerol lipase genes). When fed on cotton, a total of 6 triacylglycerol lipase genes were induced. All the corn-induced triacylglycerol lipase genes were the same as those induced by soybean, except LOC111355064. There were 2 cotton-induced triacylglycerol lipase genes that were the same as soybean-induced genes, but there were no shared triacylglycerol lipase genes with corn.

During carbohydrate digestion and absorption process in the *S. litura* midgut, amylases and glucosidases were the main observed differentially expressed genes. A total of 2 amylase and 12 glucosidase genes showed higher expression (FPKM >100). We found that 1 alpha-amylase was induced in corn and cotton fed insects. However, there were no observed induced alpha-amylase genes in other diet treatments. In corn and cotton fed samples, there was 1 alpha-amylase gene with higher induced expression. No other diets showed induced alpha-amylase gene expression (Supplement figure 5).

Quantitative real-time PCR validation

To verify the transcriptome data, we examined the relative expression levels of *P450* (LOC111350062, LOC111358240, LOC111351731), UDP-glucosyltransferase (LOC111348983, LOC111348863, LOC111348860) and GST (LOC111354038, LOC111351682, LOC111352663, LOC111351550). The qRT-PCR of these unigenes showed that the results were consistent with the DGE results (Figure 6).

Discussion

Insect herbivores can feed on their host plants for development and survival. Due to host plant nutrition and allelochemicals, polyphagy insects show differential fitness to the plant-hosts [32]. Studies have shown that plant secondary metabolites can have positive effects on the survival and growth rates of insects [33, 34, 35]. Our larval development assays suggested that cabbage is the best host plant for *S. litura*, with a higher growth rate and final weight than other host plants. *S. litura* fed on artificial diets with tannin had a lower growth rate and obtained a lighter final weight in our study.

In the study, we chose host-plants that contain various kinds of secondary metabolites. Gossypol, tannin, and flavonoids are the major secondary metabolites in cotton plants, and glucosinolates and isoflavones are rich in Chinese cabbage and soybean. Derivatives of 1, 4-benzoxazin-3-one are the common secondary metabolites found in maize plants. The explanation for the differences of larval development on different diets could be due to the effect of differential secondary metabolites that the diets contain. A major question in plant-insect interactions is how insect herbivores cope with secondary metabolites compounds in diverse host plants [36]. As insect midgut is the main location to digestive food and detoxification [37], and the mechanisms *S. litura* use to cope with toxic compounds in diverse host plants are not well understood [38], we used a feeding assay and RNA-Seq of *S. litura* larval midgut to determine the genes used by *S. litura* to cope with secondary metabolites associated with different diets.

Based on *S. litura* genomic data [28], high-throughput sequencing was an efficient research tool to better understand the molecular mechanisms behind adoption of host-plants. Our results demonstrated that *S. litura* could develop on different diets and the transcriptional responses of midgut were related to the host diet that *S. litura* was fed on. Generally, glutathione S-transferase genes and Cytochrome *P450* genes were the most differential expressed genes involved in detoxification, while, proteinases, lipases, and carbohydrases were the most differentially expressed genes involved in the digestive system.

A total of 47 Glutathione S-transferase genes were identified in *S. litura* genomic data [28]. In this study, 17 glutathione S-transferase genes were found to be high expressing in *S. litura* midgut when fed on different host-plant. It has been demonstrated that glutathione S-transferase can detoxify many plant allelochemicals, and can be induced by plant allelochemicals [16, 39]. In this study, the expression of some glutathione S-transferase genes showed high FPKM valves, but no special response glutathione S-transferase genes were be found when fed with different diets, which indicated that glutathione metabolism pathways were the common detoxification pathway in *S. litura.* At same time, genes in glutathione disulfide produced in feed on cabbage and cotton samples also showed high FPKM valves. One possible reason for this is that more glutathione disulfide was needed for allelochemical detoxification. As the gamma-glutamyltransferase genes, which are located at last step of glutathione metabolism pathway, were higher expressed in insects fed on cotton, cotton allelochemical detoxification may require more steps than detoxification in *S. litura* midgut fed on other host-plants.

In insects, cytochrome *P450* genes are an important gene family in the detoxification of exogenous or endogenous compounds, including plant secondary metabolites [40, 41]. A total of 138 cytochrome *P450* genes were identified in *S. litura* genomic data [28]. In this study, 17 glutathione S-transferase genes were

highly expressed in *S. litura* midgut when fed on different host-plants. 21 out 61 Clan 3 cytochrome *P450* genes were highly expressed in *S. litura* midgut when fed on different host-plants, and most of these genes belong to the *CYP6* gene family. In insect, the *CYP6* gene family is mostly involved in plant secondary metabolites detoxification. Here, we selected two *P450* genes from the transcriptome that can be induced by the secondary metabolites of soybean and cabbage, respectively, and named them *CYP6AB60* and *CYP321A19*. They all showed the highest expression levels in the 4th instar larva, and the highest expression levels in the 4th instar larva midgut and fat body. When the larvae were exposed to an artificial diet containing quercetin or coumarin and soy isoflavones, the expression levels of the selected genes were significantly up-regulated. Induction of P450 enzymes has taken center stage in the discussion of P450s and host plant chemicals [24]. Studies have shown that *CYP6AE14* and *CYP6AE11* were significantly up-regulated when *H. armigera* fed on artificial diet with higher concentration of gossypol [42]. *CYP6B46* can be induced when *Manduca sexta* larvae feed on *Nicotiana* wild-type plants, which can produce nicotine [43]. The expression of *CYP314A1*, *CYP315A1*, *CYP18A1*, *CYP307A1*, and *CYP306A1* were found to be induced by 2-tridecanone [44]. The xanthotoxin can induce the expression of *CYP9A* genes from larval *Manduca sexta* midgut [45]. The above research results are similar to the results of this study.

