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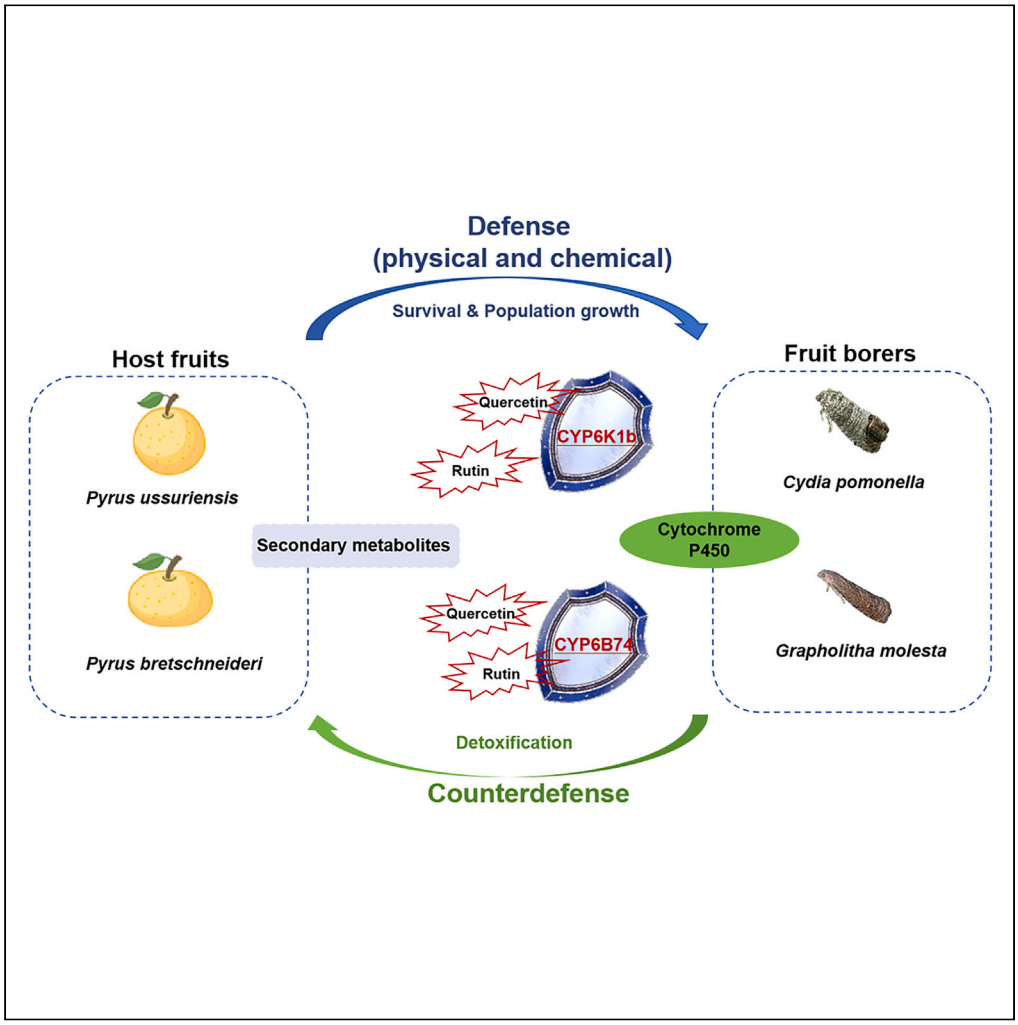

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Highlights

Content of quercetin in pears was induced by *C. pomonella* feeding

Quercetin affects the growth of *C. pomonella* and *G. molesta* populations

Cytochrome-P450-mediated counter-defense response of fruit borers to quercetin

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Secondary metabolites in host pears defense against two fruit borers and cytochrome-P450-mediated counter-defense

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SUMMARY

Herbivorous insects have evolved metabolic strategies to survive the challenges posed by plant secondary metabolites (SMs). This study reports an exploration of SMs present in pears, which serve as a defense against invasive *Cydia pomonella* and native *Grapholita molesta* and their counter-defense response. The feeding preferences of fruit borers are influenced by the softening of two pear varieties as they ripen. The content of SMs, such as quercetin and rutin, increases due to feeding by fruit borers. Notably, quercetin levels only increase after *C. pomonella* feeding. The consumption of SMs affects the growth of fruit borer population differently, potentially due to the activation of P450 genes by SMs. These two fruit borers are equipped with specific P450 enzymes that specialize in metabolizing quercetin and rutin, enabling them to adapt to these SMs in their host fruits. These findings provide valuable insights into the coevolution of plants and herbivorous insects.

INTRODUCTION

China is a prominent global contributor to pear industry, holding the largest cultivated area and highest output. Notably, Liaoning Province possesses a remarkable and competitive edge in terms of scale for pear production within China. In this province, *Pyrus ussuriensis* (Maxim) (known as Nanguo pear in Chinese) and *Pyrus bretschneideri* (Rehd) (known as Pingguo pear in Chinese) are the two primary varieties of pears cultivated. Both *P. ussuriensis* and *P. bretschneideri* belong to the Pyrus genus. *P. ussuriensis* is the most important cultivated pear in the northeastern, cold areas of China.[1] This fruit is round or oblate, yellowish green in color, and has a persistent calyx.[2] *P. ussuriensis* is typically harvested in September when they are still green, firm, and less juicy. Following harvest, the fruit ripens at room temperature and transforms into golden yellow, soft, juicy, and uniquely aromatic state.[3,4] On the other hand, *P. bretschneideri* belongs to the white pear system, which boasts excellent cold tolerance, storage capabilities, high yield, and high quality.[2] *P. bretschneideri* are crucial parent plants in breeding program, as they have contributed to the creation of 68 different varieties within the *P. bretschneideri* family. Consequently, both varieties of fruit trees hold significant economic value.

Both the codling moth, *Cydia pomonella* (Linnaeus), and the oriental fruit moth, *Grapholita molesta* (Busck), are prominent members of the Lepidoptera family Tortricidae and are recognized as significant pests of fruit trees worldwide. Although *C. pomonella* is the major agricultural invasive pest in China, its initial detection occurred in Xinjiang, China, in 1957.[5] *C. pomonella* has spread to approximately 70 countries since 1900, owing to the rapid growth of global trade and international travel, leading to an annual global fruit loss of around US$10 million.[6] *G. molesta* also poses a substantial threat in this region.[7] These two fruit borers have a similar host range, which includes pears, apples, and other nut fruits.[8] Previous studies have shown that there is a competition between these two fruit borers. Notably, *C. pomonella* was able to rapidly increase its population after exposure to quercetin by adopting an "accelerated burst" of oviposition strategy.[9] *C. pomonella* and *G. molesta* consume fruit with their larvae, causing a significant number of fruit drops, severely impacting fruit quality and resulting in substantial economic losses to the global pear fruit industry (Yang and Zhang 2015).[10] The safety and security of *P. ussuriensis* and *P. bretschneideri* are seriously compromised by both of these fruit borers.

Plants have developed intricate defense mechanisms to safeguard themselves against insect herbivory. These mechanisms can be categorized as constitutive and inducible defenses.[11] Constitutive defense refers to the inherent physicochemical traits of plants that impede the feeding activities of phytophagous insects, such as fruit fuzz, surface waxes, and hardness.[12] For instance, the trichomes found on *Arabidopsis* leaves have

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been observed to negatively influence the feeding and egg-laying behaviors of *Plutella xylostella*.[13] Additionally, an increase in silica cell content has been linked to enhanced plant resistance against insect pests by host plant hardness.[14] When faced with insect herbivory, plants synthesized a diverse array of secondary metabolites (SMs) or release volatiles as a part to their induced defense. These SMs can be broadly classified into phenolics (e.g., lignin, flavonoids, and tannins), terpenes, and sulfur (S)- (e.g., glutathione, glucosinolates, and defensin) and nitrogen (N)-containing compounds (e.g., alkaloids, cyanogenic glycosides).[15] In recent years, several studies have indicated that flavonoids can impact the nutrient accumulation of Lepidoptera larvae, leading to their inability to complete the moulting process and resulting in their death.[16] Plants produce important flavonoids such as quercetin and rutin, which have diverse effects on herbivorous insects. These effects include regulating growth and development, inhibiting immune defense, and inducing detoxification enzymes.[17] After feeding on branches containing quercetin and rutin, mortality rates for *Eriosoma lanigerum* larvae reached up to 80%.[18] Furthermore, rutin has been found to decrease the survival rate of *Ostrinia nubilalis* and has the ability to reduce pupal weight and prolong the pupal period in the *Spodoptera litura*.[19] However, the effects of consuming quercetin and rutin on the development and growth of *C. pomonella* and *G. molesta* populations are less known.

In order to cope with SMs, insects have developed a range of adaptive mechanisms, and metabolic detoxification is usually one of the most important ways in which insects adapt to exogenous substances.[7] One such mechanism involves the utilization of cytochrome P450 monooxygenases (P450), which are widely distributed throughout the organism and play a crucial role in the primary metabolism of exogenous substances, leading to the adaptation of insects to SMs.[20] As a phase I detoxification enzyme, P450 directly participates in the metabolism of secondary plant metabolites. In the case of Lepidoptera, Clan 3 represents the largest gene family of P450, within which the CYP6, CYP9, and CYP321 gene subfamilies have demonstrated the ability to metabolize various phytotoxins.[21] For instance, research revealed the potential of *CYP6AB14* from *Spodoptera litura* in the detoxification of plant allelochemicals.[22] In *Spodoptera frugiperda*, numerous P450 genes in the CYP6B, CYP321A, and CYP9A subfamilies are responsive to phytochemicals and are involved in the detoxification.[23] Additionally, it was observed that xanthotoxin and flavone induced the expression of *CYP321A1* in *Helicoverpa zea*.[24] However, knowledge on the role of individual P450 gene in response to the presence of SMs is lacking in *C. pomonella* and *G. molesta*.

