Genome-wide scans and transcriptomic analyses characterize selective changes as a result of chlorantraniliprole resistance in *Plutella xylostella*

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Abstract

Pesticide resistance in insects is an example of adaptive evolution occurring in pest species and is driven by the artificial introduction of pesticide. The diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae), has evolved resistance to various insecticides. Understanding the genetic changes underpinning the resistance to pesticides is necessary to the implementation of pest control measures. For this reason, we sequenced the genome of 6 resistant and 6 susceptible DBMs, and inferred the genomic regions of greatest divergence between strains using two indices, Fst and θπ. Among several genomic regions potentially related to insecticide-resistance, a P450 gene, *CYP6B6-like*, was observed with significant divergence between the resistant and susceptible strains, with, among other SNPs, a missense mutation located near the substrate recognition site (SRS). To characterize the relative effects of directional selection via insecticide tolerance ('strain') as compared to acute exposure of insecticide ('treatment'), four pairwise comparisons were carried out between libraries to determine the differentially expressed genes (DEGs). Most resistant-related DEGs were identified from comparison between strains, and enriched in pathways for exogenous detoxification including cytochrome P450 and ABC transporter. Further confirmation came from the weighted gene co-expression network analysis (WGCNA), which indicated that genes in the significant module associated with chlorantraniliprole-resistance were enriched in pathways for exogenous detoxification, and that *CYP6BG1* represented a hub gene in this module. Our study thus provides a genetic foundation underlying selection for pesticide resistance and plausible mechanisms to explain fast evolved adaptation through genomic divergence and altered gene expression in insects.

Keywords: chlorantraniliprole resistant; sweep selection; MAPK signaling pathway; metabolic resistance; cytochrome P450 monooxygenase.

Introduction

Pesticide resistance in insects represents an excellent genetic model to explain how insects adapt to the relative short-term and strong selection pressure exerted by non-native chemicals. Understanding the genetic mechanisms underlying pesticide resistance is thus advantageous for pest control management. As a major pest of *Brassica* vegetable and oilseed crops throughout the world, the diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae), is estimated to cause approximately US$0.77 billion management tool and crop losses annually in China alone (Li et al., 2016). Chemical insecticides remain the main management route to control the DBM. However, the abuse of various insecticides, coupled with a short generation time and the largely overlapping of generations in DBM (Talekar & Shelton, 1993), have promoted DBM field resistance to all major kinds of insecticides, including organophosphates, carbamates, pyrethroids, and *Bacillus thuringiensis* (Bt) Cry toxins (Furlong, Wright, & Dosdall, 2013). For example, Chlorantraniliprole was the first commercialized anthranilic diamide insecticide and widely used for pest control, especially for Lepidoptera pests (Selby, Lahm, & Stevenson, 2017). However, within three years after its introduction in Guangdong in 2008, DBM had evolved a high level of resistance to chlorantraniliprole in the field (Hu et al., 2012). In general, insecticide-resistance mechanisms mainly include metabolic resistance that involves overexpression and elevated catalytic activity of detoxification enzymes, target resistance involving mutation of the insecticide target-site, and penetration resistance that involves modifications of the cuticle (Balabanidou,

Grigoraki, & Vontas, 2018; Khan et al., 2020). Previous research on the chlorantraniliprole-resistance mechanism in DBM mainly has focused on the mutations in the target-site, the ryanodine receptors (RyR) (Troczka et al., 2012; Guo et al., 2014; Guo, Liang, Zhou, & Gao, 2015; Steinbach et al., 2015). More recently, the role of metabolism resistance in DBM that accompanies chlorantraniliprole-resistance has been highlighted (Lin et al., 2013; Li, Zhu, Gao, & Liang, 2017; Etebari et al., 2018; Li, Li, Zhu, Gao, & Liang, 2018). However, these studies were not informed from unbiased genome scans. And to the authors' knowledge, no selective sweep analysis has been performed to identify resistant-related candidates in chlorantraniliprole-resistant insects.

The development of high-throughput sequencing technology, combined with the availability of genome-scale genetic data and statistical methods is increasingly providing a general framework for genome-wide scans of selection (GWSS) to identify positive selected loci associated with several phenotypic traits. Methods based on population genetics' statistics such as including F_{ST}, π, iHS, Tajima's D, XP-CLR and XP-EHH, have been widely used to seek out the genetic targets of artificial selection. Examples include, genes associated with domestic yak's behavior and tameness (Qiu et al., 2015), fat tail genes of Chinese indigenous sheep (Yuan et al., 2017), meat and milk quality traits in cattle (Zhao, McParland, Kearney, Du, & Berry, 2015; Mei et al., 2019), and other economic traits of domestic animals (Petersen et al., 2013; Wilkinson et al., 2013; Xu et al., 2015; Bertolini et al., 2018; Zhang et al., 2018). In addition to domesticated traits, GWSS also has been used to identify adaptation to natural selective pressures, such as adaptation of Dehong humped cattle to heat stress (Li et al., 2020), Yanbian cattle to a cold climate (Shen et al., 2020), goats and

sheep to a hot, arid environment (Kim et al., 2016), and Tibetan pig to high altitude (Dong et al., 2014).