General observations indicate that herbivorous insects feeding on different host plants show significantly different P450 [24]. Plant secondary metabolite induction can be shown to affect insect behavior, in fact, it affects the consumption of toxic plant compounds. For example, in *Manduca sexta*, nicotine is metabolized by the P450 enzyme [46], and the induction of P450 by nicotine appears to be the key to the ability of *M. sexta* to feed on tobacco [47]. Nicotine inhibits *M. sexta* larvae from eating tobacco until P450 enzymes are induced, at which point the larvae resume feeding. This induction not only affects the metabolism of nicotine, but also the metabolism of nine other seemingly unrelated model substrates [48].

In the detoxification of plant secondary metabolites, cytochrome *P450* can be classed into specialists and generalists [16]. In this study, most high expressing cytochrome *P450* genes may be specialists to different host-plant, and those cytochrome *P450* gene family may be involved in primary detoxification metabolism. As specialists have a highly efficient and specialized detoxification system, *S. litura* has a wide host range and significantly impacts agricultural production. The most famous characteristic of P450 enzymes is the metabolism of foreign substances, especially pesticides and allelochemicals. The specialist cytochrome *P450* genes may represent a potential target site for the development of pest controls.

RNAi technology has been widely used to reveal the role of cytochrome *P450* in drug resistance, secondary metabolites and pesticide detoxification [49]. Studies have shown that when *Manduca sexta* larvae are fed plant material expressing *CYP6B46*-specific dsRNA, the level of this transcription decreases and larval growth is hindered [50]. In addition, studies have shown that RNAi silences *CYP307A1* and blocks molting steroid synthesis, suggesting that this gene is required for molting steroid biosynthesis in anopheles gambiae. Silencing *CYP6B7* alone or *CYP6B7* in combination with *CPR* and/or *Cyt-b5* increased the sensitivity of bollworm to fenvalerate, indicating that *CYP6B7*, *CPR* and *cyt-b5* were

synergistic in the metabolic enhancement of fenvalerate and played an important role in the resistance of bollworm to fenvalerate [51]. After RNAi silencing of *CYP321E1*, *Plutella xylostella* has increased sensitivity to chloroaniline, with a mortality rate of up to 70% [52]. In our study, RNA interference (RNAi) was used to investigate the function of selected target genes. After injection of ds*CYP6AB60* and ds*CYP321A19*, the tolerance of the 4th instar larva of *Spodoptera litura* to plant allelochemicals (quercetin, coumarin, soy isoflavones) was significantly reduced.

Conclusion

In our study, some *P450* genes of *S. litura* fed on different host plants and artificial diets with tannin were up-regulated compared with those fed on artificial diets, but all glutathione S-transferase genes were down-regulated. The bioassay data showed that tannin can inhibit the growth of *S. litura*, which indicated that when *S. litura* faced harmful allelochemicals, its primary detoxification metabolism optoins were narrowed to Cytochrome *P450* genes, and the detoxification metabolism of glutathione metabolism was less utilized.

In addition of detoxification metabolism, digestion was another important function in *S. litura*. In this study, the expression of digestive enzymes genes was assessed, and we found that, in general, the expression of genes encoding proteinases, lipases, and carbohydrases corresponded to food nutritional composition.

Methods

Insect rearing

Insects were purchased from Henan Jiyuan Baiyun Industry Co., Ltd, and then *S. litura* were fed on artificial diet: 16.7 g of agar, 100 g of soybean flour, 100 g of wheat germ flour, 100 g of oatmeal, 60 g of yeast powder, 40 g of sucrose, 6 g of ascorbic acid, 2 g of sorbic acid, 2 g of methyl paraben, 3 g of sodium benzoate, 8.35 mL of KOH (4M), 15 mL of decavitamin (1.528 g of nicotinic acid, 1.525 g of calcium pantothenate, 0.764 g of riboflavin, 0.382 g of niacin thiamine, 0.382 g of pyridoxine hydrochloride, 0.382 g of folic acid, 0.305 g of biotin and 0.003g of cyanocobalamine per 3.3 L of water) per 1 L water. Insect rearing took place in an artificial climate chamber (26 ± 1°C, 65 ± 5 % relative humidity, Light : Dark = 14:10) and mixed populations were used in all experiments. The insects were continuous feeding for 3 generations and then the larvae were used for the feeding assays and RNA-Seq.

Feeding assays

The following plant species were used for our experiments: cotton (zhong 49, provided by institute of cotton research of CAAS), corn (Yuyu 22, Charoen Pokphand Group), soybean (Zhonghuang 35, Beijing huinong fumin technology co. LTD) and cabbage (Jingfeng 1, Fuyichun seed sales co. LTD). All plants

were planted in a climate chamber maintained at (26±1) °C, 65 ± 5% humidity, and a 14:10 (Light : Dark) photoperiod. were used for feeding assays (4 true leaves). Additionally, 15 g and 7.5 g tannin were added to artificial diets as treatment 1 (Tannin1) and 2 (Tannin2), respectively, the final concentration was 1.5% and 0.75%, respectively. 1.5% is the highest content of tannins in cotton leaves [53]. After that, three treatments of quercetin, coumarin and soybean isoflavone artificial feed (1 mg/g) were set up respectively to verify the function of *P450* gene.

S. litura larvae were reared on an artificial diet until they grew to the third instar (L3), then they were transferred to host plants (cotton, corn, soybean or cabbage) and Tannin1 or Tannin2 (T0). Each leaf or small piece of artificial diet contained one larva; there were 3 replicates for each treatment, and each treatment included 20 larvae. Larvae were allowed to feed on the excised leaves of plants or the artificial diet for 6 consecutive days. For the duration of the experiment, larval weight was recorded and fresh leaves were replaced every second day (T1,T2,T3). Growth rates [18] were calculated per unit time for each treatment.