To gain valuable insights into the coevolution of host fruit and phytophagous insects, in this study we focused on investigating the mechanisms of fruit defense and counter-defense by phytophagous insects. To test our hypothesis, we conducted an assessment of the impact of quercetin on the growth and development of fruit borers, as well as the expression level of the P450 Clan 3 gene. This assessment involved a comparison of the fruit hardness and the content of major phyto-substance in *P. ussuriensis* and *P. bretschneideri*, with quercetin serving as an illustrative example.

RESULTS

Fruit hardness and infestation rates of *P. ussuriensis* and *P. bretschneideri*

During the fruit enlargement stage to the ripening, there was a significant decrease in fruit hardness observed in both *P. ussuriensis* and *P. bretschneideri*. Conversely, the rate of fruit decay showed an opposite trend (Figure 1). *P. ussuriensis* exhibited higher fruit firmness compared with *P. bretschneideri* during the fruit enlargement period ($p < 0.0001$) and fructescence (V) ($p < 0.001$) (Figure S1). The fruit infestation rate of *C. pomonella* was significantly higher than in both fruits compared with *G. molesta* in all periods. The fruit infestation rate of both borers increased rapidly as fruit hardness decreased. Correlation analysis revealed a negative correlation between fruit hardness and infestation rate of the two borers, indicating that a harder fruit host resulted in less damage caused by the borers (Table S3).

Changes in plant secondary metabolite content in fruit development stages

In *P. ussuriensis*, except for quercetin, the content of chlorogenic acid, epigallocatechin, catechin, rutin, gallic acid, and isoquercitrin continued to decrease from fruit expansion stage I to stage III. Among these compounds, chlorogenic acid, epigallocatechin, gallic acid, and isoquercitrin content increased in stage IV, decreased in stage V, and increased in stage VI. Rutin exhibited in an opposite trend from stage IV to stage VI. Catechin content increased in stage IV and then began to decrease until maturity stage VI. Quercetin content increased initially and then decreased from stage I to stage VI, peaking in stage III (Figure 2A). In *P. bretschneideri*, there was a trend of increasing chlorogenic acid, quercetin, and rutin content and then decreasing throughout the growth and development period. Gallic acid content showed a decreasing trend throughout the period, whereas epigallocatechin, catechin, and isoquercitrin content exhibited a fluctuating trend (Figure 2B).

Changes in plant secondary metabolite content after borers feeding

SMs were examined before and after fruit borer damage at stage IV. The results showed that the content of chlorogenic acid, gallic acid, isoquercitrin, rutin, and quercetin in *P. ussuriensis* increased by 1.85-, 1.92-, 2.32-, 1.60-, and 1.85-fold, respectively, when induced by *C. pomonella* infection compared with uninfested fruit. Similarly, the levels of chlorogenic acid, gallic acid, and rutin increased by 1.71-, 1.41-, and 1.89-fold in *P. ussuriensis* induced by *G. molesta* damage. Epigallocatechin and catechin content did not show significant changes (Figure 2C). Furthermore, in *C. pomonella*-damaged *P. bretschneideri* fruit, the substances chlorogenic acid, epigallocatechin, gallic acid, rutin, and quercetin were significantly elevated by 1.81-, 2.57-, 2.73-, 1.36-, and 1.75-fold, respectively. However, the isoquercitrin content did not show significant changes after feeding by *C. pomonella*. In *P. bretschneideri* damaged by *G. molesta*, only gallic acid and isoquercitrin levels were significantly increased by 3.27- and 1.45-fold (Figure 2D). These results indicate that the content of quercetin in both pears was only induced by *C. pomonella* damage, not by *G. molesta* damage. Consequently, quercetin will be used as an example to explore the effects of SMs on the growth and development of two insect species.

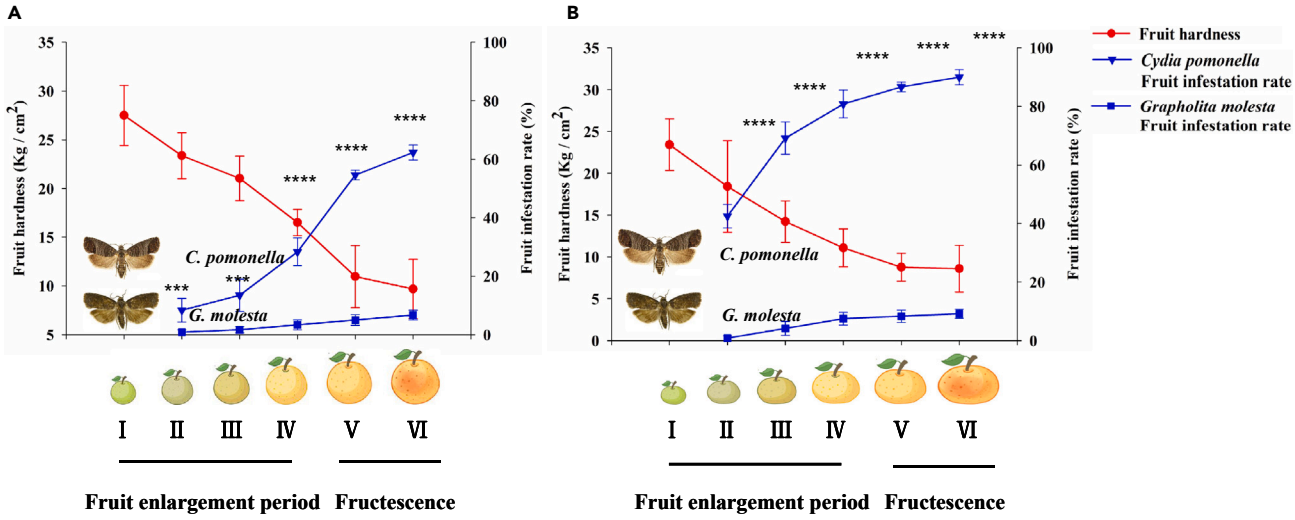

Figure 1. The infestation rate of fruit at different stages caused by *C. pomonella* and *G. molesta*

(A) The fruit hardness changes (red) within the fruit enlargement period (I–IV) and fructescence (V–VI) and the fruit infestation rate (blue) of two fruit borers in *P. ussuriensis*.

(B) The fruit hardness changes (red) within the fruit enlargement period (I–IV) and fructescence (V–VI) and the fruit infestation rate (blue) of two fruit borers in *P. bretschneideri*. * represented the difference in the infestation rate of fruit caused by *C. pomonella* and *G. molesta*. Correlation between fruit infestation rate and fruit hardness of *C. pomonella* and *G. molesta* showed in Table S3. The fruit hardness of *P. ussuriensis* and *P. bretschneideri* showed in Figure S1. All data tested for normal distribution using the Shapiro-Wilk analysis. Data shown are mean ± *SD*. Asterisks above represent statistically significant differences analyzed by Student's t test (****p < 0.001, ***p < 0.001, **p < 0.01, *p < 0.05). The software Spss Statistics 22 (IBM, Chicago) was employed for this analysis and plotted with SigmaPlot 12.

Effects of quercetin and rutin on the population parameters of two borers

In comparison to the control group, the presence of quercetin in the diet had a significant impact on various population parameters in *C. pomonella*. The group treated with 10 μg/g quercetin exhibited the highest values for intrinsic rate of population increase (r), finite rate of increase (λ), and mean generation time (T), whereas the control group (0 μg/g quercetin) had the highest net reproductive rate (R_0). The largest decrease in λ was observed in the 100 μg/g quercetin group, whereas the greatest reductions in r, R_0, and T of *C. pomonella* populations were observed in the 5,000 μg/g quercetin group compared with the control.

For *G. molesta*, the r, λ, and R_0 values were higher in the 10 μg/g and 100 μg/g quercetin groups compared with the other groups. No significant differences in r, λ, and R_0 were observed between the 1,000 μg/g quercetin group and control, except for a prolonged T in the former. The 5,000 μg/g quercetin group exhibited significantly reduced values for r, λ, and R_0, as well as significantly prolonged T when compared with the control (Table 1).

Fed on diet containing varying concentrations of rutin (20 μg/g, 100 μg/g, 200 μg/g), all *G. molesta* individuals died within 9 days. Although not all *C. pomonella* individuals died, rutin had a significant inhibitory effect on their development. All *C. pomonella* larvae were in the second and third instars and were unable to undergo pupation (Figure S2).

Effect of quercetin feeding on P450 enzyme activity of two borers

In comparison to the control group (0 μg/g quercetin), the activity of P450 enzyme in the midgut of the fourth instar larvae significantly increased when they consumed an artificial diet containing quercetin. Notably, the larvae of *C. pomonella* exhibited the highest P450 enzyme activity in their midgut when fed a diet with 1000 μg/g quercetin, which was 1.98 times higher than the control group (Figure 3A).

In contrast to the control group, apart from the artificial diet containing 1,000 μg/g quercetin, the activity of the P450 enzyme in the midgut of the fourth instar larvae of *G. molesta* was significantly increased in all other treatments. The greatest increase in P450 enzyme activity in the midgut of larvae was observed when they consumed an artificial diet containing 5,000 μg/g quercetin, which was 1.53 times higher than the control (Figure 3B).

Expression patterns of P450 genes in two borers

The results of the RT-qPCR showed that the expression levels of 20 genes belonging to the P450 Clan 3 of *C. pomonella* were found to be induced by quercetin. Out of these upregulated genes, 10 demonstrated significant upregulation across all concentrations of quercetin, whereas the remaining 10 genes showed upregulation only at specific concentrations (Figure 3C). Similarly, in the P450 Clan 3 of *G. molesta*, the expression levels of 22 genes were induced to be upregulated by quercetin. Among these genes, seven exhibited upregulation at all concentrations of quercetin, whereas the remaining 15 genes showed upregulation at their respective concentrations (Figure 3D).