In this study, whole-genome resequencing was performed of two strains of DBM (chlorantraniliprole-susceptible strain and -resistant strain), and F_{ST} for GWSS was used to detect genes with signature of positive selection. The susceptible strain has been maintained in laboratory conditions without exposure to any insecticide for 5 years, while the resistant strain originates from a chlorantraniliprole-susceptible population through six generations of chlorantraniliprole selection and backcrossing (Fig. 1A). Secondly, transcriptional data were obtained and analysis was performed to study the potential response to insecticide via altered gene expression of candidate genes. It is hypothesized that through these diverse approaches focusing on changes at the DNA and RNA level, many resistance-related genes may be detected. Such knowledge will prove essential to the future management and use of insecticides if the genetic and biochemical mechanism of insecticide resistance are better understood.

Materials and Methods

Insect Sample and Treatment

The susceptible strain (S) and the resistant strain (R) of DBM were provided by Dr Pei Liang (Department of Entomology, China Agricultural University, Beijing, P.R. China). S was initially purchased from the Pilot-Scale Base of Bio-Pesticides, Institute of Zoology, Chinese Academy of Sciences in 2014, and has been maintained in Dr Pei Liang's laboratory for 5 years without exposure to any insecticide. The resistant DBM strain was a near-isogenic line (NIL) constructed with backcross method (Fig. 1A). For more details see Zhu et al. (2015). After six times backcross

and chlorantraniliprole selection, the NIL showed high resistance (401.4-fold) to chlorantraniliprole (Table 1). R and S were used for DNA extraction, the whole body of a larvae as a sample, six samples for each strain.

Subsequently, S and R were divided into two groups, respectively. One group (treatment) was reared with cabbage leaves treated with LC_{50} concentration of chlorantraniliprole, whereas the cabbage leaves in the other group (control) was treated with water. The whole body of surviving larvae was used for RNA extraction, three replicates per treatment.

Sequencing and library preparation

Genomic DNA was extracted using TIANGEN Magnetic Universal Genomic DNA Kit. Sequencing libraries were generated using NEB Next® Ultra DNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and sequenced on an Illumina Hiseq 4000 platform. We generated 131.03 G of paired-end reads of 150bp length (Supplementary Table S1).

RNA was extracted using the TRIzol (Ambion) extraction method. The mRNA Sequencing libraries were generated using NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and sequenced on an Illumina NovaSeq 6000 platform. We generated 112.93 G of paired-end reads of 150bp length (Supplementary Table S2).

Read alignment and SNP calling

Because the quality of the NGS data is very important for the downstream sequence analysis, we filtered low-quality reads using NGS QC Tool kit (v2.3.3) with default parameters (Patel and Jain 2012). The clean data was aligned using BWA-MEM (v0.7.15) to the *Plutella xylostella*

reference genome (RefSeq accession: GCF_000330985.1). Sequence Alignment/Map (SAM) format files were dealt with using Picard tools SortSam (v2.2.4) for sorting, outputting as Binary sequence Alignment/Map (BAM) format files. Duplicate reads were removed from individual sample alignments using Picard tools MarkDuplicates (v2.2.4) (2019).

Before SNP calling, Genome Analysis ToolKit(v3.6), RealignerTargetCreator and IndelRealigner were used for global realignment. SNPs were called using GATK UnifiedGenotyper with the parameters: min_base_quality_score of 20, stand_call_conf of 30 and stand_emit_conf of 30. Then, GATK VariantFiltration was used to filter the unconfident variant sites, with the filter: a) QUAL < 30.0; b) QD < 5.0; c) FS > 60.0; d) MQ < 40.0; e) MQRankSum < -12.5; f) ReadPosRankSum < -8.0.

Selective-sweep analysis

To detect PSGs related to insecticide resistance to chlorantraniliprole, we scanned the genome for regions with population differentiation index (F_{ST}) and nucleotide diversity($\theta\pi$) ratio. F_{ST} and nucleotide diversity($\theta\pi$) were calculated with VCFtools (v0.1.13) using a 5kb window with a 1kb step. The negative and missing F_{ST} values were discarded, because these values have no biological interpretation (Akey, Zhang, Zhang, & Jin, 2002). The $\theta\pi$ ratio was calculated as $\theta\pi$(susceptible)/$\theta\pi$(resistant).

