Multiple-omics techniques reveal the difference between polyphagous Conogethes punctiferalis and oligophagous C. pinicolalis

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Abstract

Although Conogethes punctiferalis and Conogethes pinicolalis have reported on morphology and molecular identification, the molecular biology differences between the two species in different diets are still unknown. In this work, we investigated the difference in the biological process of the two species by the methods of transcriptomics, proteomics, metabolomics, and bioinformatics. The results showed that the expression of 74,611 mRNA in the transcriptome, 391 proteins in the proteome, and 218 metabolites in the metabolome had been significantly changed between the two species, and the KEGG results showed the data were mainly closely related to metabolism and redox. Moreover, based on integrating system-omics data, we found that the α-amylase and CYP6AE76 gene have mutations between the two species. Different sequence analysis results may differ significantly in their exact substrate preference and product profile, which indirectly leads to the different detoxification or metabolism ability, and the gene expression of relevant was also confirmed by the qPCR and enzyme activity test. These findings in the two species and integrated networks provide beneficial information for further exploring the molecular mechanism of polyphagous and oligophagous.

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Although *Conogethes punctiferalis* and *Conogethes pinicolalis* have reported on morphology and molecular identification, the molecular biology differences between the two species in different diets are still unknown. In this work, we investigated the difference in the biological process of the two species by the methods of transcriptomics, proteomics, metabolomics, and bioinformatics. The results showed that the expression of 74,611 mRNA in the transcriptome, 391 proteins in the proteome, and 218 metabolites in the metabolome had been significantly changed between the two species, and the KEGG results showed the data were mainly

closely related to metabolism and redox. Moreover, based on integrating system-omics data, we found that the α-amylase and CYP6AE76 gene have mutations between the two species. Different sequence analysis results may differ significantly in their exact substrate preference and product profile, which indirectly leads to the different detoxification or metabolism ability, and the gene expression of relevant was also confirmed by the qPCR and enzyme activity test. These findings in the two species and integrated networks provide beneficial information for further exploring the molecular mechanism of polyphagous and oligophagous.

Keywords:

Conogethes punctiferalis ; *Conogethes. pinicolalis* ; transcriptomics; proteomics; metabolomics; gene mutation

1. Introduction

Conogethes punctiferalis (Guenée), is an important agricultural pest of chestnut (*Castanea mollissima*), peach (*Amygdalus persica*), apple (*Malus pumila*), maize (*Zea mays*), and sunflower (*Helianthus annuus*) (Luo and Honda, 2015). In some regions of China, it has become the main pest of corn, causing more significant damage than*Ostrinia furnacalis* (Guenée), the most prevalent corn pest in China (Wang et al., 2006). *C. pinicolalis* (Lepidoptera: Crambidae) is a sibling species of *C. punctiferalis* , even though it is considered the same species at the early stage. Koizumi first identified and classified the *C. pinicolalis* as another type of*C. punctiferalis* and identified and classified the *C. pinicolalis* as another type of *C. punctiferalis* commonly known Pinaceae-feeding type in 1963 (Koizumi, 1963). Honda and Mitsuhashi identified and distinguished the difference between them in the adults, larvae and pupal stages (Honda and Mitsuhashi, 1989); Konno et al. tested and reported that they were different species from their response to different spectra of host-plant constituents (Konno et al., 1981); In the end, the pinaceae-feeding type was named *C. pinicolalis* in 2006 (Inoue et al., 1982).

The two sibling species,*C. punctiferalis* and *C.pinicolalis,*are important pest species in China. These two insects are similar in morphologically, almost indistinguishable at the egg, larval and pupal stages, and only well-trained entomologists can identify them by some morphological characteristics in the adult stage. Moreover, they are so similar that they both use (E)-10-hexadecenal (E10-16:Ald) and (Z)-10-hexadecenal (Z10-16:Ald) as their main sex pheromone components, but their foraging ranges are widely differentiated. *C. punctiferalis* is a polyphagous species posing a major threat to over 100 essential plant species (Lu et al., 2010), while *C. pinicolalis* is an oligophagous insect, mainly fed on few pine trees, especially for *Pinus massoniana* (Masson pine). Their feeding preferences may be associated with olfactory and gustatory system or digestive system, and this preference ultimately must manifest as the result of the regulation of proteins, genes, and their pathways. Therefore, it was of great interest to unveil the difference in some function genes or proteins between the two species.

