

The detoxification role of P450 genes implicated in adaptation of *Helicoverpa armigera* to walnut phytochemistry

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Abstract

In northwest China, changes in cultivation patterns and the scarcity of preferred hosts have forced *Helicoverpa armigera* to feed on the marginal host walnut (*Juglans regia*). However, the mechanisms allowing this adaptation remain poorly understood. Here, we investigated the behavioral, physiological, and molecular mechanisms underlying the local adaptation of this pest to walnut fruits. Compared to the kernel, the green husk and shell generally contained higher levels of phytochemicals and had greater impact on larval feeding preference, fitness, and cytochrome P450 activity. These negative effects were further supported by a larger number of upregulated detoxification genes in insects fed green husks or shells based on transcriptome sequencing. Together, these data suggest that P450 genes (such as LOC110371778) may be crucial to *H. armigera* adaptation to the phytochemicals of walnuts. Our findings provide significant insight into the adaptation of cotton bollworm to walnut, an alternative host of lower quality.

Key message

- *Helicoverpa armigera* is a novel host on walnut (*Juglans regia*) in northwest China
- The green husk and shell of the walnut exhibited higher phytochemical contents compared to the nutrient-rich kernel, which adversely affected feeding preferences, growth, and development
- P450 enzyme activities and P450 these genes might play important roles in *H. armigera* adaptation to walnut fruits phytochemicals.

Introduction

The cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), is a polyphagous agricultural pest that feeds on a wide range of host plants, including cotton, chickpea, tomato, tobacco, corn, sesame, hemp, sunflower, peanut, okra and soybean (Fitt 1989; Smith 1992; Naseri et al. 2010; Hemati et al. 2012; Cunningham and Zalucki 2014; Tay et al. 2017), causing serious economic losses to agriculture worldwide (Wu et al. 2017; Yang et al. 2022). Generalist herbivores such as *H. armigera* must continually shift to new, alternative, and nutritionally challenging host plants as availability or quality of other hosts declines. In China, *H. armigera* has been reported to shift onto some marginal hosts when preferred host plants become scarce either due to seasonality or changes in cultivation or land use patterns. The increased cultivation of high-value horticultural crops in areas formerly dominated by cotton production has led to greater exposure of some marginal hosts to *H. armigera* feeding (Yang et al. 2022). Southern Xinjiang Uygur Autonomous Region, China has an arid or semi-arid climate that is ideal for cultivating high-quality fruit of various species, including walnut (*Juglans regia*), jujube (*Ziziphus jujube*), apple (*Malus pumila*), grape (*Vitis vinifera*), pear (*Pyrus sinkiangensis*), and apricot (*Prunus armeniaca*). Recent changes in agricultural production in the region and advancements in farming technology have led to an increased level of exposure of these fruits to *H. armigera*. For example, since 2017, damage to walnuts by first-generation *H. armigera* has been reported in Aksu, in southern Xinjiang, resulting in significant

economic losses for the local walnut industry (Li et al. 2017; Liu et al. 2022). However, the mechanisms underlying the local adaptation of cotton bollworm larvae to walnut fruits are unclear.

Polyphagous generalists such as *H. armigera* feed on a wide range of host plants (Ruan and Wu 2001; Schellhorn 2008; Razmjou et al. 2014), and in doing so larvae encounter a wide range of plant allelochemicals, against which herbivorous insects have developed various mechanisms to overcome plant defenses (Heckel et al. 2014; Heidel and Vogel 2015; Petschenka and Agrawal 2016), including behavioral, physiological, and transcriptional changes that promote better detoxification of defense compounds (Sarfraz et al. 2006; Hemati et al. 2013; Ntalli et al. 2019; Magalhaes et al. 2020; Maurya et al. 2020; Wari et al. 2022). Metabolic detoxification of such defensive compounds is commonly categorized into three phases (Jin et al. 2019; Li et al. 2023). In Phase I, enzymes such as cytochrome P450 monooxygenases and carboxylesterases (CarE) act upon the toxin molecule, enhancing its hydrophilicity and the reactivity of its functional groups. In the second stage, Phase II enzymes, including UDP-glycosyltransferases (UGTs) and glutathione S-transferases (GSTs), facilitate the conjugation of endogenous molecules with toxins. Also, other transferases such as methyltransferases, acetyltransferases, phosphotransferases, and sulfotransferases contribute to this conjugation process (Robertson et al. 1999; Crava et al. 2016). The resulting conjugates are more hydrophilic and less reactive, diminishing their ability to pass through membranes. Finally, in Phase III, ATP-binding cassette (ABC) transporters bolster the transmembrane transport of toxins, thereby aiding their excretion. Many studies have demonstrated that in *H. armigera* key detoxification enzymes, including cytochromes P450 (P450), are involved in the detoxification of phytochemicals, helping this pest to adapt to diverse toxins produced by host plants (Shi et al. 2014; Jin et al. 2019). For example, CYP6AE14 is involved in the adaptation of cotton bollworms to gossypol, and CYP303A1 plays a role in metabolism of the plant allelochemical 2-tridecanone (Shi et al. 2014).

In recent years, with rapid development of next-generation sequencing technology, an increasing number of studies have employed RNA-seq to explore adaptive changes in insects in response to different host plants or xenobiotic phytochemicals (Heiko et al. 2014; Jeckel et al. 2022). For example, transcriptomic analysis was used to determine the mechanism of adaptation for the maize pest *Diabrotica virgifera* to different host plants. The study found that the larvae of this rootworm underwent fewer transcriptional changes when feeding on an alternate host plant than when feeding on a marginal or poor host (Coates et al. 2023). Transcriptome profiling of larvae of the weevil *Pagiophloeus tsushimanus* exposed to the plant-derived volatile compound linalool showed that linalool exposure induced the expression of genes encoding for P450s, cuticular proteins, antioxidases, heat shock proteins, and digestive enzymes (Li et al. 2023). Moreover, a review of studies that used transcriptomic analyses to investigate how genome-wide transcriptional changes contributed to adaptation over time to novel or poor-quality host plants found that during that process (Birnbaum et al. 2020), many genes involved in detoxification (P450s, CarE, UGTs, GSTs, ABC transporters), digestion, chemosensory perception, and transcription factors were dynamically expressed. For instance, transcriptome profiling of two tobacco-adapted species of the whiteflies *Trialeurodes vaporariorum* and *Bemisia tabaci* found that CYP6DP1 and CYP6DP2 (in *T.*

vaporariorum) and CYP6DP3 (in *B. tabaci*) played important roles in strong induced tolerance to the toxic allelochemical nicotine (Pym et al. 2024).

In the present study, we investigated for the first time the effects of walnut fruits (green husk, shell, or kernel fractions) on feeding preferences and fitness costs of *S. frugiperda*. Then, the detoxification activities of P450 and carboxylesterase enzymes in *H. armigera* larvae fed on the above three fractions of walnut fruits were also assessed. Finally, the molecular mechanisms underlying adaptation and detoxification of cotton bollworm larvae to these three walnut fruit fractions were examined using RNA-seq and confirmed by qPCR analysis. This study was designed to develop novel strategies for sustainable management of *H. armigera* in walnut orchards and improve understanding of the interactions between polyphagous pests and their host plants.

Materials and Methods

Plant and Insect Sources. Walnut fruits of *J. regia* cv. Wen 185 were collected from the Xinjiang Experiment Station of the Chinese Academy of Agricultural Sciences, Aksu, Xinjiang, China (80.34°E, 41.12°N). The adult of *Helicoverpa armigera* collected from the Xinjiang Experiment Station were paired for mating, the resulting offspring larvae were subsequently reared on an artificial diet under controlled conditions ($25 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH, and a 16:8 L:D photoperiod) (Liang et al. 2008).

For Total Tannin Content Determination. 2 g samples were extracted with 60% acetone, and measured using the Folin-Ciocalteu method as described previously (Nyero et al. 2023), with some modifications. The absorbance was measured at 725 nm. The total alkaloids assay was performed with berberine as a reference standard, as reported previously (Liu et al. 2022). Total polyphenols were measured by Folin-Ciocalteu method as previously reported (Złotek et al. 2014), with some modifications. Briefly, 1 g samples were extracted by 60% ethanol. The absorbance was measured at 778 nm. The gallic acid solution was used as a standard. Total flavonoid content was determined using aluminum chloride reagent as described previously (Yang et al. 2023). The absorbance was measured at 415nm. The rutin standard solution was used as a reference standard. Monomeric phenolics including gallic acid, chlorogenic acid and p-hydroxybenzoic acid (PHBA) were measured on an Agilent 1220 Infinity II gradient liquid chromatography system equipped with an Agilent ZORBAX SB-C18 column. A total of 2.0 g of sample was extracted by 75% methanol. Solvent A: 0.1% formic acid; solvent B: acetonitrile. Gradient elution was as follows: 0–5 min, 5–20%; 5–20 min, 20–100%; 20–21 min, 100–5%; 21–30 min, 5%. The flow rate was 0.5 mL/min. An injection volume of 10 μL was used. These metabolites were verified by the synthesized standards.