Sequence alignment and 3D modeling of CYP6B6-like

The multiple alignment was constructed with MEGA-X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) and Jalview (v2.11.1.0) (Waterhouse, Procter, Martin, Clamp, & Barton, 2009). 3D models of *CYP6B6-like* were generated by I-TASSER (Zhang, 2009; Roy, Yang, & Zhang, 2012;

143 Yang & Zhang, 2015) and model quality was assessed with Verify 3D, ProQ and ModFOLD
144 (Wallner & Elofsson, 2003) (Maghrabi & McGuffin, 2017). The structural changes introduced by
145 the revealed missense variant were predicted with Missense3D (Ittisoponpisan et al., 2019).

146 **RNA-seq, Data processing and Differentially expressed gene (DEG) analysis**

147 To further identify whether the potential selective-sweep regions (F_{ST}>0.5) could also affect
148 gene expression, we compared the gene expression between the susceptible and resistant strains of
149 DBM. Three susceptible and three resistant DBM samples were used for RNA-seq. Sample
150 information was listed in Table 2.

151 We used the fastp (v0.20.0) to filter out the bad reads and to cut adapters with the default
152 parameters (Chen, Zhou, Chen, & Gu, 2018). After building a HISAT2 index using hisat2-build, we
153 mapped the clean reads to the DBM reference genome using HISAT2(v2.1.0) (Kim, Langmead, &
154 Salzberg, 2015), with the output files of the SAM format. Then, the SAM format files were
155 imported to SAMtools for sorting and building index files, and the index of the fasta file of
156 reference genome was built with SAMtools (v1.9). We used the htseq-count tool to calculate the
157 counts of the reads mapping to each gene (Anders, Pyl, & Huber, 2015). These count data were
158 used to determine gene expression variation by analyzing each resistant sample to susceptible
159 sample with DESeq2 (v1.24.0) (Love, Huber, & Anders, 2014) in R (v3.6.1) (Gentleman et al.,
160 2004). Genes with $\log_2$FC >1.5 and padj <0.05 were considered to be upregulated after insecticide
161 treatment, while those with $\log_2$FC > -1.5 and padj <0.05 were considered as down-regulated genes.
162 We plotted the volcano plots and a Venn diagram of differentially expressed genes related with
163 insecticide resistance in DBM using ggplot2 (v3.2.1) and VennDiagram (v1.6.20) in R (Chen &

Boutros, 2011; Wickham, 2016), respectively.

We use the clusterProfiler(v3.12.0) (Yu et al., 2012), an R package, to determine significantly Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched within the DEG dataset using p.adjust < 0.05 as a threshold .

Weighted gene co-expression network analysis (WGCNA) and identification of hub genes in significant module

WGCNA is a systems biology method for identifying patterns that have relevance among genes in microarray samples. In the WGCNA network, interesting gene modules related to sample traits and key genes can be identified. In this study, the expression data of 12 samples were log-transformed using log2(x+1) and then used for WGCNA. The weighted co-expression network was constructed in accordance with the protocol of the WGCNA package in R (Langfelder & Horvath, 2008). We chose the power 6, which is the lowest power for which the scale-free topology fit index reaches 0.90. We merged highly similar modules (correlation > 0.75) and obtained 16 modules. To identify modules that significantly associated with the trait (resistance), we used module eigengenes (MEs) as principal components to correlate them with external traits and look for the most significant module. Gene Significance (GS) is defined as the correlation between the gene and the trait, and module membership (MM) is a measure of intra-modular connectivity. We identified hub genes as those genes with both a high Gene Significance (GS >0.95) for resistance as well as a high Module Membership (MM>0.95) in significant modules. The network of 22 hub genes was visualized with Cytoscape 3.7.1(Shannon et al., 2003).

Results

185 **Genome resequencing and genetic variation**

186 Genome resequencing of 6 resistance and 6 susceptible DBM yielded 131.03 G of clean data.
187 Sequencing data were mapped to the *Plutella xylostella* reference genome, which has a total
188 sequence length of 393.5 Mb. A total of 1.07 million high-quality SNPs were detected among all
189 samples and these SNPs were annotated using SnpEff (v4.3). Principal component analysis (PCA)
190 divided the 12 DBM genome samples into two populations, matching the two (resistant and
191 susceptible) strains (Fig. 1B). The first PC and the second PC respectively explained 20.53% and
192 11.25% of the total variation separating the two populations. Reduced genetic diversity was
193 observed among the resistant strain samples, by estimation of average $\theta\pi$ (p<2.22e-16) in both
194 strains (Fig. 1C). These results implicated higher diversity in the susceptible strain than the
195 resistant strain, whose tolerance to chlorantraniliprole differs by 401.4-fold (Table 1). This pattern
196 is in accordance with expectation of selection on the resistant strain by selecting only the most
197 pesticide-tolerant animals in subsequent generations for continuation of the strain.