Midgut tissue collection

Newly hatched larvae of *S. litura* were transferred to living plants of cotton, corn, soybean, cabbage, and artificial diet containing tannin. Then observe the growth of larvae on each host and artificial feed, and take the 4^{th} instar larvae on each treatment after 5-7 days, and the midgut tissues were dissected and collected.. Insects were dissected in 0.7 % saline solution. Dissected tissues without contents were quickly frozen in liquid nitrogen and kept at -80°C prior to RNA isolation. There were 3 replicates for each treatment and each replicate included 12 larvae.

RNA isolation and illumina sequencing

RNA-Seq experiments were carried out with RNA isolated from larvae reared on different diets. Total RNA was extracted by TRIzol® Reagent (Life Technologies, USA) according to the manufacturer's instructions. The quantity and quality of the RNA were assessed by 1.0% agarose gel electrophoresis and absorbance at 260 nm on a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). Then samples were sent to Beijing Genomics Institute (Shenzhen, China) for cDNA library construction and Illumina sequencing. Sequencing was performed using an Illumina Hi-Seq 4000 sequencer (San Diego, CA, USA).

RNA-Seq data analysis

Raw reads of fastq format were initially processed using in-house perl scripts. The clean reads were then obtained by removing reads containing adapter, poly-N, and low quality reads from raw reads. The Q20,

Q30, GC-content, and sequence duplication level of the clean reads were then calculated. These clean reads were then mapped to the reference genome sequence using hisat2 tools software. The databases of Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KO, and GO were used for gene function annotation. Gene expression levels were estimated by fragments per kilobase of transcript per million fragments mapped. Differential expression analysis of the two groups was performed using the DESeq R package (ver. 1.10.1). 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. Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R package based on Wallenius non-central hyper-geometric distribution [54]. KOBAS [55] software was used to test the statistical enrichment of differentially expressed genes in KEGG pathways.

Quantitative real-time PCR validation

Total RNA was extracted as described above and Reverse Transcription System (Takara) was used for cDNA synthesis. The relative expression levels of *P450*, UDP-glucosyltransferase and *GST* were conducted by quantitative real-time PCR (qRT-PCR).

Two differentially expressed genes, LOC111355823 and LOC111355088, were identified from the transcriptome. We named them separately *CYP321A19* and *CYP6AB60*. To clarify the expression pattern of the *CYP321A19* and *CYP6AB60* sequences, four tissues (midgut, fat body, cuticle, and hemolymph) were dissected from 1-day-old 4th instar larva of *S. litura*. For analyses of the *CYP321A19* and *CYP6AB60* expression pattern at different development stages, we collected the samples which were at day 2 of each development stage used RNase-Free centrifuge tubes, including eggs (100 eggs per tube), first to sixth instar larvae (6 larvae per tube) and pupae (6 pupae per tube) of *S. litura* (collect 6 tubes for each sample above). Three biological replications were performed.

To elucidate the expression profiles of *CYP321A19* sequences when exposed to plant allelochemicals. Newly molted fourth instar larvae of *S. litura* were fed on artificial medium containing quercetin. Take no added artificial feed as control group, each group contains 6 repetitions, each repetition contains 24 larvae. Dissecting the midgut and fat body from surviving larvae after fed on 72 h and collected with RNase-free tubes (each tube contains tissue samples from 24 larvae). The samples were then immediately frozen in liquid nitrogen and stored at −80°C to extract RNA and further expression analysis. Similarly, the midgut and fat bodies of 4^{th} instar larvae that ate coumarin and soybean isoflavones were taken to analysis the expression profile of *CYP6AB60*. Three independent RNA extractions (representing three biological replicates) were performed for all treatments.

Sample RNA extraction and cDNA synthesis in the above two experiments are as described above. Subsequent real-time quantitative PCR (RT-qPCR) were equivalent to those described above (includes 3 technical repetitions). Beacon Designer 7.7 was used for designing qRT-PCR primers of the selected

unigenes. Unigenes and primers were listed (Table S1). *GAPDH* and *RpL10* were chosen for reference genes [56]. Cycle threshold (CT) values were collected after completion of reaction and the relative expression levels were analyzed using the $2^{-\triangle\triangle CT}$ method [57].

dsRNA synthesis

Using the cloned *CYP321A19* and *CYP6AB6*0 sequences as templates, an in vitro transcription reaction template was obtained by PCR amplification. Two pairs of primers (Table S1) were used to amplify *CYP321A19* and *CYP6AB60*. The amplification conditions comprised 5 cycles of 95°C for 10s, 50°C for 15s, 72°C for 30s, 20 cycles of 95°C for 10s, 50°C for 15s (Increase 0.5°C by each cycle), 72°C for 30s, 10 cycles of 95°C for 10s, 60°C for 15s, 72°C for 30s. PCR products were purified using the Promega SV Gel and PCR clean up system (Promega, Madison, WI, USA) and DNA concentrations were determined using UV-visible spectrophotometry. According to the instructions, dsRNAs (ds*CYP321A19* and ds*CYP6AB60*) corresponding to *CYP321A19* and *CYP6AB60* were synthesized using the T7 RiboMAX ™ Express RNAi System (Promega, Madison, WI, USA). In addition, dsRNA of the *GFP* gene used as a negative control was synthesized by the same method as above, and primers used were listed (Table S1). The obtained dsRNA was detected by ultraviolet spectrophotometry and the purity and integrity were evaluated by agarose gel electrophoresis. The final concentrations of dsRNA were adjusted to 1.5 $\mu g \cdot \mu L^{-1}$ by RNase-free water and kept at -80 °C.