Feeding Preferences of Larvae on Walnut Fruit Fractions. Walnut fruit fractions (green husk, shell, and kernel) were cut into 2 mm thick portions. The area of each of the three parts was recorded before and after a 24 h period of 3rd instar feeding to determine the relative preference of larvae among parts. This experiment was replicated three times, with 30 larvae per replicate (90 per treatment). Experimental conditions were $25 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ RH, and a 16:8 L:D photoperiod.

Determination of Fresh and Dry Weights of Test Larvae. To establish breeding protocols for the cotton bollworm, we selected pre-molt 5th instars (excluding ones preparing to pupate) and placed each larva individually in sterile vials. Each larva was fed either green husk, shell, or kernel tissue, with an artificial diet serving as the control. After 48 hours, we recorded the fresh weights of 30 larvae from each treatment. The larvae were subsequently dried at 80°C until at constant weight to determine their dry weights. The water content of the larvae was calculated by subtracting the dry weight from the fresh weight (Jia et al. 2012).

Growth and Development, Pupal Weight, and Detoxification Enzyme Activities. Growth and development of *H. armigera* larvae were evaluated by providing them with green husk, shell, or kernel diets. Initially, neonate larvae were supplied with a specialized artificial diet tailored for the nutritional requirements of cotton bollworms. Upon entering the second instar, the larvae were placed individually in tubes and provided with one of the three walnut fruit fraction. There were 30 larvae in each treatment, and the whole experiment was replicated three times, for 90 larvae per treatment. The experimental conditions were $25 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH, and a 16:8 L:D photoperiod. Fresh walnut material was supplied daily, and both the survival and developmental stages of the larvae were recorded daily. Pupal weights were measured upon larval pupation.

To assess detoxification enzymes' activities, *H. armigera* larvae were reared to the 3rd instar on an artificial diet. At the conclusion of this stage, larvae were fed green husk, shell, or kernel as treatments and artificial diet as the control. We then examined the impact of these treatments 24 and 48 hrs after the exposure to diets by measuring levels of the primary detoxification enzymes. Larvae were collected at random within treatments, and whole-body tissue was collected. Twenty individuals per replicate per treatment (60 per treatment) were dissected on ice. The whole experiment was repeat three times. So, there were 60 larvae total per treatment. Protein concentrations were measured using the TP Assay Kit (A045-2) sourced from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). The activities of two key detoxification enzymes – carboxylesterase (CarE) and cytochrome P450 (P450) – were determined using the Carboxylesterase Assay Kit (A133-1-1) and the Cytochrome P450 Assay Kit (H303-1-2), respectively, adhering to the protocols provided by the Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

Total RNA Extraction and Transcriptome Sequencing. Larvae of consistent size at the end of the 3rd instar were selected and fed different walnut fruits fractions (green husk, shell, and kernel). These larvae were then collected 24 or 48 hrs after the start of the treatment. Control larvae were reared on artificial diet. Each treatment contained 30 larvae and the whole experiment was replicated three times (So, 90 [3 x 30] larvae were tested for each treatment). Subsequently, total RNA from the sampled larvae was isolated using the TRIzol reagent (Invitrogen Life Technologies, CA, USA) and subjected to RNA sequencing analysis. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). For cDNA library preparation, 3 µg of total RNA per sample was used as input material with the NEBNext Ultra II RNA Library Prep Kit for Illumina according to the manufacturer's instructions. Sample sequencing was conducted on the Illumina NovaSeq 6000 platform

(Personal Biotechnology Co., Ltd., Shanghai, China). Clean reads were aligned to the *H. armigera* reference genome available at NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_023701775.1/) using HISAT2 (v2.1.0). Genes with $|\log_2\text{FoldChange}| > 1$ and $P < 0.05$ were identified as differentially expressed genes (DEGs). GO enrichment analysis and KEGG pathway enrichment analyses were performed using the topGO (v2.50.0) and ClusterProfiler (v4.6.0) packages, respectively.

qPCR Validation. Total RNA used for transcriptome sequencing was reverse transcribed into cDNA using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa). The qPCR validation was performed using AceQ® qPCR SYBR® Green Master Mix kit (Vazyme Biotech, Nanjing, China) following the manufacturer's protocols (Huang et al. 2023). The qPCR amplification conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The actin gene of cotton bollworm was used as a candidate reference gene (Su et al. 2023). All primers used for qPCR are listed in Table S2.

Statistical Analysis. Principal component analysis (PCA) was used to indicate the distribution of secondary metabolites in various tissues of the walnut fruit- specifically, the green husk, shell, and kernel. PCA was performed by using “vegan” package (Oksanen et al. 2022) in R 4.2.1 software (R Development Core Team 2022). All values shown are means $\pm$ SE (standard errors). Significant differences for feeding preference, fresh weight, dry weigh, growth, development, pupal weight, and detoxification enzyme activities between larvae fed walnut fruit fraction and the artificial diet were analyzed using one-way ANOVA, followed by Tukey's tests for mean separation. Student's t test was applied to compare the survival rate between treatment and control groups. Statistical difference was considered significant at $P < 0.05$.

Results

Secondary Metabolites Content in Walnut Fruit. Principal component analysis (PCA) indicated the variation of secondary metabolites content in different walnut fruit tissues. The first two principal components explained 67.1% and 32.5% of the variance, respectively, with 99.6% cumulative percentage of explained total variances. Tannin, total polyphenol, total alkaloids and PHBA had high contributions to the PC1 axis, whereas gallic acid and total flavone had high contributions to the PC2 axis. For the three parts of walnut fruit (green husk, shell, and kernel), the contents of tannin, total polyphenols, and chlorogenic acid were highest in the shell, while the contents of total flavones and gallic acid were highest in the green husk. Generally, the green husk and shell contained higher levels of phytochemicals compared to the kernel (Fig. 1, Table S1).

Feeding Preferences of Larvae on Walnut Fruit Fractions. The cotton bollworm larvae demonstrated a strong preference for the kernel over the green husk or shell of the walnut fruit. At 24 hours after the onset of feeding, the consumed area of the kernel was 1.06 cm², but only 0.10 cm² and 0 cm² for shell and green husk. Consumption of kernel was significantly greater than shell or green husk ($F = 1259.0$; $df = 2, 6$; $P < 0.001$) (Fig. 2).

Development of Larvae on Walnut Fruit Fractions. Larvae fed walnut fruit fractions had developmental delays, higher mortality rates, and decreased pupal weight compared to the control group reared on artificial diets (Fig. 3). Both green husk and the shell treatments led to longer developmental periods ($F = 543.0$, $df = 3, 8$, $P < 0.001$), significantly higher mortality rates ($F = 154.0$, $df = 3, 8$, $P < 0.001$), and lighter pupal weight ($F = 317.4$, $df = 3, 8$, $P < 0.001$) compared to the kernel treatment. The green husk treatment resulted in the longest developmental period (41.5 days from the 2nd to the 6th instar, the highest mortality rate (37.8%), and the lightest pupal weight (0.08 g) ($F = 317.4$, $df = 3, 8$, $P < 0.001$).

Larval Fresh and Dry Weights. Healthy 5th instar were chosen to be fed on different walnut fruit fractions for 48 hrs, after which their fresh and dry weights were recorded (Fig. 4A). Larvae fed green husks had an average fresh weight of 0.092 g, while those consuming the shell or kernel weighed 0.144 g and 0.219 g, respectively. By comparison, larvae fed artificial feed weighed 0.284 g on average. Fresh weights of larvae fed green husks were significantly lower than all other treatments or the control ($F = 382.0$; $df = 3, 116$, $P < 0.001$).

Average dry weight (Fig. 4B) was lowest for larvae fed green husks (0.041 g), compared to 0.067 and 0.089 g for larvae fed shell or kernel, respectively. Larvae fed on the artificial diet had the highest average dry weight (0.152 g). Dry weight of larvae fed green husks was significantly lower than other treatments or the control ($F = 125.9$; $df = 3, 116$, $P < 0.001$).

The water content of larvae fed green husk was 5.0%, compared to 8.9% on shell, 13.0% on kernel, and 15.2% on artificial diet (Fig. 4C). Water content of larvae fed green husks was significantly lower than that of other treatments or the control ($F = 65.7$; $df = 3, 116$, $P < 0.001$).