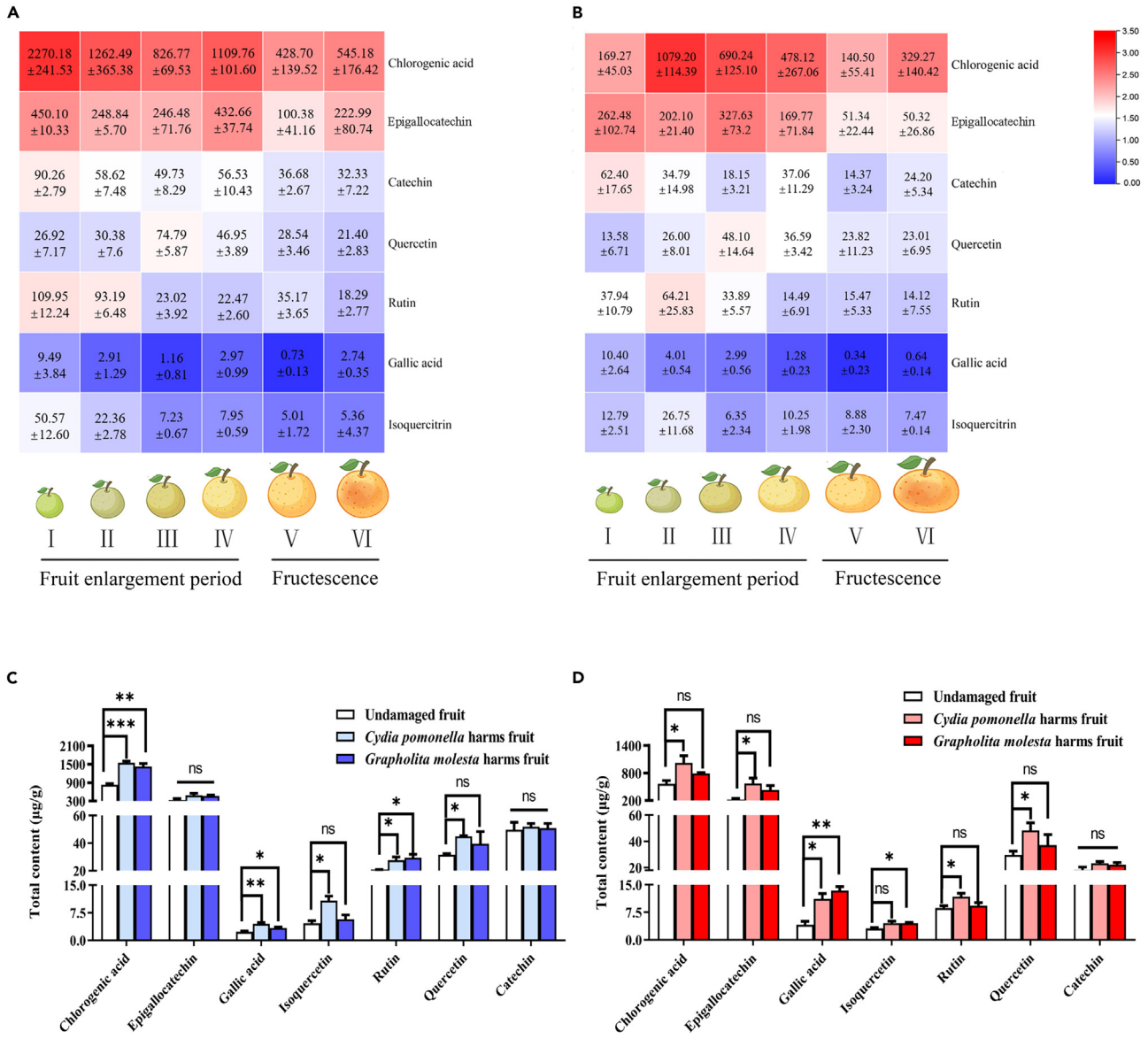

Figure 2. Analysis of the content of main SMs in *P. ussuriensis* and *P. bretschneideri*

(A) The contents of SMs were examined in *P. ussuriensis* during fruit enlargement period (I–IV) and fructescence (V–VI) using HPLC.

(B) The contents of SMs were examined in *P. bretschneideri* during fruit enlargement period (I–IV) and fructescence (V–VI) using HPLC.

(C) SMs were examined in the *P. ussuriensis* fruits collected before and after borers damage in stage IV, as μg equivalents per g of fresh weight.

(D) SMs were examined in the *P. bretschneideri* fruits collected before and after borers damage in stage IV, as μg equivalents per g of fresh weight. The results are the mean ± SD of three biological replicates. Asterisks above represent statistically significant differences analyzed by Student's t test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$) and visualized using GraphPad Prism 9 software (GraphPad Software, CA).

Based on the quantification of quercetin content in two pear samples, further investigation was carried out on the P450 genes (*CYP354A22, CYP6AW1, CYP6K1b, CYP9e2a* of *C. pomonella; CYP6A17, CYP6B74*, gm_13876, gm_16205 of *G. molesta*) that were significantly induced by 100 μg/g quercetin (Figures 3C and 3D). The expression patterns of four P450 genes in *C. pomonella* and *G. molesta* were investigated at different developmental stages. The results indicated that *CYP345A22, CYP9E2a*, and *CYP6K1b* in *C. pomonella* exhibited the highest expression levels of expression during the fourth instar larvae stage, whereas *CYP6AW1* showed the highest level of expression during the first instar larvae stage (Figures 4A–4D). In the case of *G. molesta*, the expression patterns of *CYP6B74, gm_13876, gm_16205*, and *CYP6A17* were observed throughout all developmental stages, with the highest expression occurring during the fourth instar larvae stage (Figures 4E–4H).

Furthermore, the results showed that these P450 genes were expressed in all tissues of the fourth instar larvae of both *C. pomonella* and *G. molesta* (Figure 4). The expression level of *CYP345A22, CYP9E2a*, and *CYP6K1b* in the midgut of *C. pomonella* was significant higher than

Table 1. Effects of different concentrations of quercetin on population parameters of *C. pomonella* and *G. molesta*

Species	Concentrations	r	λ	R_0	T
Cydia pomonella	0 μg/g	0.092 ± 0.005 b	1.096 ± 0.006 b	27.274 ± 4.715 a	35.798 ± 0.353 b
	10 μg/g	0.099 ± 0.006 a	1.104 ± 0.006 a	19.700 ± 3.973 c	35.997 ± 0.152 a
	100 μg/g	0.080 ± 0.005 days	1.092 ± 0.006 days	17.337 ± 3.006 days	35.534 ± 0.210 c
	1,000 μg/g	0.088 ± 0.006 c	1.0947 ± 0.005 c	23.256 ± 4.378 b	35.717 ± 0.229 b
	5,000 μg/g	0.075 ± 0.005 e	1.083 ± 0.006 e	14.175 ± 2.592 e	35.302 ± 0.242 d
Grapholita molesta	0 μg/g	0.102 ± 0.008 c	1.107 ± 0.009 c	19.010 ± 4.104 b	28.912 ±0.283 d
	10 μg/g	0.107 ± 0.008 b	1.113 ± 0.009 b	23.100 ± 4.936 b	29.341 ± 0.303 c
	100 μg/g	0.127 ± 0.013 a	1.136 ± 0.014 a	53.950 ± 24.897 a	31.314 ± 0.688 b
	1,000 μg/g	0.099 ± 0.007 c	1.104 ± 0.008 c	21.790 ± 4.768 b	31.01 ± 0.227 b
	5,000 μg/g	0.084 ± 0.009 days	1.087 ± 0.010 days	14.420 ± 3.769 c	31.84 ± 0.445 a

Note: r: intrinsic rate of increase; λ: finite rate of increase; R_0: net reproductive rate; T: mean generation time. The data in the table are represented as mean ± *SE*; Different letters indicate significant differences ($p < 0.05$) according to paired bootstrap test.

in other tissues (Figures 4I–4K). Conversely, *CYP6AW1* demonstrated higher expression in the fat body, Malpighian tubes, and midgut relative to the head and cuticle (Figure 4I). On the other hand, the midgut of *G. molesta* displayed significantly higher expression levels of four P450 genes (*CYP6B74*, gm_13876, gm_16205, and *CYP6A17*) compared with other tissues (Figures 4M–4P).

Functional analysis of P450 genes associated with quercetin metabolism of *C. pomonella* and *G. molesta*

To verify the metabolic function of P450 genes, the gene silencing technique in both borers was applied. The expression levels of *C. pomonella CYP6AW1* and *CYP6K1b* were observed to decrease significantly by 53.53% and 51.68% and 41.31% and 36.64%, respectively, after the injection of dsRNAs for 6 and 12 h (Figures 5A and 5B). Similarly, the expression of *G. molesta CYP6B74* and *gm_13876* was found to decrease significantly by 59.67% and 56.41% and 56.62% and 62.31%, respectively, after the injection of dsRNAs for 6 and 12 h (Figures 5D and 5E).

There was no significant difference in body weight gain observed in *C. pomonella* (Figure S4A) and *G. molesta* (Figure S4B) larvae within 48 h when treated with 0 μg/g quercetin (control) and 100 μg/g quercetin. Furthermore, when *dsGFP* was injected into the aforementioned treatments, the body weight gain of *C. pomonella* (Figure S5A) and *G. molesta* (Figure S5B) larvae did not differ significantly from the control group within 48 h.

The control groups consisted of dsGFP-injected larvae of *C. pomonella* and *G. molesta* fed on artificial diets containing 100 μg/g quercetin. In comparison to the control, *C. pomonella* larvae injected with *dsCYP6AW1* and *dsCYP6K1b* exhibited significantly lower body weight gain within 48 h after consuming artificial diets containing 100 μg/g quercetin (Figure 5C). The same pattern was observed for *G. molesta* larvae injected with *dsCYP6B74* and *dsgm_13876* (Figure 5F).