In this study, we applied a proteomic technique, the isobaric tags for relative and absolute quantification (iTRAQ), and RNA sequencing-based transcriptome technique. The transcripts with new exons were identified from an alternative splicing database to understand further the related proteins and transcripts involved in feeding preferences. At the same time, metabolomics was used to detect the difference between the two species. Our result provided a profound understanding of the functions of the different genes about polyphagous and oligophagous.

2. Material and methods

2.1 Insects rearing and antennae collection

C. punctiferalis larvae were collected from corn ear at Langfang Experimental Station of Chinese Academy of Agricultural Sciences, Hebei Province, China, and reared on fresh corn ear in an environmentally controlled room at 27 ± 1 °C, 70-80 % relative humidity (RH), and 16:8 light: dark (L:D).

C. pinicolalis larvae were collected from the masson pine in Quanjiao County (32.07 N 117.54 E), Anhui Province, China. Fresh masson pine branches were used to feed the larvae under ambient conditions 27 ±

0.5 °C, with 70-75 % relative humidity (RH) and a photoperiod of 16:8 h light: dark (L:D). After emergence, the moths were feed on 10% honey solution (Braccini et al., 2015).

2.2 RNA extraction and transcriptome sequencing

Total RNA of all test samples was isolated using the Quick-RNA^{TM} MicroPrep Kit (ZYMO Research, USA) according to the manufacturer's protocol. The integrity of the total RNA was analyzed using 1.5 % agarose gel electrophoresis (Cui et al., 2017). The quality and concentration were analyzed on NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The cDNA was synthesized by following the instructions from RT^{TM} All-in-One Master Mix Kit (Herogen Biotech, USA). Transcriptome sequencing was performed at Novogen Co., Ltd. Beijing, China, and the samples were sequenced on the Illumina Hiseq 2500 platform. The raw reads were curated by removing adaptor sequences and reads of low quality, then assembled into unigenes using Trinity (Huang et al., 2018; Grabherr et al., 2011).

2.3 Protein extraction and sequencing

Total proteins were extracted from the fourth instar whole body of two insects with three biological replicates according to a previously described protocol (Unwin et al., 2010) with minor modifications. Samples were ground to a powder in liquid nitrogen and lysed with 2 mL lysis buffer containing 8 M urea, 2 M thiourea, 0.1 % 3-[(3-cholamidopropyl) dimethylammonio propanesulfonate (CHAPS) (Amresco Ltd., USA) and 1 × Protease Inhibitor Cocktail (Roche, USA). The lysis solution was centrifuged at 4 °C, 13,000 × g for 15 min to collect the supernatant in a new tube and then save it at -80 °C until use. The protein concentration was determined using a 2-D Quant Kit (GE Healthcare, USA), and quality was examined with SDS-PAGE (Beyotime, China). Protein digestion was conducted using trypsin (Promega, USA) at 37 °C overnight, and peptides were dried in a centrifugal vacuum concentrator.

According to a previously described protocol, protein isolation and labeling were performed using the 8-plex iTRAQ (Applied Biosystems) according to a previously described protocol (Wang et al., 2015) with some modifications. Sample peptides were subjected to nano-electrospray ionization, followed by tandem mass spectrometry (MS/MS) in an Orbitrap Q-Exactive plus system (Thermo Fisher Scientific, USA). MS scans were obtained from m/z 350-1,800, with 40 precursors selected for MS/MS from m/z 100-1,800 using a dynamic exclusion of 40 s for the selected ions. The collision-induced dissociation (CID) energy was automatically set as 32 %. The database search strategy-based peptide matching tolerance was controlled below 10 ppm and 0.05 Da to prevent the omission of proteins.