Activities of Detoxification Enzymes among Treatments. Activities of three major detoxifying enzymes – cytochrome P450 (P450), carboxylesterase (CarE), and glutathione S-transferase (GST) (Fig. 5A) – in larvae in treatments were significantly different. After 24 hours of feeding, the P450 enzyme activity level was in larvae was 148.4 U/L for green husks, 80.5 U/L for shell, 103.1 U/L for kernel, and 83.8 U/L for artificial diet. Notably, P450 activity in larvae fed green husks was significantly greater than that in larvae fed other treatments or the control ($F = 25.1$, $df = 3, 8$, $P < 0.001$). After 48 hours of feeding, the P450 activity in larvae fed green husks and shell were both significantly higher than in larvae fed kernel (green husks: $F = 5.8$, $df = 3, 8$, $P = 0.014$; shell: $F = 6.7$, $df = 3, 8$; $P = 0.007$). However, there was no significant difference in P450 enzyme activity between larvae fed green husks versus shell ($F = 0.83$, $df = 3, 8$, $P = 0.9340$) (Fig. 5A).

The level of carboxylesterase (CarE) activity after feeding on green husks for 24 hours was 625.97 U/ml, compared to 490.28 U/ml on kernel and 435.19 U/ml on shell. The lowest activity was 232.17 U/ml in larvae fed artificial diet. The CarE activity in larvaer fed green husks was significantly higher than on shell ($F = 10.7$, $df = 3, 8$, $P < 0.001$), but was not significantly different from larvae fed on kernels ($F = 3.7$, $df = 3, 8$, $P = 0.1154$) (Fig. 5C). After 48 hours, CarE activities in larvae fed green husk and shell were both significantly higher than in larvae fed kernel (green husks: $F = 5.7$, $df = 3, 8$, $P = 0.015$; shell: $F = 7.6$, $df = 3, 8$, $P = 0.003$) (Fig. 5D).

The GST enzyme activity levels in larvae showed no consistent pattern among treatments at 24h or 48h (Fig. 5F).

Transcriptional Responses in Larvae among Treatments. To identify the molecular mechanisms of local adaptation of cotton bollworm to alternative host plants and to identify potential key genes and associated biological processes, transcriptomic changes in the larvae among treatments were monitored with RNA-seq analysis at 24 and 48 h of feeding. After 24 hrs, the number of DEGs in larvae fed green husks, shell, or kernel were 986 (623 up- and 363 downregulated), 1177 (760 up- and 417 downregulated), and 712 (380 up- and 332 downregulated), respectively (Fig. 6A). Of these, 62 DEGs were common across all treatments (Fig. 6B), while 730, 741, and 252 DEGs were specific to the green husk, shell, and kernel treatments, respectively. After 48 hours of feeding, 986 (623 up- and 363 downregulated), 1177 (760 up- and 417 downregulated), and 712 (380 up- and 332 downregulated) DEGs were identified in the green husk, shell, and kernel treatments, respectively (Fig. 6C). A Venn diagram showed that 36 DEGs were present in all three treatments (Fig. 6D), while 826, 188, and 355 DEGs were unique to the green husk, shell, and kernel treatments, respectively.

Expression Patterns of DEGs in Different Treatments. After 24 hours, a total of 2,268 genes were differentially expressed in at least one control versus treatment comparison, while 1,664 genes were differentially expressed at 48 hours. A k-means clustering analysis on expression patterns of these DEGs under different treatments showed distinct clusters. At 24 hours, three clusters (of 647 total genes) that exhibited the lowest expression levels were associated with the green husk treatment (clusters a, c and e). Similarly, three clusters (499 genes) that had the lowest expression levels were associated with the shell treatment (clusters b, g and h), and two clusters (210 genes) displayed the lowest expression level for the the kernel treatment (clusters d and f) (Fig. 7A). At 48 hours, 72 genes in one cluster that showed the lowest expression level were associated with the green husk treatment (cluster c), while 263 and 303 genes that showed the lowest expression were associated with the shell (clusters b and g) and kernel treatments (clusters a and e), respectively. In contrast, 89 genes that exhibited the highest expression were associated with the shell treatment (cluster f), and 50 genes with the highest level of expression were associated with the kernel treatment (cluster d) (Fig. 7B).

GO and KEGG Function Enrichment Analysis of DEGs. GO functional enrichment and KEGG pathway analyses revealed that DEGs were mainly enriched in the following biological processes: immune response, insect hormone biosynthesis, chitin synthesis and degradation, detoxification, and digestion (Fig. 8). At both 24 and 48 hrs, the treatments with green husk and shell diets generally induced more DEGs encoding detoxification enzymes and proteases (P450, carboxylesterase, glutathione S-transferase, UDP-glucosyltransferase and ABC transporter) and digestive enzymes (amylase, maltase, lipase, phospholipase, trypsin, chymotrypsin, carboxypeptidase, aminopeptidase and serine protease) compared to the kernel treatment (Fig. 8). Notably, the general induction of P450 in larvae in treatments with green husk, shell, or kernel at both time points highlights its importance and pivotal role in facilitating cotton bollworm adaptation to green walnut fruits. At the 24 hrs, 30 P450 genes, including 12 belong to the CYP4 family, 12 belong to the CYP6 family, and 3 belong to the CYP9 family, were

differentially expressed in at least one control versus treatment comparison, while 14 P450 showed differential expression at 48 hours, with 4 from the CYP4 family, 7 from the CYP6 family, and 1 from the CYP9 family (Fig. 9A). Green husk-exposed larvae exhibited the greatest number of differentially expressed P450s at both 24 hrs (17 genes) and 48 (8 genes) hrs. Eight P450 genes were selected and confirmed by subsequent qPCR, and their expression levels were highly consistent with those from RNA-seq, indicating the RNA-seq results are reliable (Fig. 9B).

Discussion

Helicoverpa armigera is a polyphagous pest known to damage several crops (Hemati et al. 2012; Petschenka and Agrawal 2016). While the literature on cotton bollworm is large, few reports mention their capacity to feed on walnuts. This study was started following field observations of cotton bollworm infestation in walnut orchards in the Xinjiang region of China. Its goal was to investigate the mechanisms by which cotton bollworm larvae were adapting to walnut fruit. In walnut production, the kernel, the edible part of the fruit, is primarily consumed for its nutritive value, while the by-products, particularly the green husk and shell, are widely used in traditional medicine and have received increasing interest in modern pharmacology due to their abundant phytochemicals and excellent antioxidant activities (Jahanban et al. 2019). In this study, the phytochemical contents of different parts of fresh walnut fruit (green husk, shell, and kernel) were firstly evaluated. Similar to previous publications (Jahanban et al. 2019), our findings revealed that the green husk and shell contained substantially higher quantities of phytochemical compounds compared to the nutrient-rich kernel (Fig. 1, Table S1).

We further provided cotton bollworm larvae with various parts of fresh walnut fruit (green husk, shell, and kernel) and monitored their feeding behavior and preference. Significant differences in preference for parts of the walnut fruit were noted: larvae showed the highest preference for the kernel, followed by the shell, and the green husk was the least acceptable (Fig. 2). Similar patterns of host plant-related behavioral differences have been reported in terms of development duration, survival, and reproductive rates when individuals (*Aphis spiraecola* and *Carposina sasakii*) were reared on different hosts (Tsai and Wang 2001; Kim and Lee 2002). In earlier work, cotton bollworm larvae have shown a stronger affinity for cotton leaves compared to those of tobacco, tomatoes, or peppers. Significant differences in average relative growth rates of larvae on young leaves from these four hosts were observed, and showed a descending order of cotton, tobacco, tomato, and pepper (Wari et al. 2022). In comparison to natural substrates such as okra, tomato, eggplant, pepper, and maize, cotton bollworms exhibit a pronounced preference for ovipositing on artificial diets, with maize being the least preferred option (Jallow et al. 2001; Jallow et al. 2003). In our present study, the average mortality rate of cotton bollworm larvae consuming green husk was 37.8%, followed by 26.7% for the shell, 7.8% for kernel, and only 1.1% for larvae fed on artificial diet. The developmental duration from the 2nd instar to the 6th instar was longest for larvae fed on green husks (41.5 days), followed by shell (25.0 days), and kernel (16.4 days), whereas only 11.2 days were needed on artificial feed. The time required for the cotton bollworm larvae to develop on green husks was significantly longer than on shell, kernel, or artificial diet. But there is no consistency in pupa weight, the cotton bollworm larvae to weight on green husks was significantly lighter

than on shell, kernel, or artificial diet. Shorter developmental times, higher total reproduction and higher pupal weight on a host plant are indicative of greater host suitability (Van Lenteren 1990). Our study similarly determined that larval development on walnut kernel was significantly shorter than on shell and green husk, with a resultant heavier pupal weight. Furthermore, larvae feeding on the green husk exhibited significantly lower average fresh and dry weights compared to those feeding on shell or kernel (Fig. 4). These findings suggest that cotton bollworm larvae are better adapted to walnut kernels than green husks or shell.