198 **Genomic regions under selection and candidate genes**

199 To detect signatures of positive selection associated with insecticide resistance, we searched
200 the DBM genome for regions with high F_{ST} between susceptible strains and resistant strains. We
201 initially screened genes located at a genomic region with $F_{ST} > 0.50$ region, yielding 1501 genes
202 mainly enriched in pathways associated with signal transduction (MAPK signaling pathway and
203 Wnt signaling pathway), lipid metabolism (fatty acid degradation), development and regeneration
204 (cytoskeleton proteins) (Fig. 2). Among these, we identified 63 resistant-related genes coding for
205 metabolic detoxifying enzymes, digestive enzymes, insecticide receptors, ion channels, the

206 ubiquitinase system and cuticle proteins previously reported to be potentially involved in
207 insecticide resistance (Supplementary Table S3, Supplementary Fig. S1).

208 In order to reduce false positives, we then calculated the ratio of nucleotide diversity (θπ
209 (susceptible/resistant)). Using only those regions in both the top 5% of F_{ST} (>0.44) and of θπ ratio
210 (>14.03) as threshold, we identified 208 genes (Fig. 3). Among these candidate genes, a P450 gene,
211 cytochrome 6B6-like (geneID:105395328, denoted by "*CYP6B6-like*") showed both a high F_{ST}
212 (0.65) value and log2 (θπ(susceptible/resistant)) value (6.09) (Fig. 4A). In resistant samples of
213 DBM (n=6), we identified 8 SNPs in the intragenic region of *CYP6B6-like*, including 4 intron
214 variants (SNP48704, SNP48706, SNP48716, and SNP48729), 1 missense variant (SNP48968) and
215 3 synonymous variants (SNP48570, SNP48597 and SNP48999), which were absent in susceptible
216 strains of DBM (n=6) (Fig. 4B). These results indicate that the mutation of the *CYP6B6-like* may
217 be associated with insecticide resistance. Furthermore, the genomic sequence of *CYP6B6-like* was
218 used as input in DBM-DB blastn query (http://iae.fafu.edu.cn/DBM), and the result showed that it
219 was the same as *CYP6BG5* (Gene ID:Px014216), which is over-expressed in chlorpyrifos- and
220 fipronil-resistant strains of DBM (Yu et al., 2015).

221 To further analyze the effect of mutation on the *CYP6B6-like* protein, we focused on the
222 missense variant (SNP48968). SNP48968 caused an amino acid mutation (A486V) at the protein
223 level (Supplementary Fig. S2). Sequence alignments shows that *CYP6B6-like* has five conserved
224 motifs common to insect CYP6s: the WxxxR motif, the GxE/DTT/S motif, the ExLR motif, the
225 PxxFxPE/DRF motif, and the PFxxGxRxCxG/A motif (Fig. 5A). The missense mutation A486V of
226 *CYP6B6-like* is not in the conserved region, but in the highly variable region adjacent to the SRS6

(SRS: substrate recognition site). The three-dimensional structure of *CYP6B6-like* (Fig. 5B) was then predicted using I-TASSER (C-score=0.56, TM-score=0.79±0.09). The quality assessment of VERIFY, ProQ and ModFOLD indicated that the resulting model is of good quality. The SRSs were annotated according to sequence alignment with mammalian CYP2 (Fig. 5B). We observed that the mutation A486V was located at the protein surface near the SRS6. Missense3D prediction showed that the missense mutation did not cause structural damage to the protein, suggesting that this alteration is not deleterious. Moreover, several SNPs were found in the 2000nt regions upstream of *CYP6B6-like* (Fig. 5C), and potential transcription factor binding sites at these regulation regions of *CYP6B6-like* were predicted using Jaspar (http://jaspar.genereg.net/). Interestingly, these SNPs landed at two predicted transcription factor binding sites which were in the upstream fragment of the *CYP6B6-like* (NW_011952654.1:45630-47636 and NW_011952654.1:45641-45646). These findings suggest that genetic divergence resulting from artificial selection for chlorantraniliprole resistance in DBM may influence the expression of this CYP gene to some extent.