2.4 Metabolomics analysis

MetWare (Wuhan, China) performed the extracted analysis, metabolite identification, and quantification following their standard procedures and a previous study (Yuan et al., 2018).

2.5 Correlation analysis

Correlation analysis was carried out between transcriptomic analysis revealed differentially expressed genes (DEGs) and analysis of differentially expressed proteins (DEPs). Functional annotation of transcripts and proteins data were searched using BLASTX against the non-redundant (nr) NCBI protein database (Liu et al., 2012). The calculation of unigene expression uses the FPKM method (Fragments Per kb per Million reads); In addition, using Blast2GO (http://www.blast2go.org) (Conesa et al., 2005), we predicted and classified functions of unigenes by Clusters of EuKaryotic of orthologous groups (KOG) database (Ashburner et al., 2000). In addition, the online Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (KAAS) was employed for KEGG pathway enrichment analysis following the procedure of pathway annotations for transcripts and proteins data (Kanehisa et al., 2008; Shevchenko et al., 2008).

2.6 Gene sequences verify and qPCR detection

Total RNA was reverse transcribed to cDNA using RT^{TM}All-in-One Master Mix Kit (Herogen Biotech, Shanghai, China) according to the protocol of the manufacturer, then PCR technology was used to amplify the selected gene sequences in two species. qPCR (quantitative real-time PCR) experiments were conducted

with actin as an internal ribosomal protein RP49 (Yang et al., 2017), and calculations were performed as described previously (Jing et al., 2020). All primer sequences are given in (Supplementary Table 1).

2.7 ὂμπυτατιοναλ αναλψσις οφ α-αμψλασε ανδ ˆΨΠ6AE76

The α-amylase and cytochrome P450 (CYP) monooxygenase CYP6AE76 ammino acid sequences of *C. pinicolalis* and *C. punctiferalis* were submitted to structure homology modeling using Swiss-Model server (https://swissmodel.expasy.org/) (Waterhouse et al., 2018). The UCSF ChimeraX v1.1 was used to superimpose and visualize the 3D modeled structures of α-amylase 1 and CYP6AE76 from *C. pinicolalis* and*C. punctiferalis* (Goddard et al., 2017). The ESPript 3.0 was used to compare the α-amylase 1 and CYP6AE76 ammino acid sequences of*C. pinicolalis* and *C. punctiferalis*(https://espript.ibcp.fr/ESPript/ESPript/) (Robert et al., 2014).

2.8 Preparation of recombinant protein

The methods of protein expression, purification and Western blot followed by the previously reported; more specific parameters were showed in supplement figure (Supplementary Table 2).

2.9 Enzyme activity assays

The α-amylase activity was tested using an amylase activity assay kit (Sigma-Aldrich, MO, USA) according to the manufacturer's protocol. Briefly, 20 uL of purified α-amylase expressed in the *Escherichia. coli* expression system and 30 μL of Amylase assay buffer was added to each well of the microplate. The reaction was initiated by adding 100 μL of the Master reaction mix and mixed using a horizontal shaker. After 3 min, an initial optical density was read at 405 nm. The plate was incubated at 25 °C and measured the absorbance (405 nm) every 5 minutes. One unit is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmole of p-nitrophenol (p-NP) per minute at 25 °C.

The CYP6AE76 activity was assessed according to the method reported by Qian et al. (Qian et al., 2008) and Shabbir et al. (Shabbir et al., 2021) with slight modification. A 125 μL of 2 mM p-nitroanisole (p-NA) solution and 50 μL cytochrome P450 monooxygenase expressed in *E. coli* were added to each well of a microplate and mixed. This mixture was incubated at 27 °C for 2 min, and the reaction was initiated by the addition of 25 uL of 9.6 mM NADPH. The optical density at 405 nm was recorded using a microplate reader (FlexStation 3, Molecular Devices, CA, USA).

3. Results

3.1 Transcriptomic and proteomic analysis

The results of RNA sequencing from*C. punctiferalis* and *C. pinicolalis* were a total got 203,131 assembled unigenes with a mean length of 1119 bp and N50 length of 1753 bp (Table 1). The total numbers of sequences detected by mass spectrometry of both unique spectra were 21,646, which represented 13,680 unique polypeptides, and 3,728 proteins were matched (Table 2). The total DEPs between *C. punctiferalis* and *C. pinicolalis*were 391.