Changes in gene expression in *H. armigera* larvae in the process of adapting to walnut fruits were investigated using RNA-seq analysis and subsequent qPCR validation. Functional classification revealed that the DEGs identified in this process, compared to larvae feeding on diet, were primarily involved in processes essential for growth and development, such as immunity, hormone regulation, cuticle formation, detoxification and digestion. The green husk and shell treatments induced more upregulated genes than the kernel treatment (Figs. 6A and 6C). For example, DEGs responsible for chitin synthesis and degradation were found in the green husk and shell treatments, with almost all of these genes being upregulated; however, these DEGs were absent in the kernel treatment (Fig. 7). Many DEGs responsible for cuticle protein were also identified, with almost all showing upregulation across all three treatments at both time points. Previous studies have shown that several genes encoding cuticle proteins were also induced in the midgut and fat body of *Spodoptera litura* larvae that were exposed to a botanical quinoline alkaloid compound camptothecin (Shu et al. 2021a; Shu et al. 2021b) and in *P. tsushimanus* larvae exposed to the plant-derived, volatile compound linalool (Li et al. 2023).

P450 enzymes involved in the solubilization phase of detoxification play important roles in adaptation of herbivores to the detrimental secondary metabolites of novel host plants (i.e., phytochemicals) (Song et al. 2005; Zhang et al. 2016; Lu et al. 2021; Li et al. 2023). For example, the P450 gene CYP321A9 from *S. frugiperda* was found to be induced by toxic plant metabolites and to be involved in adaptation of the corn strain of *S. frugiperda* to the defensive chemicals in rice. Similarly, the expression of CYP321B1 was significantly upregulated in the midgut and fat body of *Spodoptera litura* larvae in response to tannin levels in host plants (Zhao et al. 2022). In *Mythimna separata*, RNA-seq analysis showed that seven P450 genes were significantly upregulated and the enzyme activity of CYP450 induced by different concentrations of chlorogenic acid, a common secondary metabolite in plants (Lin et al. 2022). Similarly, in *H. armigera*, several P450 genes have been found to be induced by plant allelochemicals, indicating their potential role in the detoxification of these compounds. Several examples include (1), CYP6AE14 induction in the midgut by the cotton metabolite gossypol (Mao et al. 2007), (2) CYP6B6, CYP315A1, CYP18A1 and CYP306A1 induction by 2-tridecanone in wild tomato (Liu et al. 2006; Zhang et al. 2016), (3) CYP6B2, CYP6B6, and CYP6B7 induction in the midgut and fat body by four allelochemicals in many plants (Chen et al. 2019); and (4) CYP321A1 induction by flavone in many plants (Zhang et al. 2023). In our study on walnut, the phytochemical-rich green husk and shell increased P450 enzyme activities at both time points (Fig. 5A and 5B). Also feeding by *H. armigera* larvae on green husks, shell, or kernel treatments all upregulated multiple DEGs encoding detoxification enzymes and proteases, especially several P450s (Fig. 7). Interestingly, a P450 gene (LOC110371778) from the CYP4 family was

upregulated in all three treatments at the 24-hour time point, suggesting it may play an important role in the detoxification of green walnut allelochemicals. Additionally, several UDP-glucosyltransferases and ABC transporters, involved in the conjugation and excretion phases of detoxification were also upregulated across all three treatments, with the green husk treatment showing the most significant gene upregulation.

In addition to detoxification enzymes, digestive enzymes also play a role in the adaptation of herbivorous insects to novel plants by degrading toxic metabolites (Simon et al. 2015; Jeckel et al. 2022). In our study, feeding *H. armigera* larvae with any of the three walnut fruit treatments, at both time points, changed the expression levels of digestive enzymes (Fig. 7). The number of DEGs for the green husk treatment (50 genes at 24 hours and 63 at 48 hours) and the shell treatment (40 at 24 hours and 36 at 48 hours) encoding digestive enzymes were elevated compared to the kernel treatment (28 at 24 hours and 48 at 48 hours) at both time points, suggesting the green husk and shell treatments had a stronger negative influence on digestion, which in turn would affect larval growth and development. Many studies have demonstrated that the digestive system of insect herbivores is key to adaptation to novel host plants during host shifts, and feeding insects new host plants with novel phytochemical can result in upregulation of many digestive genes (Roy et al. 2016; Shu et al. 2021a; Shu et al. 2021b; Jeckel et al. 2022; Li et al. 2023).

Conclusions and outlook

Crop diversity in the Aksu region of Xinjiang has increased the exposure of the alternate, marginal or poor-quality crops such as walnut to cotton bollworm *H. armigera*. In this study, we provide insights into the interaction between *H. armigera* and this alternate host. Different parts of the walnut fruit exert distinct effects on feeding preferences, larval growth and development, survival rates, and enzyme activity levels of larvae, with the phytochemical-rich green husk and shell having the greater negative impacts than the nutrient-rich kernel. RNA-seq analysis showed that feeding on different walnut parts strongly affected the expression of genes involved in detoxification, digestion, immunity, and cuticle formation. More P450 genes were upregulated in the green husk and shell treatments than in the kernel treatment. These findings improve our understanding of the adaptive mechanisms being used by *H. armigera* to feed on walnut fruits specifically, and, more generally, also contribute to our understanding of host adaptation in phytophagous insects to alternate, marginal, or poor-quality hosts.

Finally, considerably more work will need to be done to determine the molecular functions of the P450 candidate genes and other genes involved in detoxification metabolism and their co-expression relationship.

Declarations

Author Contributions

Yanhui Lu conceived the experiments. Haiqiang Li, Xinzheng Huang, and Yanhui Lu conducted the experiments and wrote the manuscript. Long Yang and Haining Liu helped to collect the population of *H. armigera*. Haiqiang Li, Xinzheng Huang, Long Yang, Bing Liu and Yanhui Lu analyzed the data. All authors corrected and approved the manuscript.

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Conflict of interests The authors declare no conflicts of interest.

Data Availability

Data is provided within the manuscript or supplementary information files.