Differentially expressed genes (DEGs) and KEGG Enrichment Analysis

To investigate the effects of acute insecticide exposure (‘treatment’) and of directional selection via insecticide tolerance (‘strain’) on gene expression level of DBM, four pairwise comparisons were carried out between libraries to determine the DEGs (RT vs T, RCK vs CK, RT vs RCK and T vs CK, Table 2). The normalized RNA-Seq expression data were used to perform principal component analysis, and the PC1, PC2 and PC3 explained 91.53%, 3.83% and 2.25% of total variation, respectively (Fig. 1D). Primarily, the 12 individuals were divided into two main

248 groups, which matched the resistant and susceptible populations. Secondarily, for the susceptible
249 strain of DBM, individuals clustered also by treatment, while individuals representing the resistant
250 strain of DBM, did not show significant clustering by treatment. For further interpretation, genes
251 with |log2FoldChange|>1.5 and padj < 0.05 were recognized as differentially expressed. Our results
252 showed that, there were 518 (365 up-regulated and 153 down-regulated, CK vs RCK), 615 (453
253 up-regulated and 162 down-regulated, T vs RT), 52 (47 up-regulated and 5 down-regulated, RT vs
254 RCK) and 221 (94 up-regulated and 127 down-regulated, T vs CK) DEGs by comparison of
255 different strains or treatments (Supplementary Fig. S3). We identified 117 genes related to
256 insecticide resistance from four pairwise comparisons based previously researches (Supplementary
257 Table S4), the top 10 significantly expressed genes are listed in Supplementary Table S5. Except
258 for DEGs in the comparison of insecticide-treated susceptible DBM with control susceptible DBM,
259 the majority involved up-regulation, including the cytochrome P450s, acetylcholinesterase, ion
260 channels, and cuticle proteins. The overlap of DEGs between treatments and strains was analyzed
261 and displayed in Venn diagrams (Supplementary Fig. S4). Only 3 genes were differentially
262 expressed (DE) after acute insecticide exposure both in the susceptible and the resistant strain,
263 while 201 common genes were DE between strains with or without exposure to the insecticides.
264 Coupled with the expression pattern of DE P450s, which showed obvious expression difference
265 between strains instead of treatments (Supplementary Fig. S5), these data indicate a stronger
266 response to long-time directional selection for the susceptible strain when comparing exposure to
267 non-exposure, which was consistent with the instability of insecticide-resistance that the resistant
268 level of insects would gradually reduce once without pesticide pressure. Further analysis of the

DEGs between strains by association with known KEGG pathways, categorized DEGs into 30 groups (Fig. 6), including pathways related to metabolic regulation, exogenous detoxification (cytochrome P450, ABC transporter), and immune response (Toll and Imd signaling).

To study whether the expression level of genes under positive selection (F_{ST}>0.50) would be different between strains or between treatments, we investigated which genes with enhanced Fst were also listed as DEGs (Supplementary Table S6). 7.3% of positively selected genes (PSGs) were also DE between treatment or between strains, with more PSGs DE between strains. Among these candidate genes, we identified 8 pesticide-resistance related genes, including cytochrome P450s, ubiquitin-protein ligase, and chymotrypsin (geneID:105396609, 105395328, 105388375, 105382116, 105387005, 105397691, 105397997 and 105386494).

Identification of hub genes in significant module related to resistance

Transcript data from all 12 samples from two strains of DBM (susceptible and resistant) were used to construct the module that consists of functionally related genes of similar expression profile, according to the WGCNA algorithm (Fig. 7A). In the present study, power of β=6 was selected as the soft-threshold to ensure a scale-free network (Fig. 7B) and 16 modules were identified (Supplementary Fig. S6). Of these modules, the module eigengenes (ME) of the “magenta” module exhibited the highest positive correlation with pesticide resistance (r=0.94, P=6e-06) (Fig. 7C). Thus, this “magenta” module was considered as the most meaningful module of co-expressed genes related to resistance. This set of genes was then further explored for functional enrichment analysis and identification of key genes. 4554 genes in the “magenta” module were enriched for particular exogenous detoxification pathways including cytochrome P450 and ABC transporter,

again highlighting the importance of metabolic resistance in chlorantraniliprole-resistance (Fig. 7D). Module membership vs. gene significance (MM vs. GS) in the magenta module is highly correlated (Supplementary Fig. S7), illustrating that genes highly significantly associated with resistance are often also the most important elements of the significant module (magenta module). We found 22 hub genes with GS>0.95 and MM>0.95, including a P450 gene, *CYP6BG1*, and visualized them as a network with Cytoscape 3.7.1(Fig. 8).