Silencing of differentially genes by RNAi

For RNAi bioassays, the final concentrations of dsRNA were adjusted to 1.5 μg/μL using DEPC-treated (RNase-free) water prior to use. All dsRNA injection experiments used 4th instar larvae (day 1 and hunger for 4 h) of *S. litura*, with 2μL (3.0μg) of dsRNA injected into the distal second segment of the abdomen by manual microinjector, while the control group was injured with an equivalent volume of ds*GFP*. The treated larvae were fed with artificial feed supplemented with plant allelochemicals (The larvae injected with ds*CYP321A19* were fed with quercetin, and the larvae injected with ds*CYP6AB60* were fed with coumarin and soybean isoflavones).

For the RNAi efficiency evaluation, the midguts and fat bodies of the surviving larvae (6 larvae) were taken at 24h, 48h and 72h after the last treatment. Calculate the net weight increase (weight of each day minus weight on day 0) and weight increase per day (weight of each day minus weight of the previous day). All experiments were performed in triplicate. For each replicate, midguts and fat bodies from six larvae of *S. litura* were collected for total RNA extraction. The RNA extraction and RT-qPCR procedures were described above.

Data analysis

qRT-PCR data are presented as mean ± standard error (SE). Statistically significant differences ($p < 0.05$) of bioassay data and qRT-PCR data were identified by one-way ANOVA followed by Duncan's multiple range test.

Declarations

Ethics approval and consent to participate:

This article does not contain any studies with human participants or animals (others than insects) performed by any of the authors.

Consent for publication:

Not applicable.

Availability of data and materials:

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests:

The authors declare that they have no conflict of interest.

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Authors' contributions:

LW and PZ are co-first authors, acquired, analyzed, and interpreted the data and drafted the manuscript. JJC and SZ conceived and designed the study. SZ, JYL, CYW, XZZ, LJZ, JCJ, KXZ, DYL and JJC were

involved in data acquisition and interpretation and in manuscript revision. All authors read and approved the final manuscript.

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Supplementary Figure & Table Captions

Table S1 Primers used in this study.

Supplement figure 1 (A) Principal component analysis (PCA) for gene expression levels of each treatment. (B) The up-regulated and down-regulated gene numbers of samples feed with cabbage, corn, cotton, soybean, and artificial diet with tannin compared with artificial diet. (C) Venn diagram showing the number of up-regulated and down-regulated genes of samples fed with cabbage, corn, cotton, soybean, and artificial diet with tannin compared with artificial diet. C and E respresnt up-regulated differentially expressed genes; D and F represent down-regulated differentially expressed genes. G0: Artificial diet vs Cabbage; G1: Artificial diet vs Corn; G2: Artificial diet vs Cotton; G3: Artificial diet vs Soybean; G4: Artificial diet vs Artificial diet with tannin.

Supplement figure 2 The up-regulated and down-regulated pathways of samples fed with cabbage, corn, cotton, soybean, and artificial diet with tannin compared with artificial diet alone.

Supplement figure 3 The metabolic pathway of Glutathione. Gene expression of glutathione S-transferase when *S. litura* feed with different diets. Values are based on log2-transformed FPKM values relative to the median intensity of all contigs (red = up-regulation; green = down-regulation).

Supplement figure 4 Gene expression of cytochrome P450 when *S. litura* was fed with different diets. The plot is based on log2-transformed FPKM values relative to the median intensity of all contigs (red= up-regulation; green = down-regulation).

Supplement figure 5 Gene expression of digestive enzymes when S. litura were fed with different diets. Plots are based on log2-transformed FPKM values relative to the median intensity of all contigs (red= up-regulation; green = down-regulation).