As controls, *C. pomonella* and *G. molesta* larvae injected with *dsGFP* and fed with artificial diet containing 100 μg/g quercetin were used. The relative growth rate (RGR) and relative consumption rate (RCR) of *C. pomonella* larvae injected with *dsCYP6K1b* showed a significant decrease compared with the control, whereas no significant differences were observed in the efficiency of the conversion of ingested food (ECI) and efficiency of the conversion of digested food (ECD). The RCR, RGR, ECI, and ECD of *C. pomonella* larvae were significantly reduced compared with the control after *dsCYP6AW1* injection. Similarly, the RCR, RGR, ECI, and ECD of *G. molesta* larvae were significantly reduced compared with the control after *dsCYP6B74* and *dsgm_13876* injection (Table 2).

Verification of the recombinant bacmid DNA

Bacmid DNA from five colonies was utilized to perform PCR verification. The PCR products for CYP6K1B, CYP6AW1, CYP6B74, and CYP341B14 from all five colonies exhibited the expected size, indicating successful transposition. Additionally, four colonies of gm_13876 were confirmed to contain the correct recombinant bacmid (Figure S6). Thus, the recombinant bacmid DNA harboring the P450s was suitable for subsequent recombinant protein expression.

The western blot analysis (Figure S7) revealed the successful detection of CYP341B14 and CPR, with molecular mass of 58.9kDa and 77.9kDa, respectively, in Sf9 insect cells, indicating their successful expression. Considering that CYP6K1B, CYP6AW1, CYP6B74, and gm_13876 were cotransfected with the same CPR lacking a His tag, parallel to the positive control, it can be inferred that these four P450s and the CPR lacking a His tag were also successfully expressed. Consequently, the microsomal fractions containing these four P450s and the corresponding CPR were prepared for enzymatic assays.

Metabolic analysis of recombinant P450 proteins of *C. pomonella* and *G. molesta* against quercetin and rutin

The retention time for quercetin was observed to be between 6.227 and 6.341 min, whereas for rutin it was between 6.92 and 7.138 min. Quercetin was detected with a peak area of 257.18 ± 4.47, 227.36 ± 4.74, 212.13 ± 4.47, and 391.75 ± 4.88 mAU*s, after incubation with

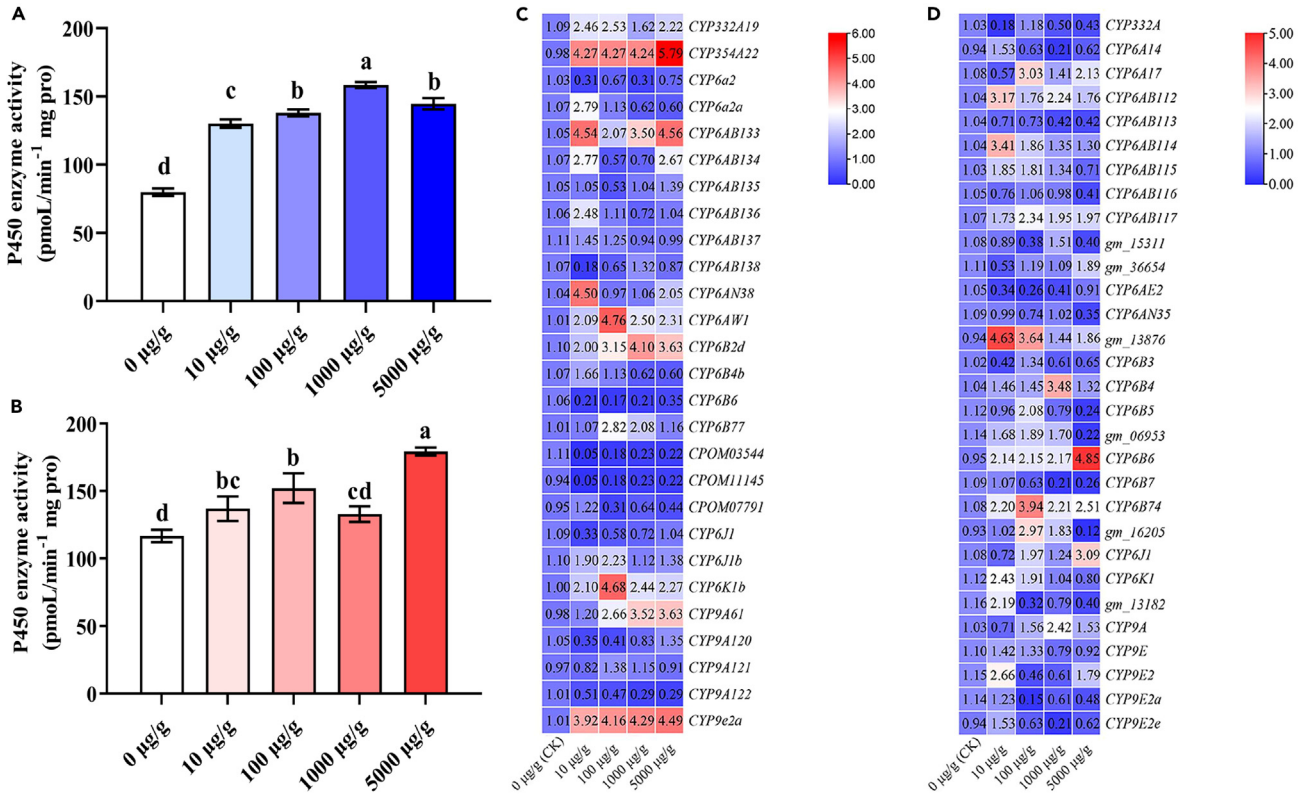

Figure 3. Changes in the P450 enzyme activities and expression levels of P450 genes in *C. pomonella* and *G. molesta* by quercetin

(A) The activity of P450 enzyme in the midgut of the fourth instar larvae of *C. pomonella* when they consumed an artificial diet containing different concentrations of quercetin was investigated.

(B) The activity of P450 enzyme in the midgut of the fourth instar larvae of *G. molesta* when they consumed an artificial diet containing different concentrations of quercetin was investigated.

(C) Using RT-qPCR, the expression levels of P450 Clan3 gene in *C. pomonella* were examined in two fruit borers fed with different concentrations of quercetin of fourth instar larvae.

(D) Using RT-qPCR, the expression levels of P450 Clan3 gene in *G. molesta* were examined in two fruit borers fed with different concentrations of quercetin of fourth instar larvae. Data shown are mean ± *SD* (n = 3). Different letters indicate significant differences ($p < 0.05$) according to Tukey's test and visualized using GraphPad Prism 9 software (GraphPad Software, CA).

recombinant CYP6AW1, CYP6K1b, CYP6B74, and gm_13876 for 90 min, respectively. However, except for CYP6AW1, which showed no significant difference in peak area with or without NADPH, all other P450s exhibited a significant decrease in peak area when NADPH was present (Figures 6A–6D). All P450s demonstrated metabolic activity toward rutin (Figures 6E–6H). The depletion rates of quercetin by CYP6AW1, CYP6K1b, CYP6B74, and gm_13876 were 1.56% ± 0.04%, 13.72% ± 0.24%, 22.39% ± 0.37%, and 5.07% ± 0.07%, with CYP6B74 being the most efficient (Figure 6I). The depletion rates of rutin by CYP6AW1, CYP6K1b, CYP6B74, and gm_13876 were 4.48% ± 0.53%, 27.78% ± 0.10%, 7.90% ± 1.69%, and 4.23% ± 0.31%, with CYP6K1b being the most efficient (Figure 6J).

DISCUSSION

Plants have developed intricate defense mechanisms to protect themselves against phytophagous insects, which can be categorized into two types: constitutive and inducible defenses.[25] Constitutive defense refers to the inherent physicochemical properties of the plant that counteract attacks from pests, which act as the initial barrier against phytophagous insects by inducing structural changes such as the formation of spines, trichomes, waxes, lignification, and thickened cell walls.[26] Sousa-Lopes et al. (2020)[27] reported that the severity damage caused by the bean weevil is influenced by the softness, lightness, and size of the seeds. It was discovered that the hardness of pears is determined by the presence of stone cells within the fruit, and as the fruit matures, these stone cells are absorbed, resulting in a decrease in fruit hardness.[28] Consequently, this led to a higher rate of infestation by *C. pomonella* and *G. molesta* (Figure 1). The infestation rate of both insects exhibited a negative correlation with fruit hardness. Both borers showed a preference for feeding on host fruits with lower hardness, possibly due to the larva's ability to penetrate the pericarp.[29] *C. pomonella* demonstrated a significantly higher infestation rate compared with *G. molesta* on both pear species (Figure 1), which could be attributed to the distinct mouthpart structure of the two fruit borers.[30] This further validates that fruit hardness is a crucial factor in deterring phytophagous insects.