Discussion

Insect resistance to pesticides is an urgent problem in integrated pest management. In the present study, we compared whole genome data of DBM from a chlorantraniliprole resistant strain with a chlorantraniliprole susceptible strain, and identified (a) lower nucleotide diversity in the resistant strain, consistent with directional selection, and (b) several PSGs related to insecticide resistance using population differentiation index (F_{ST}). From this comparison, several detoxification enzyme genes, pesticide target genes, digestive enzyme genes, ubiquitin-protein ligase and cuticle protein genes were found to be subject to positive selection. After further correction for false positives using the nucleotide diversity ratio ($\theta\pi$ ratio, $\theta\pi$(susceptible/resistant)), a cytochrome P450 gene (geneID:105395328) was identified as showing among the highest differentiation between resistant strain and susceptible strain, implicating it was under strong selective pressure. Transcriptome results also indicated that expression of many resistance genes was upregulated in the resistant strain and DEGs were enriched in pathways related to detoxification metabolism.

The KEGG pathway analysis of positive selected genes filtered with F_{ST}>0.50 demonstrated that many candidate genes were enriched in the mitogen-activated protein kinase (MAPK)

311 signaling pathway. The MAPKs regulate various cellular programs in response to extracellular
312 signals, and have many endogenous and exogenous substrates (Cargnello & Roux, 2011). The
313 MAPK-signaling pathway is involved in drug-resistance of cancer cells (Kuroshima et al., 2020;
314 Liao et al., 2020; Zhao, Lu, Chen, Cheng, & Zhang, 2020), a crucial factor of which is reactivation
315 of the MAPK-signaling pathway (Corcoran et al., 2012; Ahronian et al., 2015). Although studies on
316 the role of the MAPK signaling pathway in insecticide resistance are few, some exist. For example,
317 the MAPK p38 pathway has been implicated in insect defense against Bt Cry toxins
318 (Cancino-Rodezno et al., 2010). Furthermore, another study indicates that the MAPK signaling
319 pathway confers *Bacillus thuringiensis* Cry1Ac resistance by altering expression of midgut ALP
320 and ABCC genes (Guo et al., 2015). Additionally, recent research has revealed the role of the
321 MAPK-signaling pathway in regulation of P450-mediated insecticide resistance (Yang et al., 2020).
322 Our findings further confirm the potential role of MAPK signaling pathway in chlorantraniliprole
323 insecticide resistance, and also specify P450-mediated insecticide resistance. Similarly, studies
324 have indicated the role of the Wnt signaling pathway in chemoresistance of numerous cancer types
325 via regulating the expression of resistance genes (He et al., 2018; Yamamoto et al., 2019; Yang et
326 al., 2019). More specific to insecticide resistance, studies have shown differential expression of
327 genes associated with Wnt signaling pathway in chlorantraniliprole- and flubendiamide-resistant
328 DBM (Lin et al., 2013; Wang, Sun, Xu, Zhao, & Xue, 2018). The present GWSS analysis provides
329 valuable information at the genome level regarding role of signal transduction in development of
330 chlorantraniliprole resistance, although biological functions of the pathways linked to insecticide
331 resistance need to be confirmed experimentally and regulation mechanisms in resistant strains also

need further elucidation. Our results from the transcriptomic analysis show that DEGs are enriched in multiple pathways associated with resistance, including metabolism of xenobiotics by cytochrome P450, drug metabolism-other enzymes, drug metabolism-cytochrome P450, ABC transporters and glutathione metabolism. Detoxification metabolism to exogenous toxic substances is the most commonly described mechanism of insecticide resistance (Hemingway, Hawkes, McCarroll, & Ranson, 2004; Liu, Zhu, Xu, Pridgeon, & Gao, 2006). Strong correlations between the detoxifying enzyme activity and chlorantraniliprole resistance have been confirmed (Hu et al., 2014), and several studies have indeed also observed their overexpression in resistant DBM and verified the role of some detoxifying enzymes in chlorantraniliprole resistance of DBM by RNAi (Hu et al., 2014; Li et al., 2017; Li et al., 2018). These findings are consistent with our result of selective-sweep analysis and transcriptome analysis (Fig. 6, Supplementary Table S3, Supplementary Table S4) by pointing to the same pathways involved in pesticide resistance.