Figures

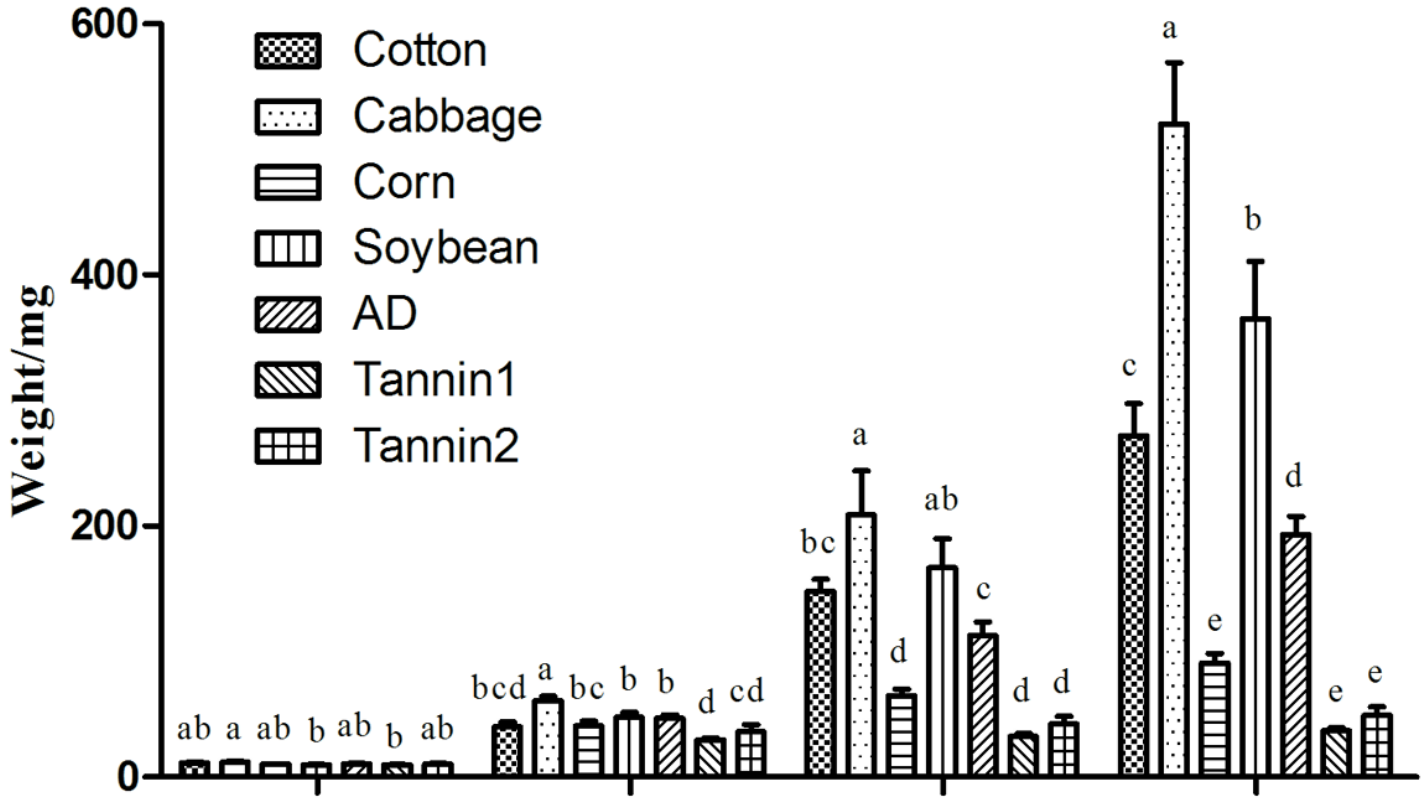

Figure 1

The weight of S. litura larvae fed on different diets. The data are mean ± SD and different letters indicate significant difference at the 0.05 level by the Duncan's multiple range test. AD: artificial diet. T: timepoint.

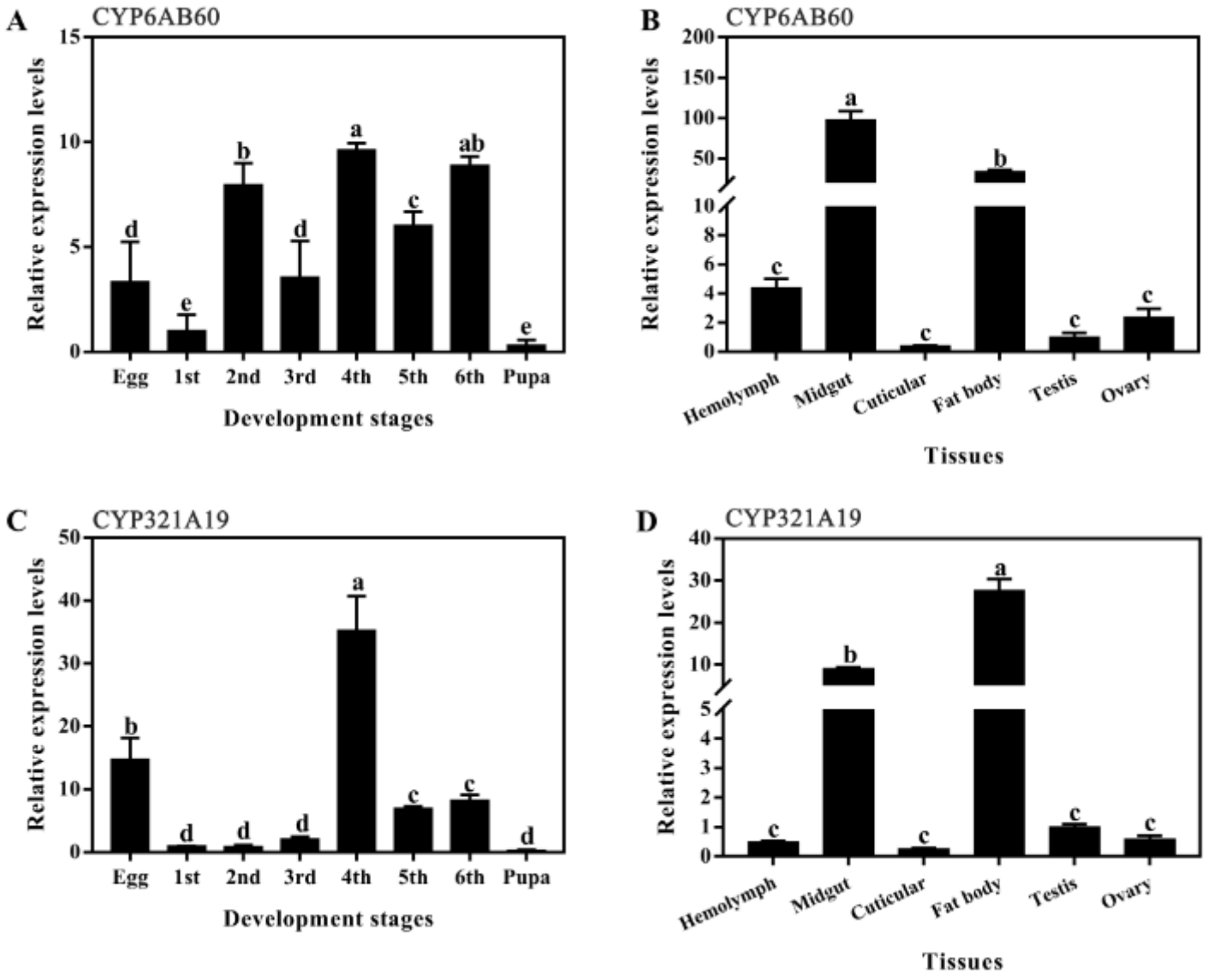

Figure 2

Expression levels of CYP6AB60 in different developmental stages (A), in different tissues (B), and CYP321A19 in different developmental stages (C), in different tissues (D). All larvae were fed an artificial diet. RT-qPCR analysis was used to determine the relative transcript levels for each gene. Data shown as means ± SE derived from three biological replicates. Different letters above bars indicate significant differences ($p < 0.05$) according to the Duncan's multiple range test (same as below).