3.2 Correlation analysis between DEGs and DEPs

Totally, 74611 DEGs and 391 DEPs were correlated and analyzed according to their difference multiples (Figure 1A). GO analysis was used to classify the Biological process, Molecular function and Cellular components after the transcriptomic and proteomic correlate analysis (Supplementary Figure 1A). The results showed that these genes were mainly closely related to metabolism and redox (Supplementary Figure 1B and C). From KEGG annotation, the data as well as mainly closely related to metabolism and redox (Supplementary Figure 2). After correlation analysis, we found that 249 of the transcriptome and proteome data are overlapped, and 142 proteins are differential proteins after correlation analysis (Figure 1C). Next, we selected all the proteins (30 proteins) related to digestion and metabolism from 142 different proteins for further study (Supplementary Table 3). Then based on whether using PCR can obtain the open reading frame (ORF) of the genes form the transcriptome data, a total of 9 proteins were selected (Table 3).

3.3 Superimpositions and sequence comparison

The ESPript 3.0 computed the structure elements, α-amylase is highly conservd, and no amino acid mutations have been found in the homologous sequence regions and active sites (Figure 2A-left). CYP6AE76 has high sequence identities in Six substrate recognition sites (SRS) 1, SRS3, SRS4 and SRS5, and SRS2 with SRS6 shows the lowest identity. In addition, both two sequences had mutations at the WxxR site, but no mutations were found at the ExxRxxP and Heme-binding sites (Figure 2A-right). Superimpositions of each model with the template using UCSF ChimeraX v1.1 software showed a very low RMSD value of 0.130 Å for α-amylase 1 from *C. pinicolalis* and*C. punctiferalis* (Figure 2B). Similarly, a low RMSD value of 0.224 Å was observed between the superimposed CYP6AE76 3D structures of*C. pinicolalis* and *C. punctiferalis* , respectively (Figure 2B). In addition, it can be seen from the 3D diagram that although the amino acid sequence is partially different, it does not affect the overall structural change.

3.4 Detection of the expression levels of two genes

The results show that both genes (α-amylase and CYP6AE76) are highly expressed in *C. punctiferalis* and reached a significant level compared with *C. pinicolalis* (Figure 3). This result is the same as the trend in the transcriptome data (Figure 1C).

3.5 α-αμψλασε ανδ"ΨΠ6AE76 αςτιιτψ

Four proteins were successfully expressed in *E. coli*(Supplementary Figure 3), and the protein obtained after subsequent purification was used for the enzyme activity test. The amount of substrate, ethylidene-pNP-G7, cleaved by the purified α-amylase from *C. pinicolalis* showed higher activity than *C. punctiferalis* (Figure 4A). Furthmore, conversion of p-NA to p-NP by CYP6AE76 was significantly higher in *C. punctiferalis* (Figure 4B).

3.6 Differentially changed metabolites in two species

To further understand the difference in metabolic leading to contrasting polyphagous and oligophagous in the two species, we compared the metabolite in*C. punctiferalis* and *C. pinicolalis* . After the comparative metabolome analysis, a total of 583 differential genes were annotated (76 down accumulated, 142 accumulated, Figure 5A), and top 20 down- and up-accumulated differential metabolic were shown in Figure 5B. From KEGG annotation, the data as well as mainly closely related to metabolism, detoxication and redox (Supplementary Figures 4 and 5).

4. Discussion

The study of the multiple-omics techniques of polyphagous *C. punctiferalis*and oligophagous *C. pinicolalis* can initially, from a particular aspect reveal the reasons of the different dietary habits in the two species. In this study, the genes related to carbohydrate metabolism were selected and some of those genes were different in highly expression and sequence mutations. Actually, the differential expression or mutation of these genes is an important reason for their different eating habits.