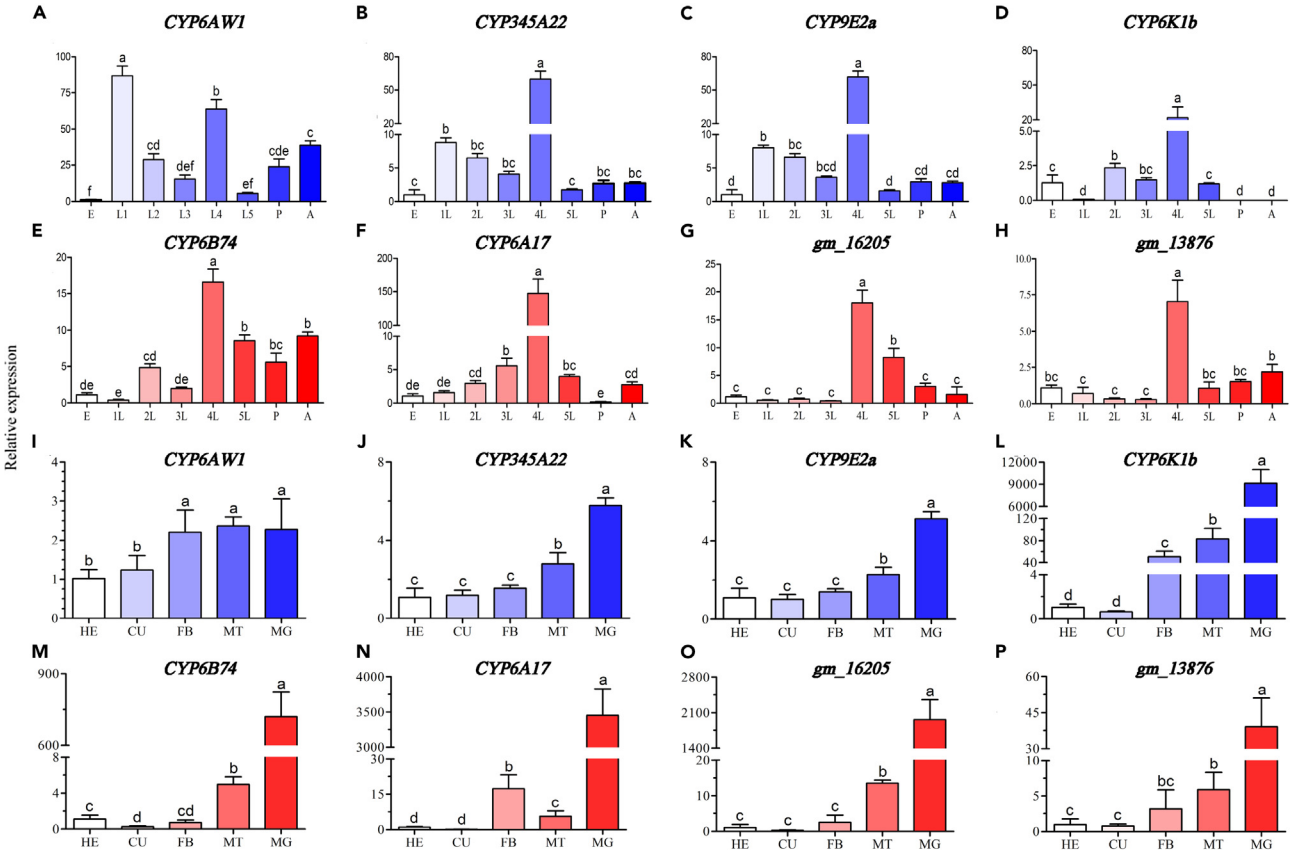

Figure 4. The expression levels of P450 genes in different development stages and tissues of *C. pomonella* and *G. molesta*

(A–D) The expression levels of P450 genes in different development stages of *C. pomonella*.

(E–H) The expression levels of P450 genes in different development stages of *G. molesta*.

(I–L) The expression levels of P450 genes in different tissues of *C. pomonella*.

(M–P) The expression levels of P450 genes in different tissues of *G. molesta*. E, egg; L, larval; P, pupa; A, adult. Tissues of fourth instar larvae. HE, head; CU: cuticle; FB, fat body; MT, Malpighian tubes; MG, midgut. Data shown are mean ± *SD* (n = 3). Data shown are mean ± *SD* (n = 3). Different letters indicate significant differences ($p < 0.05$) according to Tukey's test. The data were presented as the mean of at least three independent experiments ± SE and visualized using GraphPad Prism 9 software (GraphPad Software, CA).

The phenomenon of induced defense in plants has gained significance due to its ability to produce compounds that combat pathogens and insect herbivores.[31] SMs play a crucial role in this induced defense mechanism.[32] Our findings indicate that the content of SMs in both *P. bretschneideri* and *P. ussuriensis* exhibited fluctuating changes during fruit ripening, which could potentially be influenced by abiotic factors such as moisture, temperature, and light, etc.[33] Except for epigallocatechin, all detected SMs were significantly impacted by damages caused by *C. pomonella* and/or *G. molesta*. This suggests that the effects of pests feeding on SMs are complex and vary depending on the specific pest and plant species. The content of quercetin in the host fruits increased significantly after feeding by *C. pomonella*, whereas there was no significant change when fed upon by *G. molesta* (Figures 2C and 2D). Quercetin accumulation was also observed in tea plants as a response to feeding by *Ectropis grisescens*.[34] These findings imply that quercetin could potentially function as a crucial plant secondary metabolite involved in the defense of host plants against *C. pomonella*.

Flavonoids have a wide distribution in various plant species and play a significant role in biological processes, particularly in defense against phytophagous insects.[35] One notable flavonoid, quercetin, has been found to enhance plant tolerance to phytophagous insects and exert effects on insect growth and development.[36] The presence of quercetin on leaves treated with it has been shown to attract *Spodoptera frugiperda*, leading to increased feeding behavior.[37] Moreover, quercetin has been observed to impede the growth and development of *Spodoptera litura* larvae at low concentrations and significantly elevates larval mortality at high concentrations.[38] Similarly, our findings indicate that the different concentrations of quercetin treatment have an impact on the population parameters (r, λ, R_0, and T) of *C. pomonella* and *G. molesta* (Table 1). Specifically, the treatment with 100 μg/g quercetin has distinct effects on the populations of *C. pomonella* and *G. molesta*, suppressing the former while expanding the latter. These results suggest that quercetin is an important secondary metabolite in defending *C. pomonella* in pear fruits but does not exhibit the same defense against *G. molesta*.

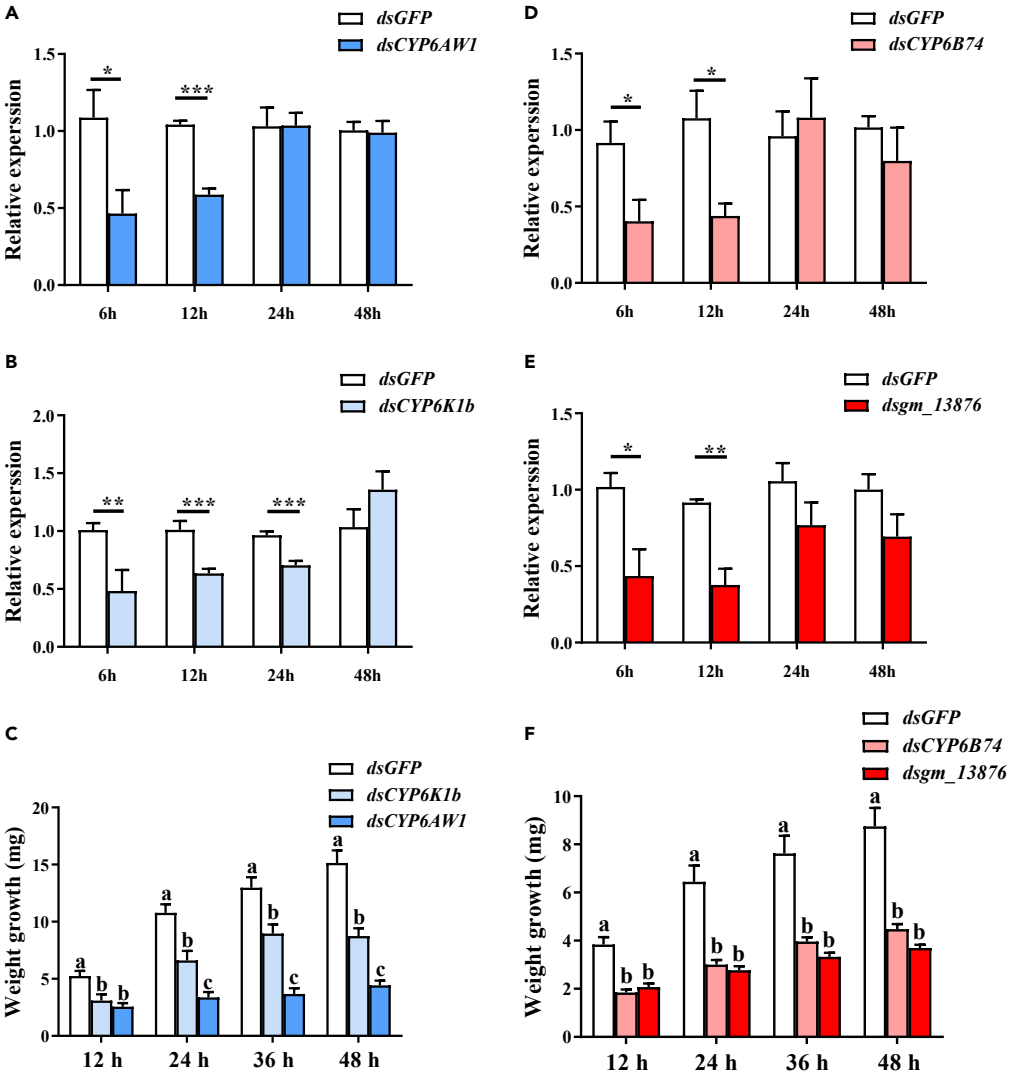

Figure 5. Knockdown of P450 gene using on the weight growth of *C. pomonella* and *G. molesta*

(A) RNAi efficiency of *CYP6AW1* in *C. pomonella*.

(B) RNAi efficiency of *CYP6K1b* in *C. pomonella*.

(C) Effect of RNAi with *CYP6AW1 and CYP6K1b* on weight growth of quercetin feeding by *C. pomonella*.

(D) RNAi efficiency of *CYP6B74* in *G. molesta*.

(E) RNAi efficiency of *gm_13876* in *G. molesta*.

(F) Effect of RNAi with *CYP6B74* and *gm_13876* on weight growth of quercetin feeding by *G. molesta*. The fourth instar larvae of both borers were injected with dsRNA and dsGFP. Samples were collected after 6, 12, 24, and 48 h of injection and assayed for interference efficiency using RT-qPCR. Asterisks above represent statistically significant differences analyzed by Student's t test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$). The data were presented as the mean of at least three independent experiments ± *SD* and visualized using GraphPad Prism 9 software (GraphPad Software, CA).