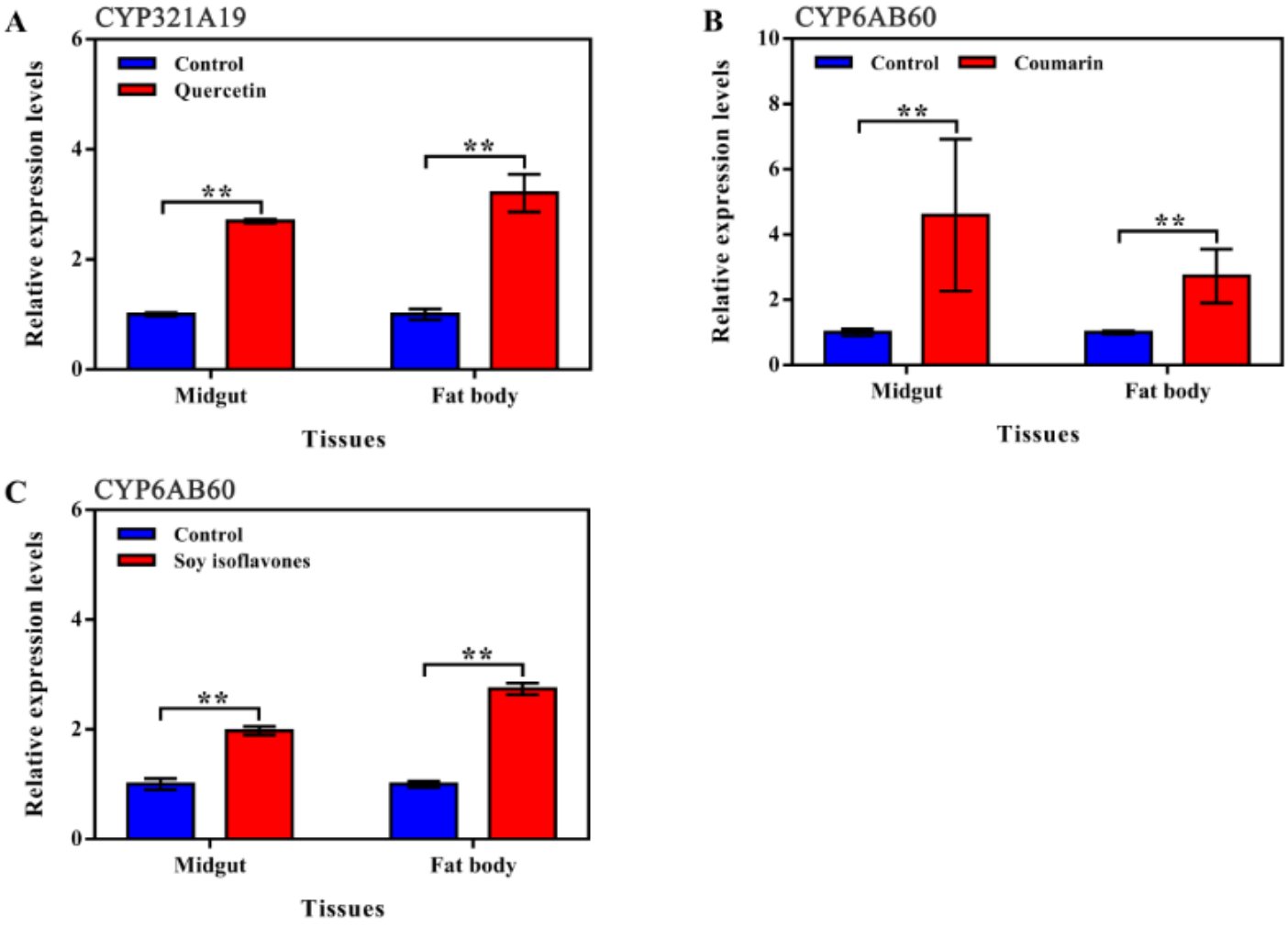

Figure 3

Expression levels of CYP321A19 sequences when exposed to quercetin (A), and CYP6AB60 sequences exposed to coumarin (B), soy isoflavones (C). Data shown as means ± SE derived from three biological replicates (Student's t-test, ** $p < 0.01$, * $p < 0.05$, same as below).