A role for gene duplication or amplification in resistance or detoxicate has now been demonstrated for some species, both as a route of enhanced production of metabolic enzymes (Devonshire et al., 1991; Bass and Field, 2011). The increased production of metabolic enzymes, which can break down or bind to (sequester) the pesticide (Bass and Field, 2011). However, in this study, the differential expression of the two genes in different species is more closely related to their food. α-amylase is an oligosaccharide endoglycosidase, an enzyme that cleaves an internal glycosidic bond within a poly or oligosaccharide helps digest carbohydrates (starch and glycogen) into simple sugar [glucose (monosaccharide) and maltose (disaccharide)] for energy. α-amylase is highly expressed in *C. punctiferalis* may relationship with the kind of hosts. *C. punctiferalis* larvae have been reported to attack more than 100 essential plant species, including peach, durian, chestnut, citrus, papaya, cardamom, ginger, etc (Lu et al., 2010), and those plant can support more carbohydrates. On the contrary, the host of*C. pinicolalis* is only Masson pine. The literature shows that Masson pine needles

contain a lot of cellulose, fat and protein, etc (Tian, 2006; Nie et al., 2020). Therefore, the type of food is relatively simple, and the nutrients in it are limited, and the demand for amylase will be relatively low.

The genomes of phytophagous insects usually contain large numbers of P450s, especially within the CYP3 clan. Within this clan, CYP6 subfamily members help detoxify plant host secondary metabolites (Feyereisen, 2012; Mittapelly et al., 2019; Tzou et al., 2000). Knockout of the CYP6AE cluster does not affect the viability of the insect, but it results in increased susceptibility to both plant toxins and synthetic insecticides (Wang et al., 2018). In this study, *C. punctiferalis*as a polyphagous insect, CYP6AE76 gene is not only highly expressed in larvae (Figure 3), but the enzyme activity level is also significantly higher than oligophagous *C. pinicolalis* (Figure 4). Moreover, Mittapelly *et al* . (Mittapelly et al., 2019) reported that the CYP6 gene expression in polyphagous insects is not based on host diet, however, they might use a cocktail of broad-spectrum detoxification enzymes that interact with a variety of compounds encountered in their diets and these CYP6Bs may be part of that cocktail. Those results showed that polyphagous *C. punctiferalis* needs more CYP6AE76 to metabolize or detoxify substances from a variety of foods. On the contrary, oligophagous *C. pinicolalis* only eats pine needles, so there is no need for multiple detoxification and metabolism compared to*C. punctiferalis* . In addition, previous studies showing induction of some CYP6AE genes by specific chemicals or different host plants (Zhou et al., 2010; Celorio-Mancera et al., 2011). However, the pine needles may contain a small amount of specific chemical substances mentioned above, and the food source of *C. pinicolalis* is relatively single, so the expression of the CYP6AE76 gene will be relatively low.

Environmental conditions are not always suitable for survival, and insects employ multiple strategies for adaptation (Zhai et al., 2019). After long-term evolution, the two species of *C. punctiferalis* and *C. pinicolalis* have become more distinct in adults and can be distinguished by the mitochondrial cytochrome c oxidase gene (Wang et al., 2014; Jeong et al., 2021). In this study, after a multi-omics joint analysis, α-amylase and CYP6AE76 were found to have mutations. However, no mutations were detected in the homologous conserved regions and enzyme active sites in α-amylase (Figure 2A), and the sequence similarity could reach 94% (Table 3). On the contrary, mutations appeared in some other regions. Although these mutations do not cause structural changes, they may also differ significantly in their exact substrate preference and product profile (Janeček and Gabriško, 2016). Therefore, those mutations may have caused the high expression of α-amylase and its enzyme activity in *C. pinicolalis* , thus affecting their metabolism or detoxification of food. Although the P450 super-family has a wildly divergent sequence and the overall homology may be less than 40% even within the same family, particularly in insects (Wang et al., 1995), there are function-critical sequence motifs preserved during evolution heme-binding sequence motif (FxxGxxxCxG) universal among CYP enzymes. In this study, no mutations in the heme-binding site were detected which suggested the main function has not changed. However, all SRS sties of the two species have mutations. Some studies have demonstrated that amino acids in SRSs affect the protein folding and substrate range of cytochrome P450s, especially SRS1 in a loop region, close to the active site heme, has proven to be the most crucial SRS that affects multiple properties of P450s (Domanski and Halpert, 2001; Schuler and Berenbaum, 2003; Shi et al., 2020). Recently, Zuo et al (Zuo et al., 2021) revealed that the mutation located in the SRS1 region of CYP9A186 of*Spodoptera exigua* , causes resistance to both emamectin benzoate and abamectin. In addition, target-site resistance involves alterations (e.g., mutations) in the insecticide target protein that reduce its sensitivity to insecticides (Zimmer et al., 2016). Therefore, due to these mutations, the binding ability is different, which indirectly leads to the different detoxification ability, and it has been verified by the qPCR and enzyme activity test. However, the difference of feeding habits of insects is result of their long-term adaptation to the environment, and genetic mutation is one of many factors. Therefore, further research is needed.