Among these detoxifying enzymes above, genetic variation and expression difference of P450s are particularly prominent in the present study. P450s play an important role in endogenous and exogenous substance metabolism, the latter including drugs, pesticides and plant toxins (Scott, 1999). P450s in DBM are distributed in four main groups including CYP2, CYP3, CYP4 and a mitochondrial cluster. Members of the CYP3, mainly CYP6s and CYP9s, are shown to participate in xenobiotics metabolism and insecticide resistance, and members of the CYP4 clan are known to be induced by xenobiotics (Yu et al., 2015). Our study comes to similar conclusions; the positively selected P450s and differentially expressed P450s are mainly CYP6s, CYP9s and CYP4s (Supplementary Table S3, Supplementary Table S4). The transcriptional up-regulation of P450

353 genes and metabolic capacities of P450 proteins were regarded as important mechanisms of
354 enhanced metabolic detoxification to insecticide (Schuler & Berenbaum, 2013). At the transcript
355 level, both P450 constitutive overexpression caused by mutations in promoter sequence, and the
356 inducible expression caused by mutations in trans-acting factors or by their signaling cascades have
357 been specifically associated with insecticide resistance (Liu, Li, Gong, Liu, & Li, 2015). At the
358 protein level, mutations in catalytic site residues (SRS1, SRS4, SRS5 and SRS6), in substrate
359 access channel residues (SRS2 and SRS3), in proximal surface residues and in interacting partners
360 may account for the enhanced metabolic capacities of P450s (Schuler & Berenbaum, 2013). In
361 DBM, overexpression of *CYP6BG1* in resistant strains has been reported to be involved in
362 chlorantraniliprole-resistance (Li et al., 2018). In the present study, we not only also observed
363 overexpression of *CYP6BG1* in resistant DBM but also identified it as the hub gene in a significant
364 module of co-expression associated with resistance. In other lepidoptera, like *Chilo suppressalis*
365 (Xu et al., 2019) and *Spodoptera exigua* (Wang et al., 2018), constitutive overexpression of
366 multiple P450s in chlorantraniliprole-resistance strains has been confirmed. In our study, the
367 expression pattern of P450s also shows constitutive overexpression of P450s in resistant strains
368 (Supplementary Fig. S5), and we suggest the potential role of these overexpressed P450s in
369 resistance to chlorantraniliprole, because their overexpression has been reported in chlorpyrifos-
370 and fiprinol-, and chlorantraniliprole-resistance in DBM. However, acute
371 chlorantraniliprole-treatment does not induce increased expression of P450s, it could be connected
372 with the insect growth stage, insecticide exposure time, insecticide type and insecticide
373 concentration (Terriere, 1984). These enzymes may not be sensitive to the inducer or do not have

374 enough time controlling their biosynthesis to respond to insecticide.

375 Combined with the result of F_{ST} and θπ, we found that a P450 gene,
376 *CYP6B6-like*(geneID:105395328), was under strong positive selection and had a high genetic
377 divergence between resistant and susceptible DBM strains. As a member of CYP6s, it may
378 participate in the metabolic detoxication to insecticide. Previous study has shown that *CYP6B6-like*,
379 also named as *CYP6BG5*, was transcriptionally up-regulated in the chlorpyrifos- and
380 fiprinol-resistance strains compared to susceptible strains (Yu et al., 2015). Although the target site
381 of chlorantraniliprole is different from chlorpyrifos and fiprinol, cross-resistance in insects is a
382 common phenomenon as metabolic mechanisms of resistance could confer broad-spectrum
383 resistance (Furlong et al., 2013). Moreover, *CYP6B6-like* shares a high identity with *CYP6BG1*,
384 which has a confirmed involvement in chlorantraniliprole resistance in DBM. Interestingly, it was
385 also predicted as one of the target genes of 11 lncRNAs linked to chlorantraniliprole-resistance in
386 DBM (Zhu, Xu, Shi, Gao, & Liang, 2017). Taken together, these data may be regarded as evidence
387 that *CYP6B6-like* is involved in detoxification metabolism of chlorantraniliprole. Furthermore, we
388 uncovered a missense mutation in the resistant strain, causing an amino acid mutation A486V,
389 which is located at a highly variable region near the SRS6. SRSs are reported to be located at
390 regions of extensive sequence variation and even a single amino acid mutation in the SRS regions
391 could affect metabolic capacity (Gotoh, 1992; Schuler & Berenbaum, 2013). For example, a variant
392 in SRS6 (A484V) of CYP6B3 has been reported to force substrates to bind closer to the reactive
393 oxygen on heme (Wen, Rupasinghe, Niu, Berenbaum, & Schuler, 2006). However, catalytic
394 properties are not only associated with SRSs. Three point-mutations in CYP6A2, located between

SRS4 and SRS5 (R335S and L336V) and before SRS6 (V476L) are reported to have a prominent role in DDT-insecticide resistance (Amichot et al., 2004). In our study, the missense mutation A486V is located before SRS6, but its potential role in chlorantraniliprole-resistance needs further study.