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Figures

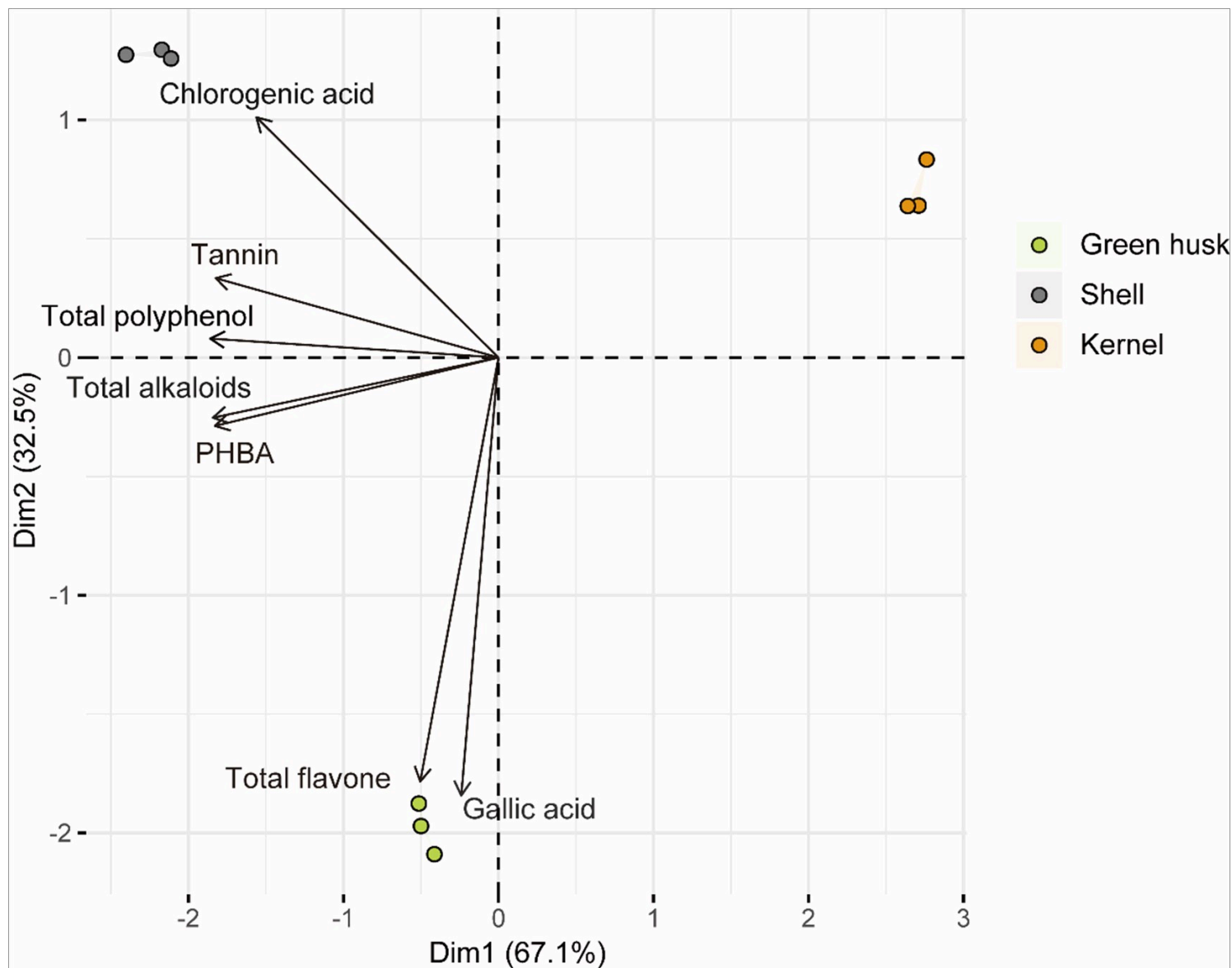


Figure 1

Biplot of principal component analysis (PCA) demonstrated the variation of seven secondary metabolites in the green husk, shell, and kernel of walnut fruit. The sample points with different colors indicated different position of walnut fruit- green husk (green), shell (gray), and kernel (orange),

respectively. Solid lines with arrows represent the secondary metabolites, the cosine of the angle between them and the PC axis indicated the correlation for they on the PC axes (smaller indicated higher correlation).

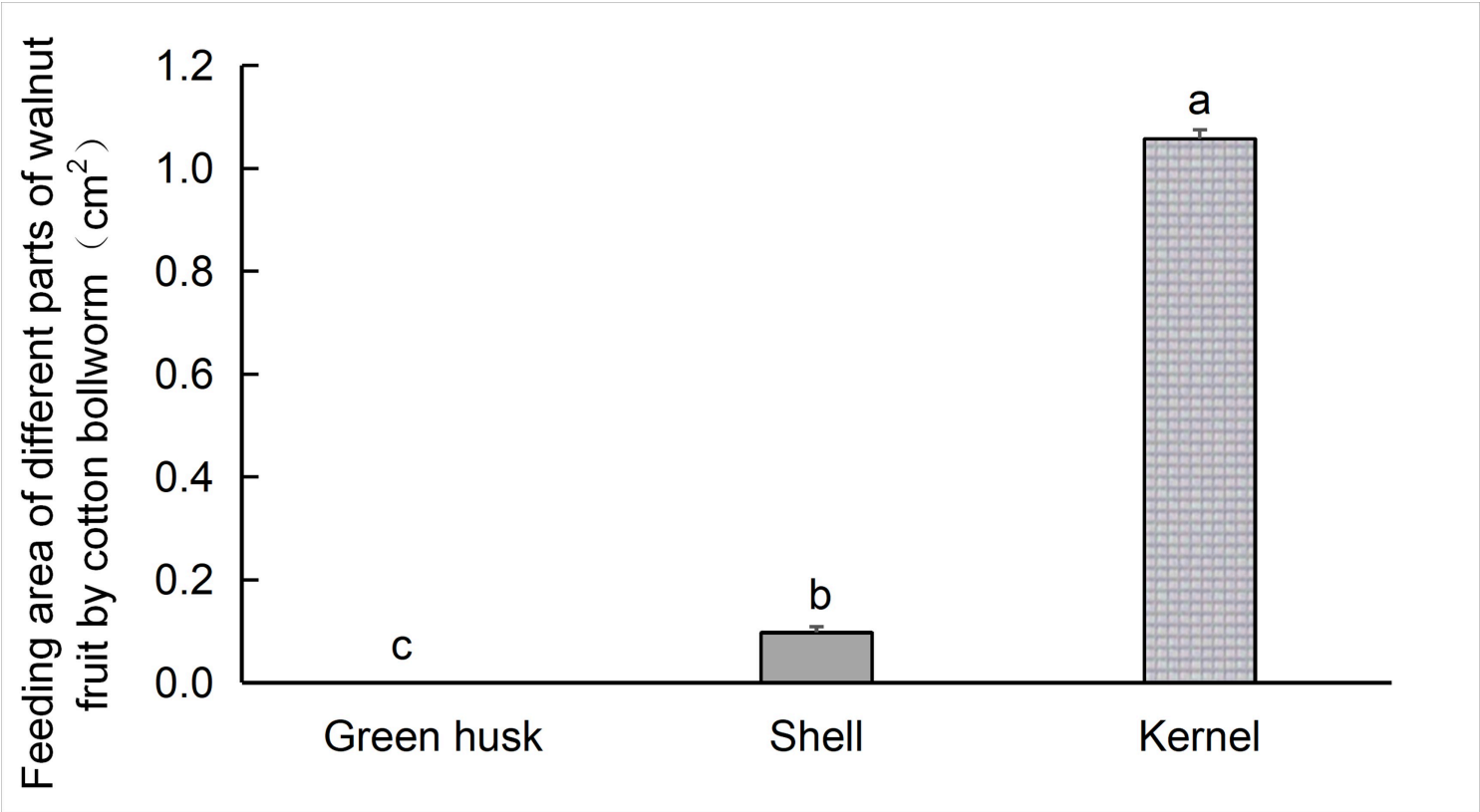


Figure 2

Feeding preferences of *Helicoverpa armigera* on different parts of walnut fruits (green husk shell, kernel) at 24 h. The different letters indicate significant differences analyzed by one way ANOVA followed by Tukey’s tests, $P < 0.05$.