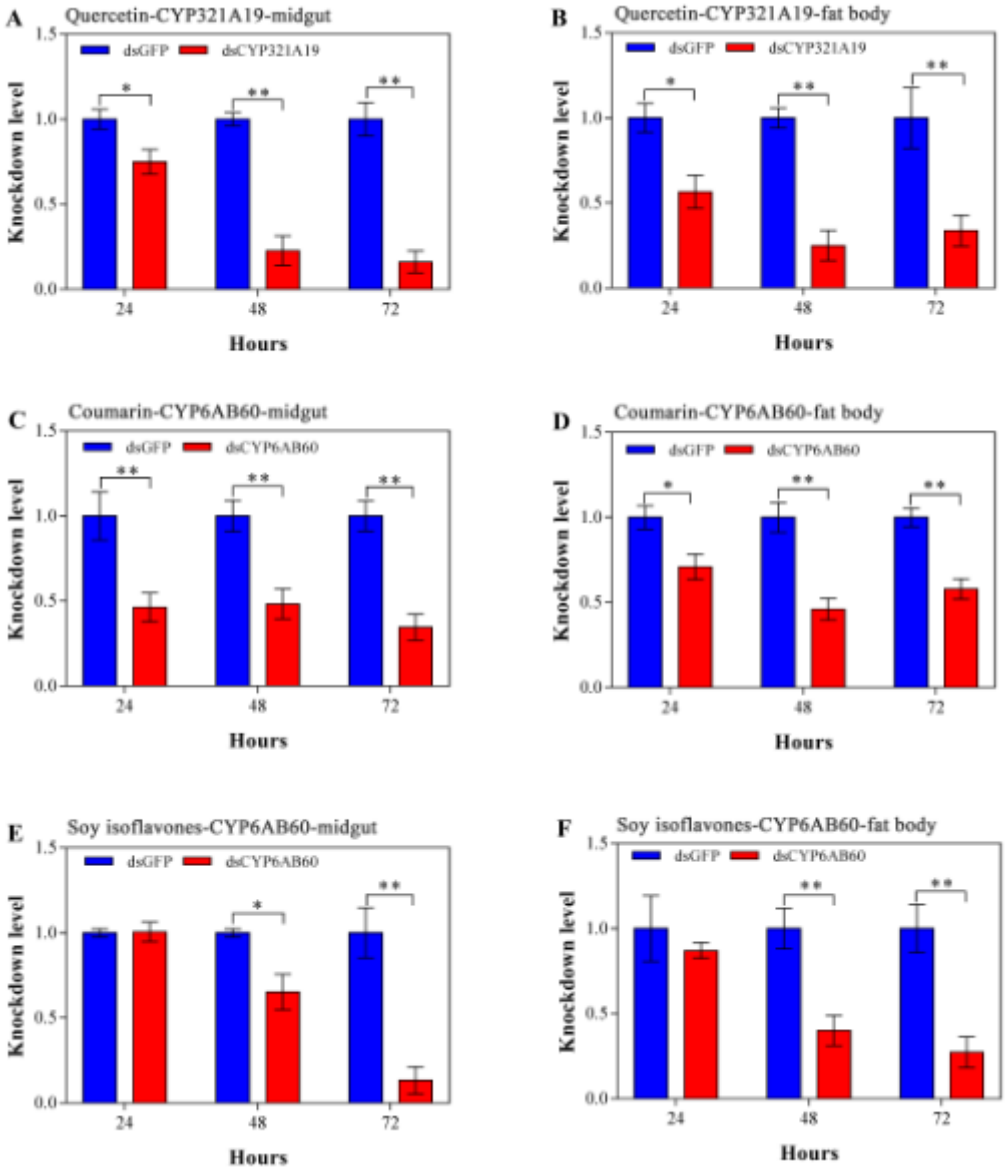

Figure 4

Effect of CYP321A19 and CYP6AB60 silencing on S. litura resistance to allelochemicals. Knockdown reduction rates of CYP321A19 in midgut (A) and fat body (B) after injection of dsRNA. Relative expression of CYP6AB60 in the midgut and fat bodies of larvae exposed to coumarin (C, D) and soy isoflavones (E, F) after dsRNA injection. Control larvae were injected with dsGFP.