Metabolites are the end products of cellular regulatory processes. Therefore, it is necessary to understanding the final metabolites difference in dietary habits between the two species. The detection results showed more metabolic difference substances in *C. punctiferalis* than *C. pinicolalis* . Among the top 20 down-and up-accumulated metabolites, *C. punctiferalis* is mainly metabolizes of amino acids, organic acids, and alcohols; while *C. pinicolalis* mainly metabolizes of lipids, organic acids, and terpenes (Figure 5B). These differences can also reflect their different foods resource, especially in *C. pinicolalis* . The pine needles contain many

lipids (Tian et al., 2006), so these enzymes are needed for metabolism, this may also be the reason why its α-amylase activity is stronger than that of *C. punctiferalis* . The KEGG classification indicates that *C. punctiferalis* is enriched in more metabolic pathways than *C. punctiferalis* . For example, biosynthesis of amino acids, pyrimidine metabolism, ATP binding cassette transporters (ABC) transporters, etc (Supplementary Figure 4). However, ABC as a transporter has been increasingly recognized with resistance to cancer chemotherapy in humans, drug resistance in protozoa, antibiotic resistance in bacteria, and pesticide detoxification in nematodes, arthropods and Lepidoptera pests in recent years (Lage, 2003; Porretta et al., 2016; Zhou et al., 2020). In the current study, although esterases, glutathione S-transferases and P450 as three prominent enzyme families are well known implicated in metabolic and digestive (Li et al., 2007), no significant differences were found in the metabolites of the two species. More interestingly, ABC transporters are higher in polyphagous *C. punctiferalis* than in oligophagous *C. pinicolalis* , suggesting polyphagous insects need more metabolism and transport pathways to degrade or digest different foods or toxins in foods. Therefore, more in-depth research is needed on ABC transporters.

5. Conclusion

To summarize, the present study showed that the mRNA levels, proteins and metabolites had significantly altered in polyphagous *C. punctiferalis* and oligophagous *C. pinicolalis* by the multi-omics techniques and all the data were mainly closely related to metabolism and redox. In particular, the mutations of α-amylase and CYP6AE76 gene lead to differences in gene expression levels and enzyme activities, resulting from long-term evolutionary selection between the two species. These findings increase our understanding of the two species and offer new perspectives for revealing the molecular mechanisms of polyphagous and oligophagous.

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Tables

Table 1 Summary of assembled contigs and unigenes

Type (bp)	Contig	Unigene
Total number	257,639	203,131
Total length	241,804,378	227,279,444
Min length	201	201
Mean length	939	1119
Maximum length	25005	25,005
N50	1,638	1,753
N90	355	469
DEGs	-	74,611

Table 2 Summary of iTRAQ metrics

Metrics	Number
Unique polypeptide	13,680
Unique spectra	21,646
Matched proteins	3,728
DEPs	391