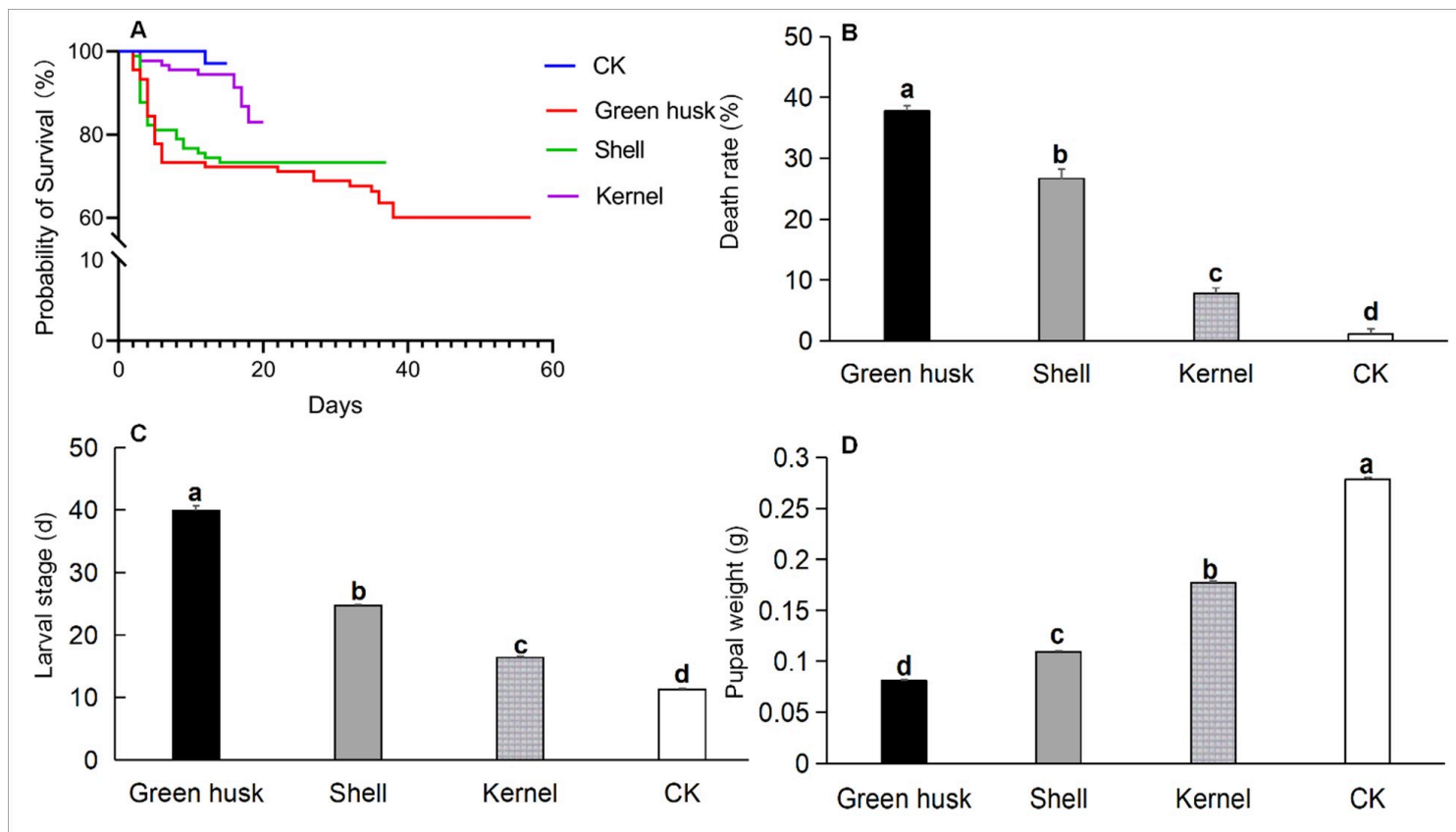


Figure 3

The survival rate (A), mortality rate (B), growth and development (C), and pupal weight (D) of *Helicoverpa armigera* fed on different parts of walnut fruits. The different letters indicate significant differences analyzed by one way ANOVA followed by Tukey's tests, $P < 0.05$.

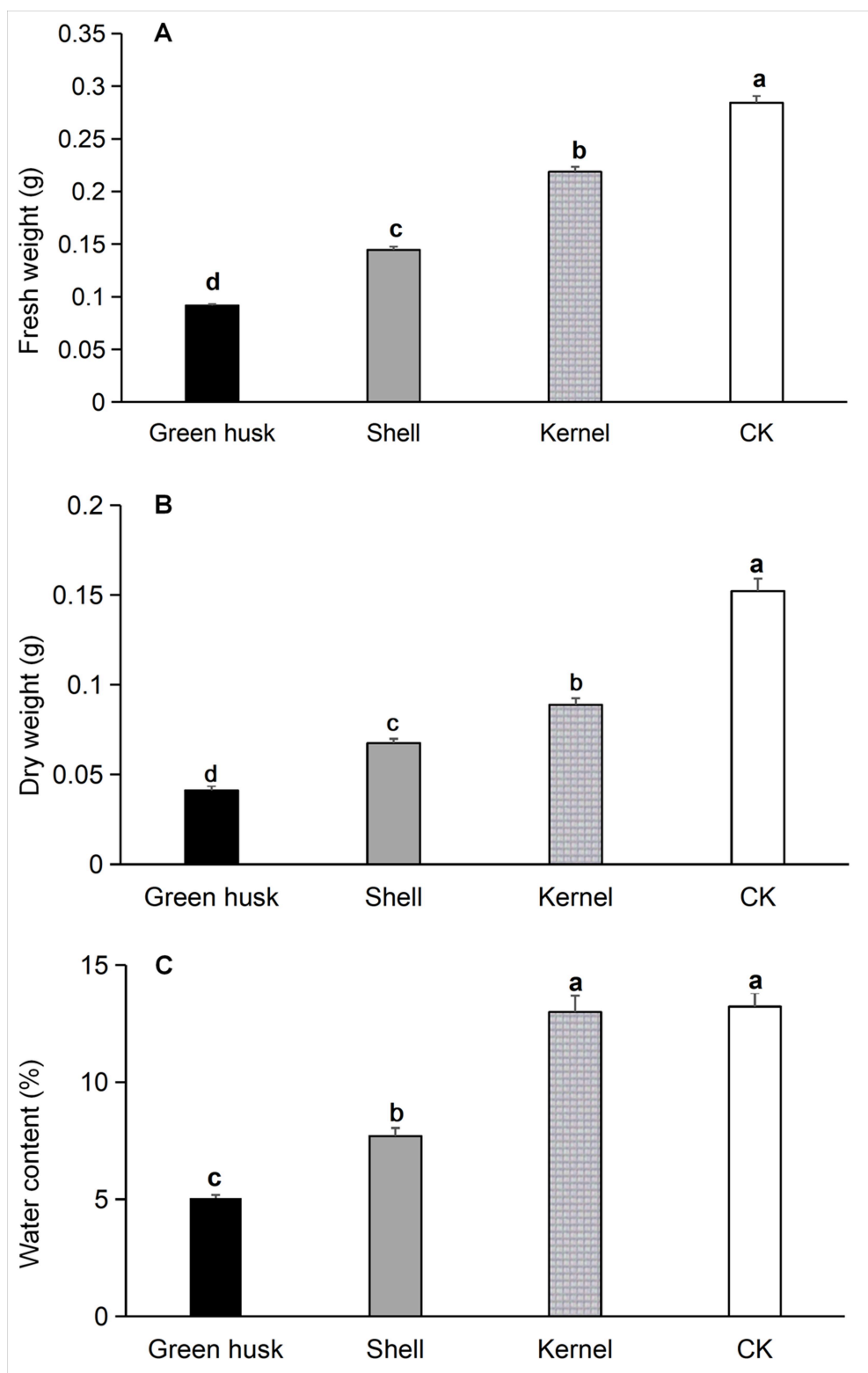


Figure 4

Fresh weight (A), dry weight (B), and water content (C) of *Helicoverpa armigera* fed on different parts of walnut fruits at 48 h. The different letters indicate significant differences analyzed by one way ANOVA followed by Tukey's tests, $P < 0.05$.