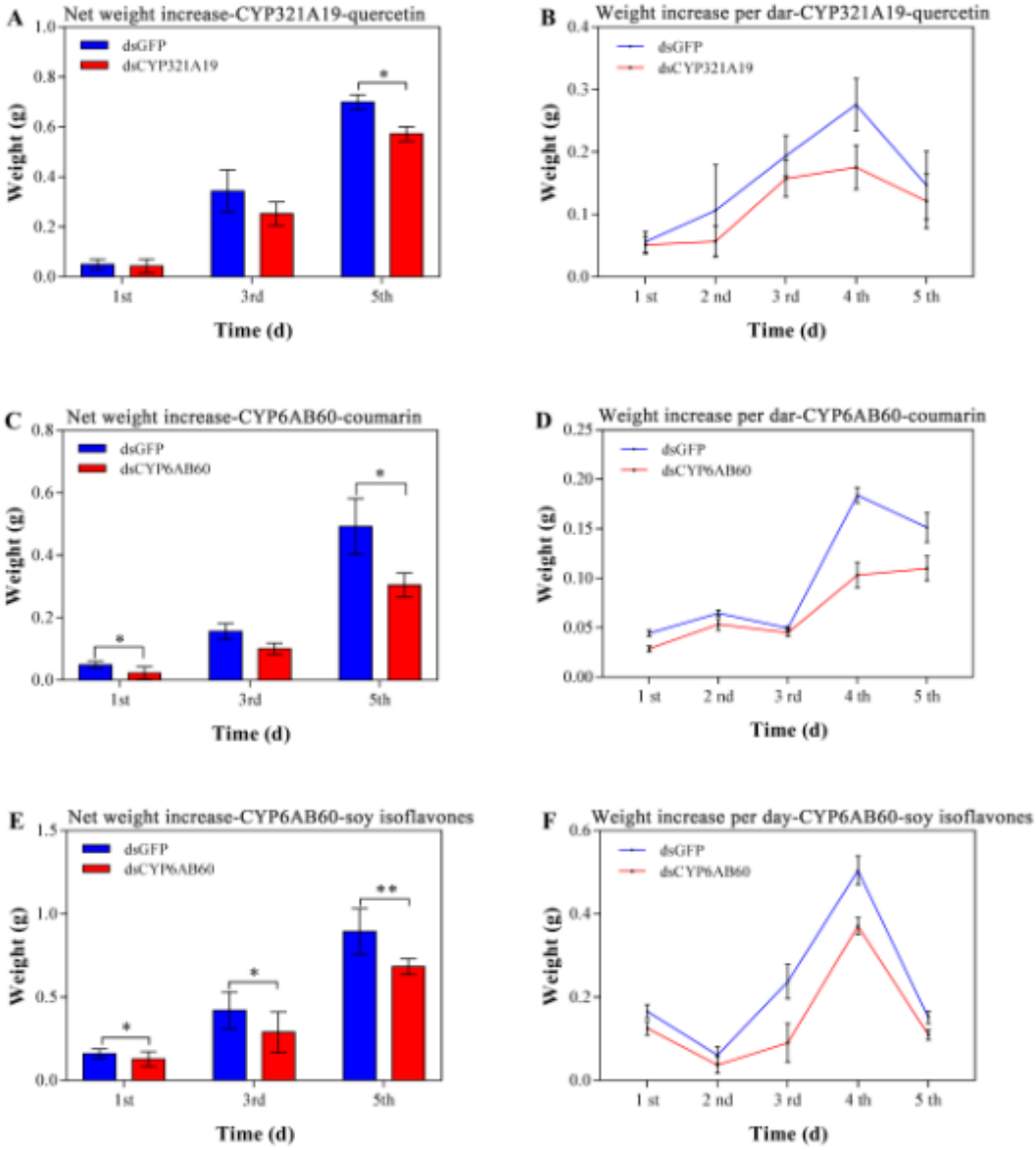

Figure 5

Changes in larval body weight of S. litura after dsRNA injection. After dsCYP321A19-injected larva, the net weight increased (A) and the weight increased per day (B) after feeding with quercetin. After dsCYP6AB60-injected larva, the net weight increased (A) and the weight increased per day (B) after feeding with coumarin (C, D) and soy isoflavones (E, F).

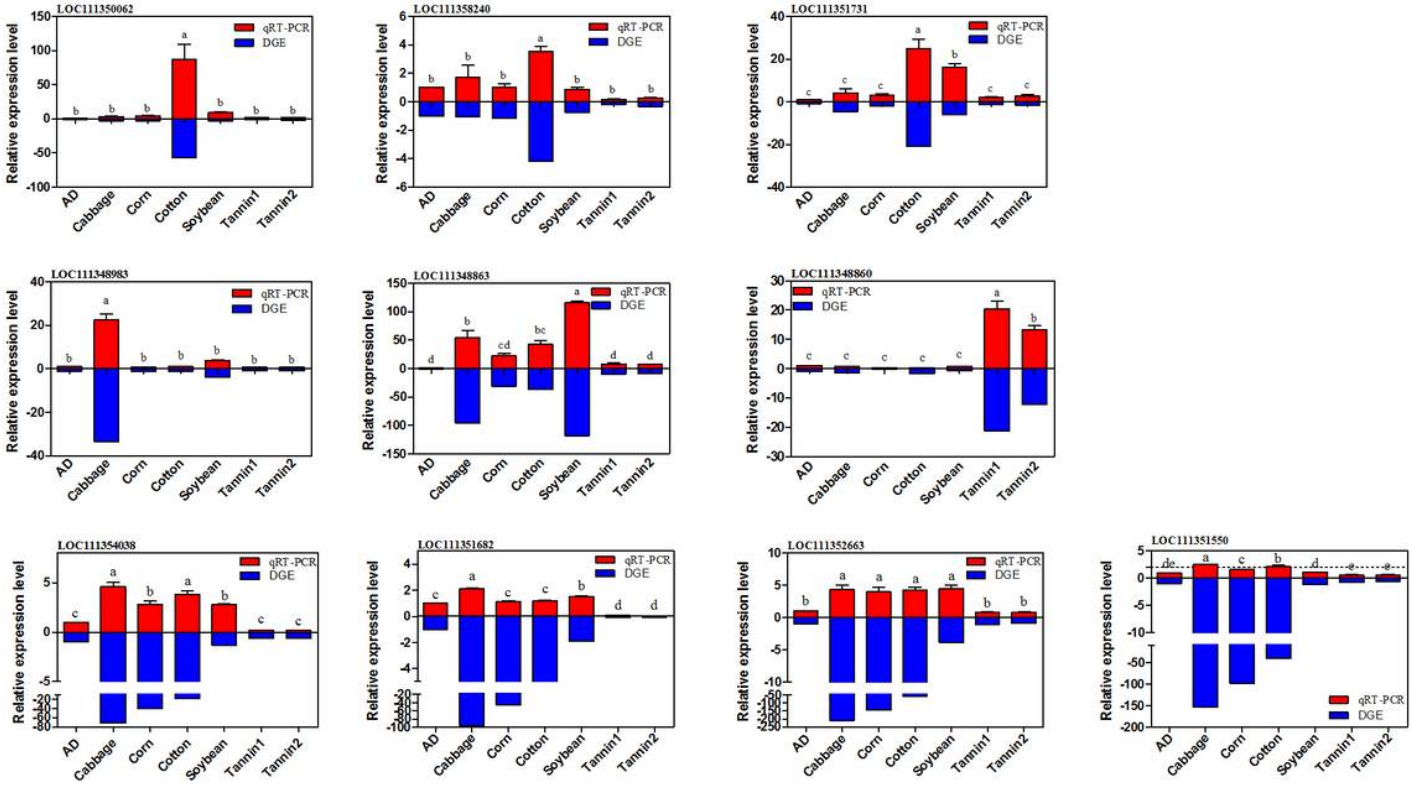

Figure 6

Verification of P450 (3), UDP-glucosyltransferase (3) and GST (4) genes in tissue of Spodoptera litura midgut. Gene expression analysis of 10 genes for which DGE data were available. Blue, DGE data; red, qRT-PCR results.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.xls
- Supplementfigure3.TIF
- Supplementfigure2.TIF
- Supplementfigure1.TIF
- Supplementfigure4.TIF
- Supplementfigure5.TIF