Insect P450 plays a crucial role in the detoxification of exogenous substances, including chemical insecticides and SMs. Its function is to enhance the reactivity and water solubility of toxic substances, thereby reducing the toxicity of phytotoxins and minimizing the harm caused to insects.[39] Previous studies have demonstrated that activity and gene expression of P450 enzymes, such as *CYP321A1*, *CYP6B6*, and *CYP6B8*, significantly increase when exposed to quercetin in *Helicoverpa armigera*.[40] Similarly, the CYP6AS subfamily of *Apis mellifera* is capable of metabolizing quercetin found in pollen.[41] Our findings also indicate that 20 and 21 P450 Clan 3 genes are highly expressed in *C. pomonella* and *G. molesta*, respectively, when subjected to quercetin treatment (Figures 3C and 3D). These results suggest that P450 genes in *C. pomonella* and *G. molesta* may be involved in responding to SMs, specifically quercetin.

The RNAi technology is a widely employed method for the analysis P450 function. The utilization of RNAi to silence specific target genes can enhance the susceptibility of insects to SMs.[42] For instance, Wang et al. (2015)[22] observed an increase in the susceptibility of larvae to quercetin when the *S. litura CYP9A40* gene was knocked down using RNAi. Furthermore, injection of *dsCYP6AB60* and *dsCYP321A19* into

Table 2. Effect of feeding quercetin on nutrient utilization index of *C. pomonella* and *G. molesta* after knockdown of P450 genes by RNAi

Species	Processing groups	Relative growth rate (RGR)%	Relative consumption rate (RCR)%	Efficiency of the conversion of ingested food (ECI)%	Efficiency of the conversion of digested food (ECD)%
Cydia pomonella	*dsGFP*	81.98 ± 11.36 a	203.13 ± 31.14 a	44.78 ± 8.50 a	50.44 ± 9.04 a
	dsCYP6K1b	47.73 ± 10.14 b	104.23 ± 13.58 b	37.68 ± 8.17 ab	43.34 ± 8.88 a
	dsCYP6AW1	30.84 ± 8.75 c	88.69 ± 9.97 b	31.52 ± 8.14 b	36.01 ± 9.39 b
Grapholita molesta	*dsGFP*	88.02 ± 7.73 a	203.73 ± 36.82 a	50.44 ± 5.85 a	64.05 ± 7.59 a
	dsCYP6B74	36.56 ± 5.39 b	151.13 ± 17.97 b	26.08 ± 5.54 b	26.42 ± 2.22 b
	dsgm_13876	32.79 ± 6.43 b	121.19 ± 9.50 c	26.25 ± 5.98 b	28.71 ± 4.91 b

Note: the data in the table are represented as mean ± *SD*; different letters showed significant differences.
Related to Table S2

fourth instar larvae of *S. litura* resulted in a decrease in tolerance to quercetin.[22] In *H. armigera*, flavonoids have been found to induce significant upregulation of the *CYP6B8* and *CYP321A1* genes.[43] This study demonstrated that interference with key P450 genes (*CYP6AW1* and *CYP6K1b* of *C. pomonella*; *CYP6B74* and gm_13876 of *G. molesta*) led to a significant decrease in the weight gain of fourth instar larvae when fed artificial diets containing quercetin within 12–48 h (Figures 6C and 6F). This decrease in weight gain indicated the adaptation of both insects to quercetin, as evidenced by a notable decline in relative consumption rate (RCR), relative growth rate (RGR), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD). The involvement of both P450 genes in the detoxification of quercetin in the two fruit borers was established. However, in this study, we employed RNAi technology to elucidate the role of specific P450 genes in the insect-mediated metabolism of plant toxins, focusing on *C. pomonella* and *G. molesta*. Given the limited effectiveness and short duration of Lepidopteran RNAi interference, it is essential to employ more intuitive research tools.[44]

Many studies have shown that SMs can be metabolized by P450s in insects.[41,45] For instance, CYP6B8 in *Helicoverpa zea* has the ability to metabolize xanthotoxin, flavone, quercetin, and other phytochemicals.[46] *H. zea* also utilizes CYP321A1 to metabolize similar phytochemicals as CYP6B8, indicating that this insect species has developed systems for detoxifying phytochemicals.[47] However, the ability to detoxify toxic compounds is complex and not solely reliant on a single specialized P450 detoxification enzyme. Multiple upregulated P450s are often responsible for enhancing the metabolic detoxification of phytochemicals and are implicated in the insect's adaptation to their host plant's defense mechanisms.[48] In this study, we discovered that *G. molesta* has two P450s (CYP6B74 and gm_13876) that metabolize quercetin, whereas *C. pomonella* has only one (CYP6K1b). Among these, CYP6B74 exhibited the highest metabolic capacity, depleting 22.39% ± 0.37% of quercetin in 90 min. This may explain why *G. molesta* did not experience significant negative effects from quercetin and instead saw its population grow. Although it possesses CYP6AW1 with limited ability to metabolize quercetin, the presence of CYP6K1b, which exhibits a metabolic rate of 13.72% ± 0.24%, allows *C. pomonella* to avoid population extinction when exposed to quercetin. Interestingly, the ability of *C. pomonella* to metabolize rutin, as exhibited by the presence of the CYP6K1b, allows it to adapt to this plant secondary metabolite. However, *G. molesta* is unable to overcome the toxicity imposed by rutin, resulting in a decline in its population. The ability of insect populations to overcome toxicity conferred by SMs like quercetin and rutin determines their outbreak potential. The varying adaptations of insects to SMs can also lead to competition among different insect species in the same vicinity.[36] These results imply that the capability of *C. pomonella* to metabolize both quercetin and rutin may contribute to its global invasiveness. However, it is crucial to recognize that this study only investigated a few P450 protein metabolisms *in vitro* for restricted range of SMs. Consequently, it remains unclear whether there are additional P450 enzymes capable of metabolizing quercetin and rutin or if these four P450s have the capacity to metabolize other SMs.

Limitations of the study

In this study, we exclusively assessed the metabolic capability of counter-defense-related P450s in two fruit borers toward the SMs, quercetin and rutin, and did not identify the metabolite(s). Future studies will entail investigating the P450 genes response to successive generations of SMs stress in two fruit borers, as well as elucidating their adaptive strategies. This will contribute to enhancing the molecular understanding of the coevolution between herbivorous insects and plants.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

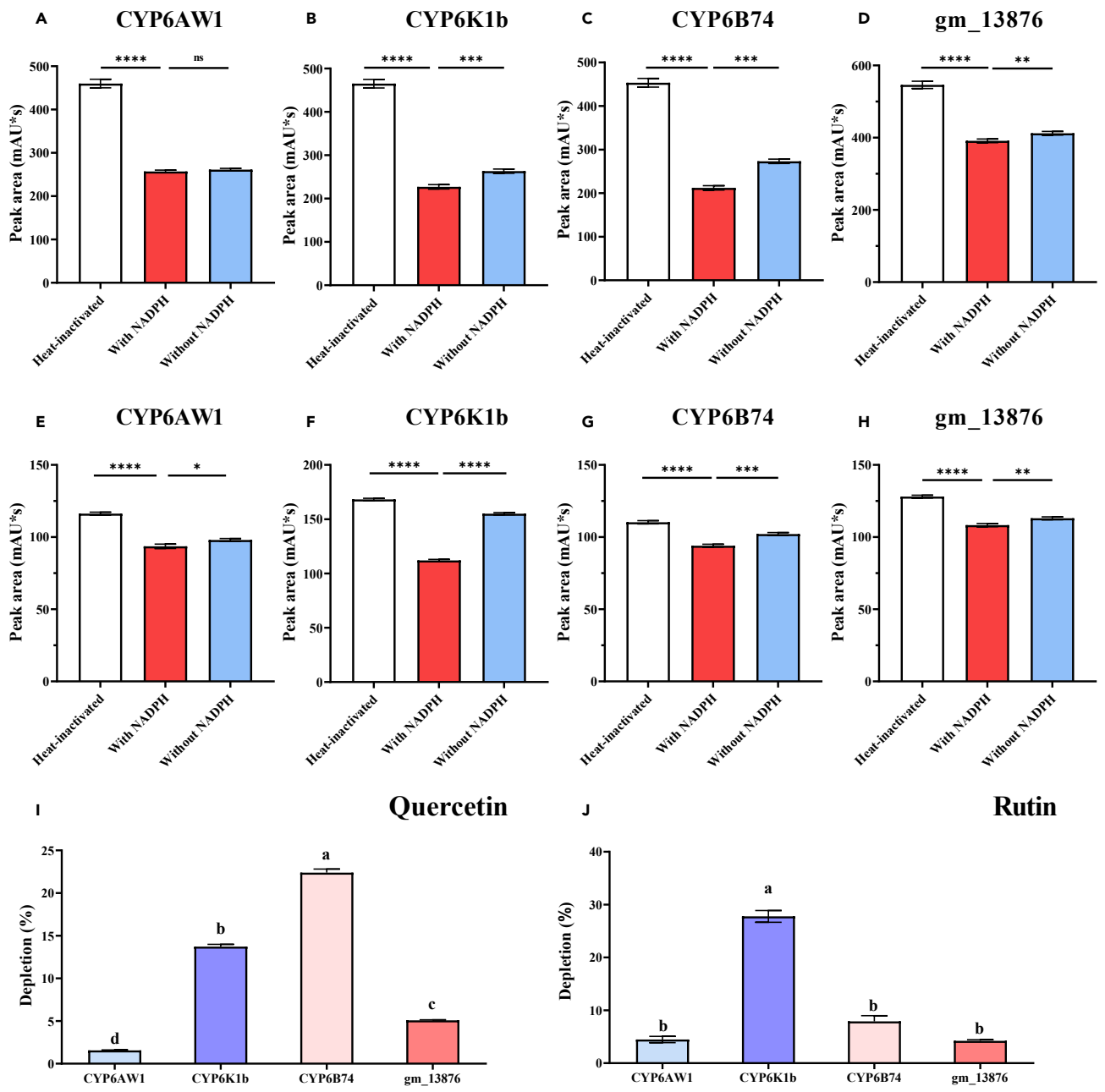

Figure 6. Metabolic analysis of recombinant P450 proteins of *C. pomonella* and *G. molesta* against quercetin and rutin

(A–D) Peak area of recombinant P450 proteins against quercetin.