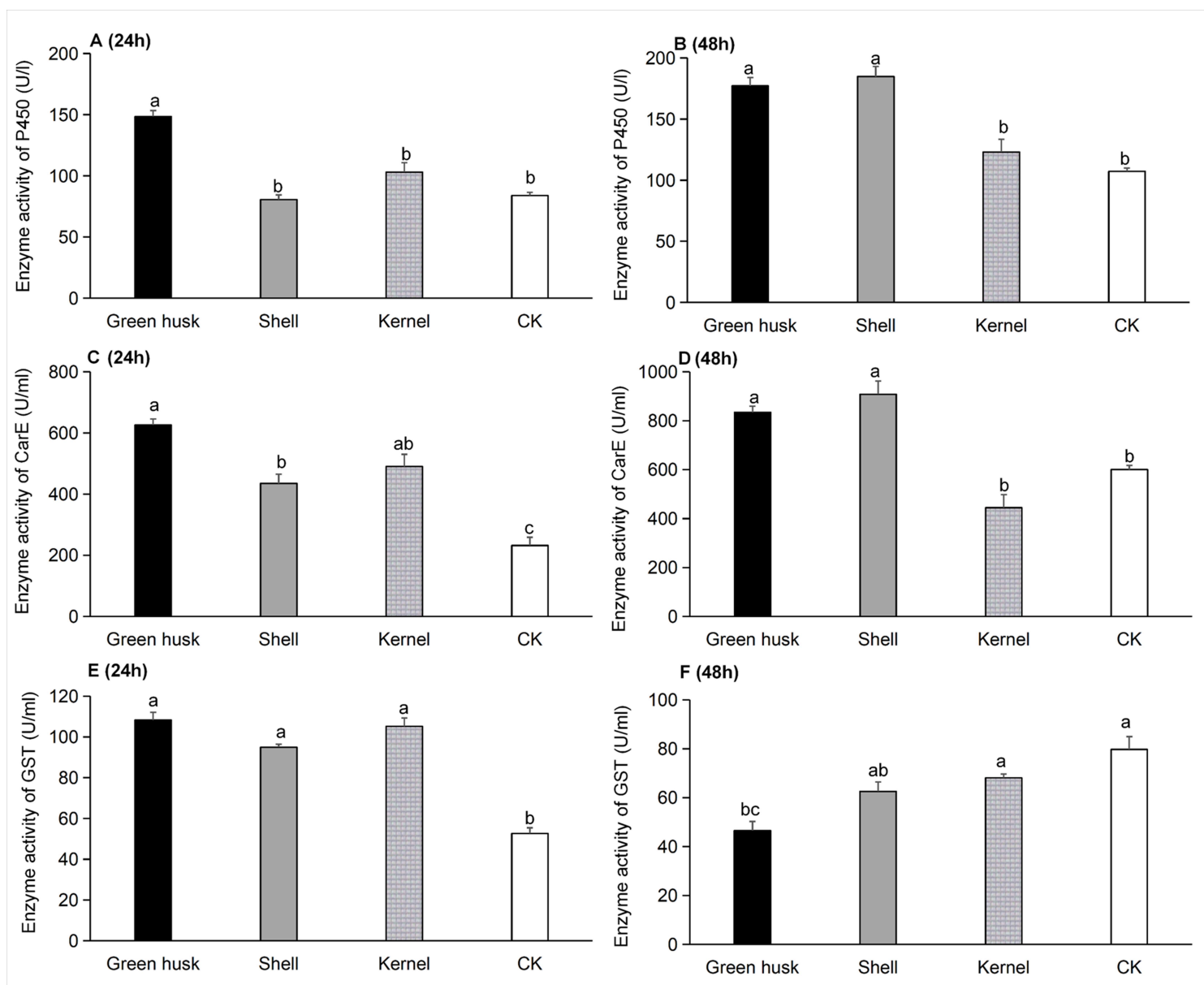


Figure 5

Detoxification enzyme activities of *Helicoverpa armigera* fed on different parts of walnut fruits at two different time points. The enzyme activity of P450 at 24 h (A) and 48 h (B), the activity of CarE at 24 h (C) and 48 h (D), and the activity of GST at 24 h (E) and 48h (F). The different letters indicate significant differences analyzed by one way ANOVA followed by Tukey's tests, $P < 0.05$.

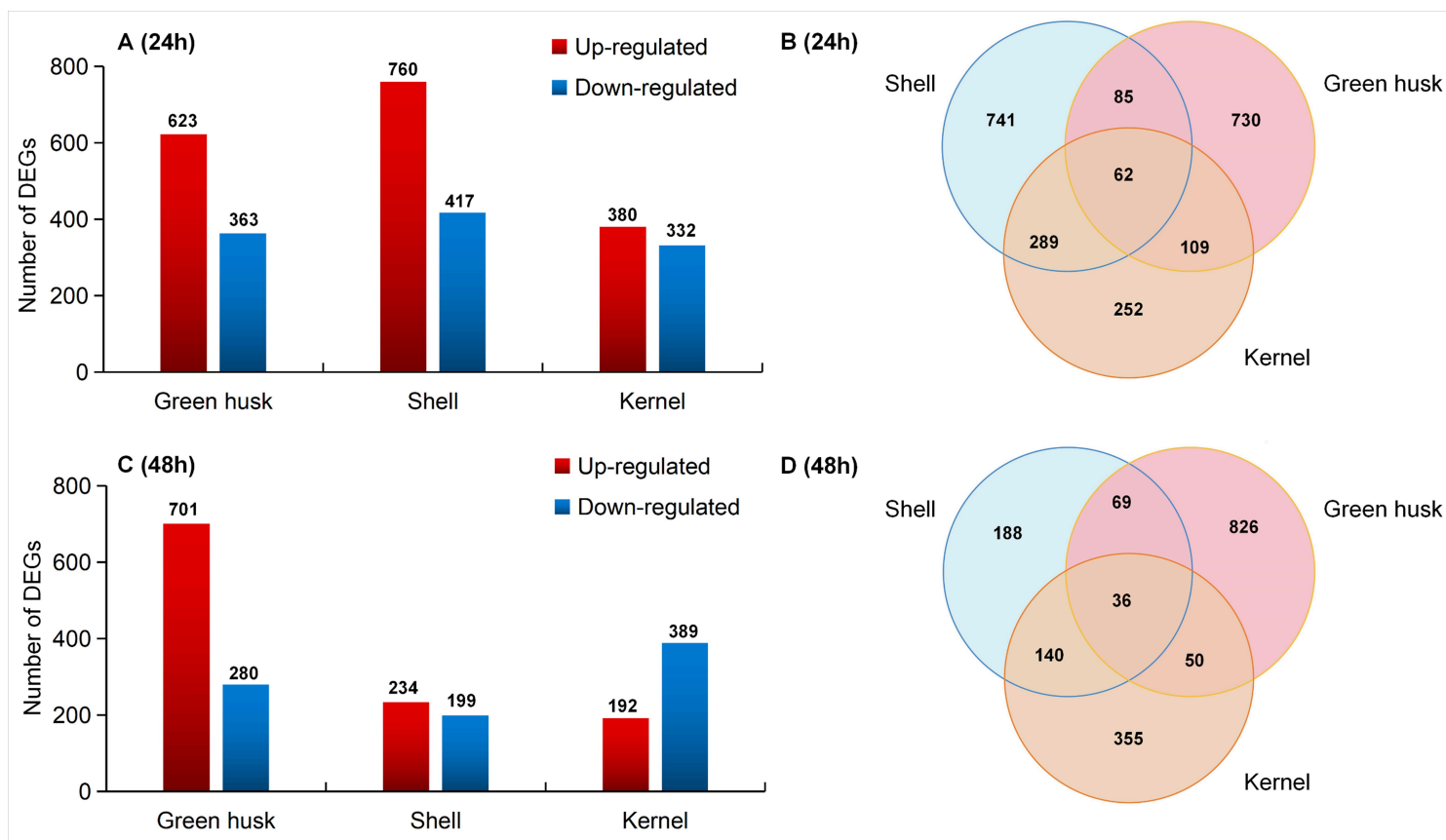


Figure 6

Differentially expressed genes (DEGs) in *Helicoverpa armigera* fed on different parts of walnut fruits at two different time points. Number of up- and downregulated DEGs (A) and Venn diagram of common and unique DEGs (B) at 24 h. Number of up- and downregulated DEGs (A) and Venn diagram of common and unique DEGs (B) at 48 h.

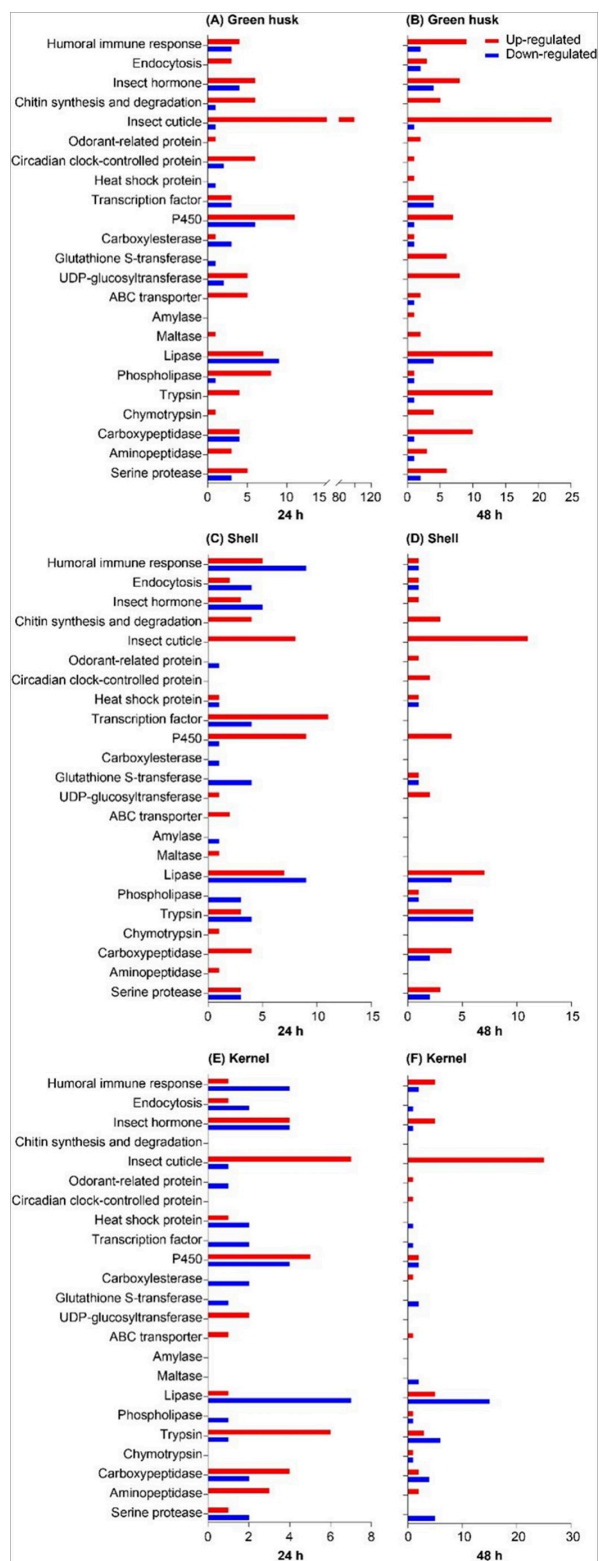


Figure 7

Functional classification of differentially expressed genes (DEGs) in the green husk at 24 h (A) and 48 h (B), in the shell at 24h (C) and 48 h (D) and in the kernel on 24 h (E) and 48 h (F).