Table 3 Summary of genes selected from DEGs and DEPs

Gene name	Sequences similarity rate (%)	Gene description
alpha-amylase	94	Hydrolyses alpha bonds of large, alpha-linked polys
acetyl-CoA acetyltransferase	100	Catalytic enzyme
glutathione S-transferase	100	Detoxification
chymotrypsin BII-like	100	Diverged evolution, including digestive process
P450 monooxygenase CYP6AE76	92	Detoxification
pancreatic triacylglycerol lipase	100	Digestive enzyme
cytochrome P450 6B2	100	Detoxification cytochrome P450 6B2-like
beta-fructofuranosidase	100	Hydrolyze sucrose aiming to produce inverted suga
protein dj-1beta-like	100	Antioxidants protein dj-1beta-like isoform X1

Figure legends

Figure 1. Interaction analysis of DEGs and DEPs. **(A)**Correction plot analysis based on DEGs and DEPs. **(B)** Venn diagram of DEGs and DEPs. **(C)** Heat map based on FPKM value of DEGs and DEPs.

Figure 2 . Diagram of the secondary and tertiary structure of the mutant gene. **(A)** Comparison of amino acid sequences of alpha-amylases and CYP6AE76. Homologous sequence regions 1, 2, 3 and 4 are surrounded by purple rectangles. The amino acid sequence described above the rectangular regions was taken as representative of regions 1 to 4, respectively. Active sites and those of substrate binding proposed by Matsuura et al. (Matsumura et al., 1984) for Taka-amylase A from are indicated by the purple triangle and black oval, respectively; SRS is represented in green line boxes, heme-binding signature motif (FxxGxxxCxG) (black-dotted rectangular frame), helix C motif (WxxxR) (navy blue box), and PxPF motif are in lightboxes.**(B)** superimpositions of predicted models of α-amylase and CYP6AE76 from *C. pinicolalis* and *C. punctiferalis* with their respective templates.

Figure 3. Gene expression of α-amylase and P450 monooxygenase CYP6AE76 in *C. pinicolalis*and *C. punctiferalis* . **(A)** The relative expression of α-amylase in *C. pinicolalis* and *C. punctiferalis* .**(B)** The

relative expression of P450 monooxygenase CYP6AE76 in *C. pinicolalis* and *C. punctiferalis* . The gene expression level between the two species was statistically significant (t -test, $^{***}P < 0.001$).

Figure 4. The comparison of enzymatic activity of recombinant α-amylase and CYP6AE76 from two species. **(A)** The amount of ethylidene-pNP-G7 (substrate) cleaved by the purified α-amylase from *C. pinicolalis* and *C. punctiferalis* . **(B)** Conversion of p-nitroanisole to p-nitrophenol in by recombinant cytochrome P450 from *C. pinicolalis* and *C. punctiferalis* . The α-amylase and CYP6AE76 enzyme activities were statistically significant (t -test, $^{**}P < 0.01$).

Figure 5. Association analysis the different genes between the two species (*C. pinicolalis* vs *C. punctiferalis*).**(A)** Volcano map of all differential genes;**(B)** Top 20 down- and up-accumulated metabolites between the two species.

Figure S1. Transcriptome and proteomics association analysis.**(A)** Differential gene GO enrichment. **(B)** and**(C)** Differential gene GO enrichment of biological process and molecular function, respectively.

Figure S2. KEGG pathway enrichment scatter plot of the different genes.

Figure S3. Candidate gene recombinant expression protein.**(A)** SDS-PAGE analysis of purified recombinant Candidate proteins. The four SDS-PAGE diagrams are Coomassie Blue staining of purified α-amylase (1 and 2) and CYP6AE76 (3 and 4) protein samples from left to right in *C. pinicolalis* vs *C. punctiferalis* , respectively. M: Protein marker; Lane 1,2,5,6,9,10,13 and 14: Sonicated bacterial pellet; 3,4,7,8,11,12,15 and 16: purified protein.**(B)** Western blot analysis of α-amylase (Line 1 and 2) and CYP6AE76 (Line 3 and 4) protein samples from left to right in *C. pinicolalis* vs *C. punctiferalis* , respectively. M: Protein marker.

Figure S4. Metabolite KEGG classification map.

Figure S5. KEGG pathway enrichment scatter plot of the different metabolite.