(D–H) Peak area of recombinant P450 proteins against rutin. Data shown are mean ± *SD*. Asterisks above represent statistically significant differences analyzed by Student's t test (***p < 0.001; **p < 0.01; *p < 0.05).

(I) Depletion of quercetin by P450 proteins.

(J) Depletion of rutin by P450 proteins. Depletion (%) = (peak area of without NADPH- peak area of with NADPH)/peak area of without NADPH*100%. Data shown are mean ± *SD* (n = 3). Different letters indicate significant differences (p < 0.05) according to Tukey's test and and visualized using GraphPad Prism 9 software (GraphPad Software, CA).

- Construction of population parameters
- Determination of P450 enzyme activity
- Total RNA extraction, cDNA synthesis, and RT-qPCR
- RNA interference (RNAi) of P450 genes
- Effect of knock down of P450 genes on the growth
- Construction of the recombinant plasmids
- Heterologous expression of P450s
- Western blot
- Metabolism assay

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109518.

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AUTHOR CONTRIBUTIONS

S.-P.Z. wrote the manuscript. X.-Q.Y. conceived and designed research. B.B. undertook the revision of the manuscript. N.-X.F. and X.-F.L. provided p450 protein. Y.-T.L., P.G., G.-M.C., Y.-Q.W., and C.H. gave some suggestions for this paper. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-His antibody	Beyotime	Cat#ZRB2297; RRID: AB_10775216
Bacterial and virus strains		
MAX Efficiency® DH10Bac ™	Thermo Fisher Scientific	Cat#10361012
Bac-to-Bac baculovirus	Thermo Fisher Scientific	Cat#10359016
Chemicals, peptides, and recombinant proteins		
Gallic acid (98% purity)	Solarbio	Cat#SG8040
Isoquercitrin (98% purity)	Solarbio	Cat#SI8270
Rutin (98% purity)	Solarbio	Cat#SR8250
Chlorogenic acid (98% purity)	Solarbio	Cat#C8960
Epigallocatechin (98% purity)	Solarbio	Cat#SE8130
Catechin (98% purity)	Solarbio	Cat#SE8100
Quercetin (98% purity)	Solarbio	Cat#SQ8030
Methanol(chromatography grade)	Macklin	Cat#67561
Penicillin/streptomycin	Walkersville	Cat#516106
D-glucose-6-phosphate	Sigma-Aldrich	Cat#D810527
Glucose-6-phosphate dehydrogenase	Sigma-Aldrich	Cat#G822621
T7 RiboMAXTM Express RNAi System	Promega	Cat#P132
Critical commercial assays		
PrimeScriptTMRT reagent Kit with gDNA Eraser	Takara	Cat#RR047
RNAiso Plus kit	Takara	Cat#9108
BCA protein assay kit	Takara	Cat#T9300
TB Green Premix Ex Taq 2 (TliRNaseH Plus)	Takara	Cat#RR420
DNA polymerase I	Invitrogen	Cat#EP0041
Taq DNA polymerase	Invitrogen	Cat#1034205
Pfu DNA polymerase	Invitrogen	Cat#12344024
Sf-900 II SFM medium	LifeTechnologies	Cat#12659017
Fetal bovine serum	Invitrogen	Cat#A5669401
Bradford Protein Assay Kit	Beyotime	Cat#2740-OP
Experimental models: Cell lines		
Sf9 insect cells	Invitrogen	Cat#12552014
Software and algorithms		
Prism version 9.0	GraphPad Software	http://www.graphpad.com/scientificsoftware/prism/
Systat Software	SigmaPlot 12.0	https://systatsoftware.com/sigmaplot/
IBM, Chicago	SPSS Statistics 22	https://spss.en.softonic.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xue-Qing Yang (sling233@hotmail.com).

Materials availability

This study did not generate new unique reagents. All key resources are listed in the key resources table.

Data and code availability

- This paper does not report original code.
- All data are included in the article and/or supplemental information.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Fruits and chemicals

The *P. ussuriensis* and *P. bretschneideri* used in the experiment were gathered from the orchard located in Xiha Village, Hartau Town, Zhangwu County, Liaoning Province, China (122.14°E, 42.48°N). The orchard spans an area of 13,340 m^2, with plant spacing set as 6 m × 4 m.

The chemicals used for the extraction and detection of SMs were of chromatographic grade. The standards, namely gallic acid (98% purity), isoquercitrin (98% purity), rutin (98% purity), quercetin (98% purity), chlorogenic acid (98% purity), epigallocatechin (98% purity), and catechin (98% purity), were purchased from Solarbio (China). Methanol (chromatography grade) was purchased from Macklin (China).

Large fragment DNA polymerase I (9 U/μL), Taq DNA polymerase (5 U/μL), cloned Pfu DNA polymerase (2.5 U/μL), BamH I/Kpn I (10 U/μL), Sf9 insect cells, SF-900 serum-free medium, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Penicillin/streptomycin was purchased from Bio-Whittaker (Walkersville, MD, USA). D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase (360 U/mg protein) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Insects

The susceptible strain (SS) of *C. pomonella* and *G. molesta* were bred for more than 50 generations in a controlled environment without exposure to any insecticide or plant secondary material. The rearing method used in this study follows the protocol described by Hu et al. (2023).[49] Both species were raised in a climatic chamber maintained at a temperature of 26 ± 1°C, with a photoperiod of 16:8 (L: D) and a relative humidity of 60 ± 5%. Adults were provided with honey water at a concentration of 10% as their food source. Samples of *C. pomonella* and *G. molesta* were taken at random, regardless of gender.

METHOD DETAILS

Fruit hardness and infestation rate investigation

To assess fruit hardness and infestation rates, a survey was conducted every 20 days between May and September 2021, encompassing the stages of fruit expansion (I-IV) and fruit ripening (V-VI), as defined by Liu (2014).[50] Six pear trees of each species were randomly selected at each time point, then five fruits were randomly chosen from each tree, resulting in a total of 30 fruits selected for fruit hardness testing. Fruit hardness was measured using a GY-4 fruit hardness tester (Saiyas, China), with each fruit being tested four times. Additionally, 2 g of each fruit, including the flesh, peel, and kernel, were rapidly frozen in liquid nitrogen and stored at −80°C for further analysis.

In order to record the fruit infestation rate caused by the two fruit borers, five trees of both Pear species were randomly selected in the orchard, and five fruits of each tree were surveyed at each of the four cardinal directions (east, west, south, and north).

Extraction and detection of SMs

To investigate the alterations in SMs present in fruits after being infested, three *P. bretschneideri* and three *P. ussuriensis* that were harmed by *C. pomonella* and *G. molesta* were selected for analysis. This experiment was repeated three times, with unaffected fruits serving as the control group. Upon returning to the laboratory, the damaged sections of the fruit, including the pulp, skin, and core, were excised and rapidly frozen using liquid nitrogen, and stored at −80°C for further analysis. The extraction and detection of SMs were conducted according to Li et al. (2012)[51] with slight modification.

The samples were ground to a powdered form using liquid nitrogen in a mortar and subsequently transferred to a 50 mL centrifuge tube. Then, 30 mL of 80% chromatographic grade methanol was added to the tube, and the mixture was subjected to ultrasonication at 25°C for 30 min (using an ultrasonic power of 40 kHz). The tube was then placed in a shaker at a speed of 200 rpm for an additional 30 min, followed by centrifugation at 8500 rpm for 20 min. The resulting supernatant was carefully transferred to a new tube, and the aforementioned steps were repeated once. The crude extract was then subjected to rotary evaporation at a temperature of 35°C to remove the methanol, leaving behind a nearly dry residue. This residue was subsequently adjusted to a final volume of 10 mL using 80% chromatographic grade methanol and filtered through a 0.22 μm membrane prior to analysis. The amount of chlorogenic acid, epicatechin, catechin, quercetin, rutin, gallic acid, and isoquercitrin in pears was expressed as μg equivalents per g of fresh weight.

The samples underwent analysis using high-performance liquid chromatography (HPLC) (Agilent 1260, USA). The detection parameters were as follows: a 10 μL injection volume was utilized, and an XDB-C18 liquid chromatographic column from Agilent (USA) was employed. The mobile phase consisted of a mixture of a 0.1% formic acid aqueous solution (A) and chromatographic grade methanol (B). The mobile phases eluted in a gradient manner at a flow rate of 0.1 mL/min and a temperature of 30°C. The concentration gradient elution conditions were as follows: 40% B for 20 min, 50% B for 30 min, and 80% B for 40 min. Detection was performed at wavelengths of 280 nm, 320 nm, and 360 nm. Each experiment group was repeated 3 times.

Construction of population parameters

In order to construct a life table, an artificial diet was prepared for the rearing of larvae of *G. molesta* and *C. pomonella* using different concentrations of quercetin (10 μg/g, 100 μg/g, 1000 μg/g, and 5000 μg/g) and rutin (20 μg/g, 100 μg/g, 200 μg/g) according to quercetin and rutin contents measured in *P. ussuriensis* and *P. Bretschneideri*, respectively. The first instar larvae of both species that hatched within 24 h were reared in separate diets until they reached the pupal stage. To ensure proper ventilation and prevent escape, the rearing tubes were plugged with absorbent cotton. The development stages and survival rates of each species were recorded. During the pupal stage, the males and females were distinguished based on their pupal morphology (Feng et al. 2019).[52] After the emergence of adults, ten individuals consisting of five males and five females were placed together in an inverted clear plastic cup (7.2 cm in diameter and 7.9 cm in height) for mating. The cup was covered with plastic wrap to facilitate oviposition and sealed with a rubber band. To ensure air permeability, holes were made in the plastic cups, and cotton was placed at the bottom to provide a 10% honey solution. The fecundity and survival rate of the adults were recorded on a daily basis until the death of all individuals. Each concentration of quercetin and rutin was treated with 100 larvae and the experiment was repeated three times for each concentration. A control group without quercetin and rutin were also included.