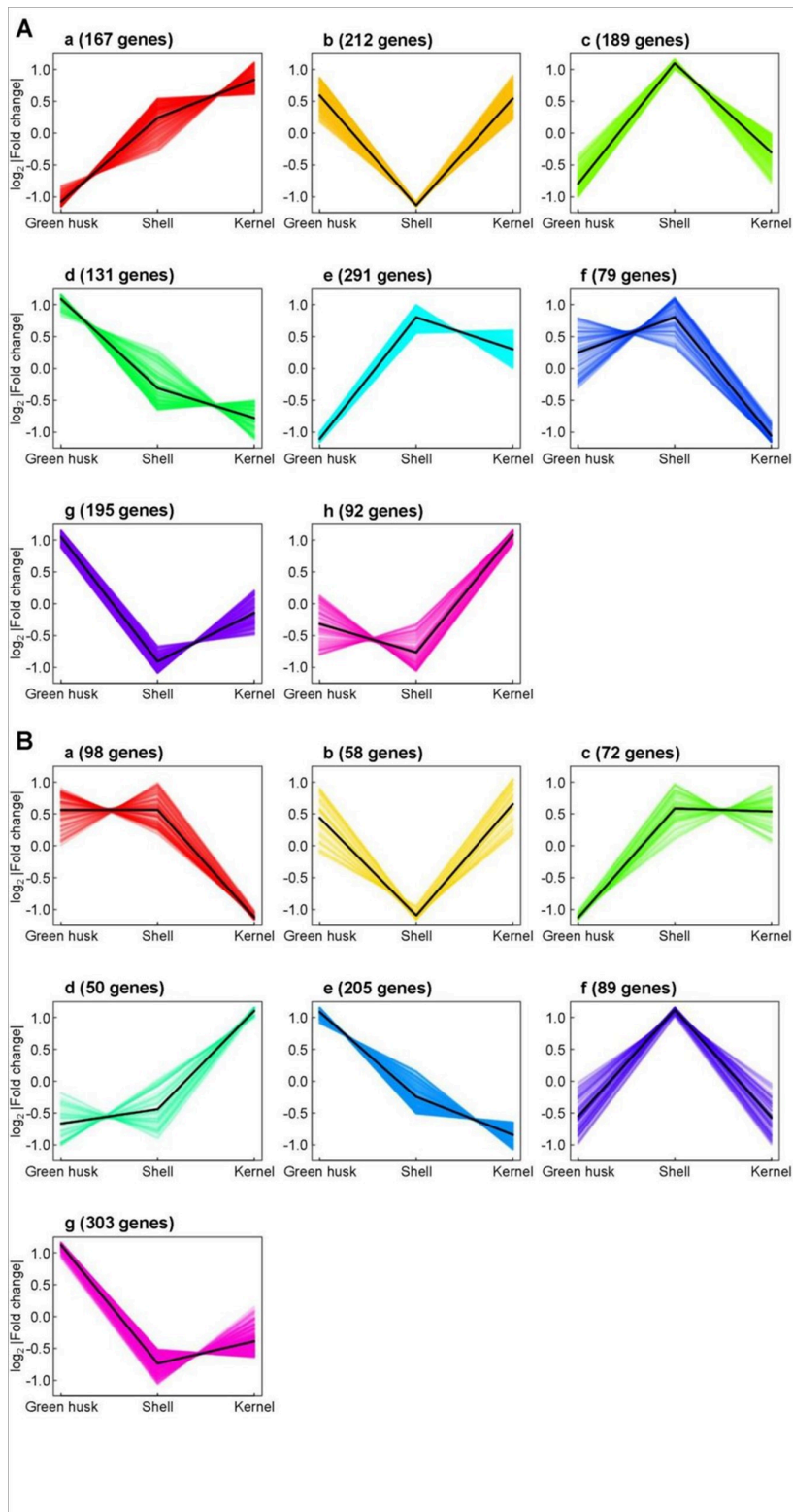


Figure 8

Expression profiles based on K-means with clustering of genes differentially expressed in at least one control-treatment comparison at 24 (A) and 48 hours (B).

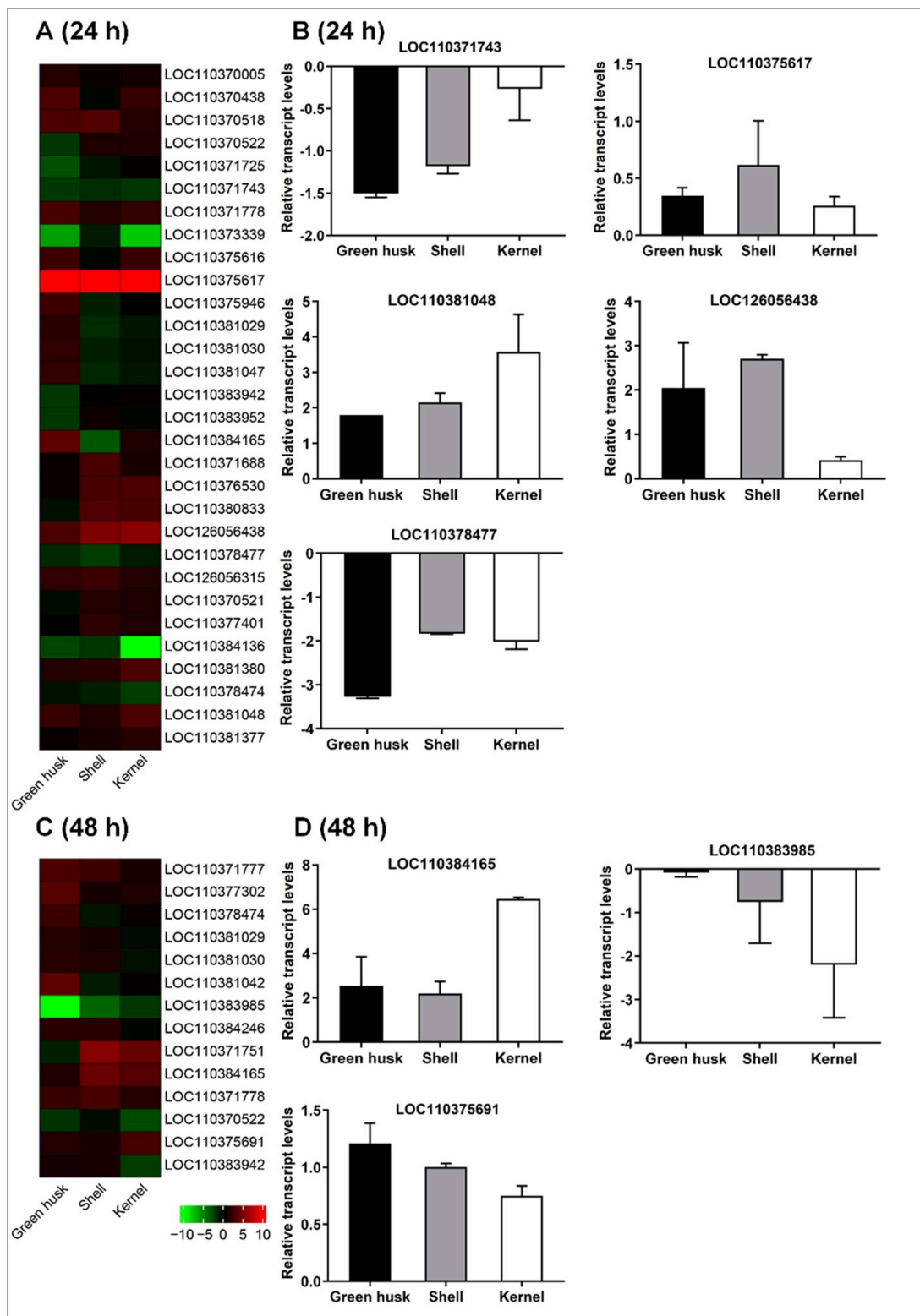


Figure 9

P450 genes differentially expressed in at least one control-treatment comparison in RNA-seq (A) and selected P450 genes in qRT-PCR (B) at 24 h. P450 genes differentially expressed in RNA-seq (C) and selected P450 genes in qRT-PCR (D) at 48 h. Downward pointing bars depict down regulation and upward pointing bars depict up regulation.

Supplementary Files

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