Determination of P450 enzyme activity

Fifteen fourth instar larvae were collected from each treatment, and their intestines were dissected to detect P450 enzyme activity. This process was repeated three times for each sample. To extract the P450 enzyme, the test insects were ground into powder and placed in a 1.5 mL centrifuge tube. Then, 1 mL of pre-cooled P450 enzyme extraction buffer was added and mixed thoroughly. The mix was centrifuged at 14000 rpm for 30 min at 4°C. The supernatant of each sample was carefully transferred to a new enzyme-free centrifuge tube for further use.

The protein concentration was determined using the Takara BCA Protein Assay Kit (Takara, China) according to the provided instructions. The P450 enzyme activity was assessed following the method described in Li et al. (2023)[39] with some minor adjustments. In brief, the extracted P450 enzyme solution was mixed with 7-ethoxy-coumarin substrate (2 mM) and NADPH (10 mM) in a centrifuge tube. The volume of the mixture was then increased to 200 μL by adding 100 mM sodium phosphate buffer (pH 7.8). After an incubation period of 10 min at 30°C, trichloroacetic acid (15%) was added to the mixture (60 μL) to stop the reaction. The resulting mixture was then centrifuged at 4°C for 10 min, and 200 μL of the supernatant was transferred to opaque microplate wells containing glycine/sodium hydroxide (1.6 mM, pH = 10.5) in a volume of 90 μL. The absorption value of the mixture was measured at an excitation wavelength of 358 nm and an emission wavelength of 456 nm. The P450 enzyme activity was determined by quantifying the amount of 7-hydroxycoumarin (ECOD) produced within a 10-min time frame. A standard curve was established with various concentrations of the ECOD standard, and their absorbance values were correlated.

Total RNA extraction, cDNA synthesis, and RT-qPCR

The RNA extraction procedure was performed using the RNAiso Plus kit (Takara, China) according to the manufacturer's instructions. The concentration of the extracted RNA samples was determined using the NanoDrop 2000 (ThermoFisher Scientific, USA). Subsequently, the first strand cDNA synthesis was performed using 1 μg of total RNA, following the instructions provided by the PrimeScriptTMRT reagent Kit with gDNA Eraser (Takara, China).

There are 72 and 77 P450 genes in *C. pomonella* and *G. molesta*, respectively. *C. pomonella* possessed 31 Clan 3 genes,[53] whereas *G. molesta* had 30.[54] In this study, the expression levels of all P450 Clan 3 genes in *C. pomonella* and *G. molesta* were analyzed. The expression levels of P450 genes were quantified using real-time quantitative PCR (RT-qPCR) on a Bio-Rad CFX96 (BioRad, USA). The reaction mixture consisted of 1 μL of cDNA template, 10 μL of TB Green Premix Ex Taq 2 (Takara, China), 0.8 μL of each primer, and 7.4 μL of sterile water. The *EF-1α* and *β-actin* genes were set as internal reference genes for *C. pomonella*[55] and *G. molesta*, 56 respectively. The specific primers used in the RT-qPCR are listed in Table S1. The reaction conditions included an pre-denaturation step at 95°C for 30 s, followed by denaturation at 95°C for 10 s, annealing at a temperature determined by primer requirements for 30 s, and extension at 72°C for 30 s. The amplification was carried out for a total of 40 cycles. A no template control was included by replacing the cDNA in the reaction mixture with ddH_2O. Each sample was analyzed in triplicate. The gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).[57]

RNA interference (RNAi) of P450 genes

Double-stranded RNA (dsRNA) synthesis was performed following the instructions provided by the T7 RiboMAXTM Express RNAi System (Promega, USA). The resulting dsRNA solution was diluted to a concentration of 2000 ng/μL. Prior to injection, both test fourth-instar larvae were briefly exposed to ice for 5 min to induce temporary anesthesia, after which they were promptly transferred to a Petri dish for injection. A volume of 1 μL of dsRNA was injected into the three-to-five segment at the end of the posterior end of each larva' abdomen, while an equal amount of dsGFP was injected as a control. Subsequently, the injected larvae were returned to their diet without quercetin and kept under appropriate rearing conditions. At time intervals of 6 h, 12 h, 24 h, and 48 h post-injection, samples were collected from each treatment group. For each time point, three larvae were selected from each group for analysis.

Effect of knock down of P450 genes on the growth

Fourth-instar larvae of *C. pomonella* and *G. molesta* underwent a period of 12 h of fasting before being subjected to injection with *dsRNA* and *dsGFP* of the target genes. These injected larvae were then provided with artificial diets containing 100 μg/g quercetin, which similar to the

content in their host pears. The weight of the insects was measured at 12 h intervals over a duration of for 48 h. For each treatment, a total of fifteen larvae were selected and the experiment was replicated three times to ensure reliability. Prior to and after the experiment, the weights of diets, feces, and larvae were recorded after undergoing a drying process. The Relative growth rate (RGR), Relative consumption rate (RCR), Efficiency of the conversion of ingested food (ECI), and Efficiency of the conversion of digested food (ECD) were calculated using the formulas presented in Table S2.

Construction of the recombinant plasmids

The open reading frame sequence of *CYP6K1B*, *CYP6AW1*, *CYP6B74*, *gm_13876*, and a NADPH-dependent cytochrome P450 reductase (CPR) were synthesized directly by Tsingke Science (Beijing, China) following the method described in Lu et al. (2021).[58] They were then subcloned into the pFastBac1 vector using restriction enzyme digestion. The resulting recombinant plasmids were analyzed and verified through PCR and sequencing, and were subsequently stored at −20°C. To generate the recombinant Bacmid DNA, the recombinant pFastBac1 constructs were transformed into the MAX Efficiency DH10Bac chemically competent cells (Thermo Fisher Scientific, Langenselbold, Germany). Positive DH10Bac cells containing the recombinant bacmid DNA were confirmed through PCR using M13 forward and reverse primers. The recombinant bacmid DNA was then isolated, quantified, aliquoted, and stored at −20°C in TE Buffer at a concentration of approximately 500 ng/μL.

Heterologous expression of P450s

Recombinant P450 proteins were produced using Sf9 cells. The bacmids containing CYP6K1B, CYP6AW1, CYP6B74, and gm_13876 were transfected into the Sf9 insect cells using a Bac-to-Bac baculovirus expression system (Thermo Fisher Scientific, Langenselbold, Germany) following the manual instructions. The titer of the recombinant virus was determined following the manufacturer's instructions. Sf9 cells were co-infected with recombinant baculoviruses expressing P450s and CPR, with a multiplicity of infection (MOI) of 1 and 0.1, respectively. The Sf9 cells were maintained at 27°C using Sf-900 II SFM medium (LifeTechnologies, Carlsbad, CA, USA), supplemented with 2.5 μg/mL hemin and 0.3% (v/v) fetal bovine serum. To detect successful expression of the recombinant protein using western blot, a positive recombinant control CYP341B14/CPR (accession number: LC326250.2) underwent the same experimental procedure. After 72 h, cells were harvested to isolate the microsomal fraction, which was then aliquoted and stored at −80°C after protein quantification using the Bradford Protein Assay Kit (Beyotime, Shanghai, China).

Western blot

Microsomal fractions containing the recombinant CYP341B14/CPR positive control, fused with the C-terminal overhang of the His tag, were denatured by incubation at 70°C for 5 min and separated using SDS PAGE. The membrane proteins were then blotted onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany) using a Bio-Rad blotting system. The membrane was blocked with a 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween 20 (TBST) buffer) for 1 h, followed by overnight incubation with Anti-His antibody (1:2000; Beyotime, Shanghai, China) in a 0.25% (w/v) non-fat dry milk TBST buffer at 4°C.After three washes with TBST buffer, the membrane was briefly incubated with SuperKine enhanced chemiluminescence (ECL) solution (Abbkine, Wuhai, China), and visualized using the Tanon 5200 chemiluminescent imaging system (Tanon, Shanghai, China).

Metabolism assay

The metabolism assay was assessed following the method described in Mao et al. (2009)[41] with some minor adjustments. Reaction mixtures for quercetin were set up with 100 pmol P450, 5 μL of 5 mM stock solution for tested quercetin, 5 μL of 2.5 mM stock solution for tested rutin, 0.5 mg of D-glucose-6-phosphate, 0.5 μL of glucose-6-phosphate dehydrogenase, 50 μL of NADPH (1 mg/mL in 0.1 M phosphate buffer (pH 7.8) or 50 μL of phosphate buffer (for the no NADPH control). The total volume was adjusted to 500 μL with 0.1 M phosphate buffer (pH 7.8). The reaction mixtures were incubated at 30°C for 90 min in a shaking metallic bath. Each 500 μL reaction was then combined with an equal volume of acetone and centrifuged at 10,000g for 10 min at room temperature. The reaction products were analyzed with a reverse phase XDB- C18 column (AG120, 5 μm, 4.6 × 150 mm; Agilent Technologies Ltd., USA). For quercetin, a mobile phase of 70% water containing 0.1% phosphoric acid and 30% acetonitrile was used. For rutin, a mobile phase of 83% water containing 0.1% phosphoric acid and 17% acetonitrile was used. The absorbances of quercetin were monitored at 372 nm and rutin at 376 nm using a Waters 996 photodiode array detector. The analyses for quercetin and rutin metabolism were repeated three times. Quercetin standard was dissolved diluted to five different concentrations (5, 10, 20, 50, and, 100 μg/mL) in methanol, while rutin standard was dissolved diluted to five different concentrations (12.5, 37.5, 50, 62.5, and 87.5 μg/mL) in methanol for kinetics assay.

QUANTIFICATION AND STATISTICAL ANALYSIS

The data were presented as the mean of at least three independent experiments±SD and visualized using GraphPad Prism 5 software (GraphPad Software, CA). The expression levels of P450 genes in different development stages and tissues of *C. pomonella* and *G. molesta*. Different letters indicate significant differences ($p < 0.05$) using one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) tests ($p < 0.05$) using SPSS Statistics 22 (IBM, Chicago). RNAi efficiency of P450 genes in *C. pomonella* and *G. molesta* were analyzed by Student's t-test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$).