Alongside the possible change of CYP6B6-like protein function in the resistant strain due to directional selection, we also observed its overexpression in resistant strains, which may be associated with mutations in the transcriptional regulatory region (2000nt upstream region of gene) (Supplementary Fig. S3). As mentioned above, the promoter variations of P450s are related to constitutive overexpression (Schuler & Berenbaum, 2013). For example, some transposon insertions of P450s are reported to be associated with insecticide resistance in *Drosophila melanogaster* (Chen & Li, 2007; Schmidt et al., 2010). Here, several substitutions (SNP45630, SNP45634, SNP45635 and SNP45641) were observed to be located at 2 predicted transcription factor binding sites (NW_011952654.1:45630-45636 and NW_011952654.1:45641-45646) (Fig. 5C). Among predicted transcription factors, the B-H1, C15, Gsc, Hmx and lbe are involved in negative regulation of transcription (Supplementary Table S7). Thus, their specific role in transcriptional regulation of P450s needs further confirmation.

Alongside several P450s, we also identified several other genes with strong signs of selective sweeps (Supplementary Table S3, Supplementary Fig. S1). This finding is not entirely surprising, since metabolism resistance is a complex physiological process involving a number of enzymes and transporters. P450s, esterases and UDP–glycosyltransferases (UGTs) are three critical enzyme categories participating in metabolism of xenobiotics and ABC transporters transfer and excrete

their products (Li, Schuler, & Berenbaum, 2007; Merzendorfer, 2014). In DBM, overexpression of *UGT2B17* has been reported to be involved in chlorantraniliprole-resistance (Li et al., 2017). Contribution of ABC transporters in chloramphenicol-resistance is rarely reported, but a recent study revealed that ABC transporters were involved in transport of chlorantraniliprole (Meng et al., 2020). Interestingly, the overexpression of P450s and ABC transporters are also known to change the thickness and composition of insect cuticle (Balabanidou et al., 2016; Pignatelli et al., 2018; Chen, Pei, Li, Fan, & Liu, 2019), which may be caused also by overexpression of cuticle proteins (Sun et al., 2017; Huang et al., 2018). Insect cuticle is a critical determinant of insecticide resistance through reducing insecticide penetration, which has been reported in various insects (Lin, Jin, Zeng, & Lu, 2012; Fang et al., 2015; Simma et al., 2019), though studies about this aspect are fewer than those that support metabolism resistance. It also appears that proteasomes are associated with the response to insecticide, because they increased energy and amino acid production (Hou et al., 2014; Wilkins, 2017). In addition, trypsin has been confirmed to catalyze the degradation of deltamethrin (Xiong et al., 2014), which may lead to a deeper understanding of the role of proteasomes in insecticide-resistance. These studies mentioned above are in agreement with the possible role of candidate genes found in our study in development of chlorantraniliprole-resistance.

In summary, we provide insights into the genomic basis of chlorantraniliprole resistance of DBM. Abundance of information from genome and transcriptome analysis could facilitate further investigations of the detailed resistance mechanisms of DBM against chlorantraniliprole.

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Data Accessibility

The 12 DBMs used in whole-genome resequencing analysis and the 12 DBMs used in RNA–seq analysis are accessible at the National Center for Biotechnology Information (NCBI) under Bio-Project accession numbers PRJNA664309. The whole-genome resequencing data and the transcriptome data have been deposited in SRA under SRR12674258-SRR12674281.

Tables

Table 1 Toxicity of chlorantraniliprole to two strains of *Plutella xylostella*

Strain	LC_{50} (95% CL) (mg L^{-1})	Slope± SE	χ^2 (df)[a]	RR[b]
Susceptible	0.042(0.032-0.055)	3.35±0.55	8.86(8)	1
Resistant	16.86 (13.41-21.63)	2.04±0.23	14.46(12)	401.4

CL, confidence limit.

a Chi-square value and degrees of freedom (df) as calculated by POLOPLUS

b Resistance ratio, equal to LC_{50} of resistant population/LC_{50} of susceptible population.

Table 2 Sample information of *Plutella xylostella* RNA-Seq

Sample name	strain	treatment
RT	Resistant	Exposed to chlorantraniliprole (LC_{50}) for 24 h
RCK	Resistant	Without exposure to any insecticide
T	Susceptible	Exposed to chlorantraniliprole (LC_{50}) for 24 h
CK	Susceptible	Without exposure to any insecticide

Figures

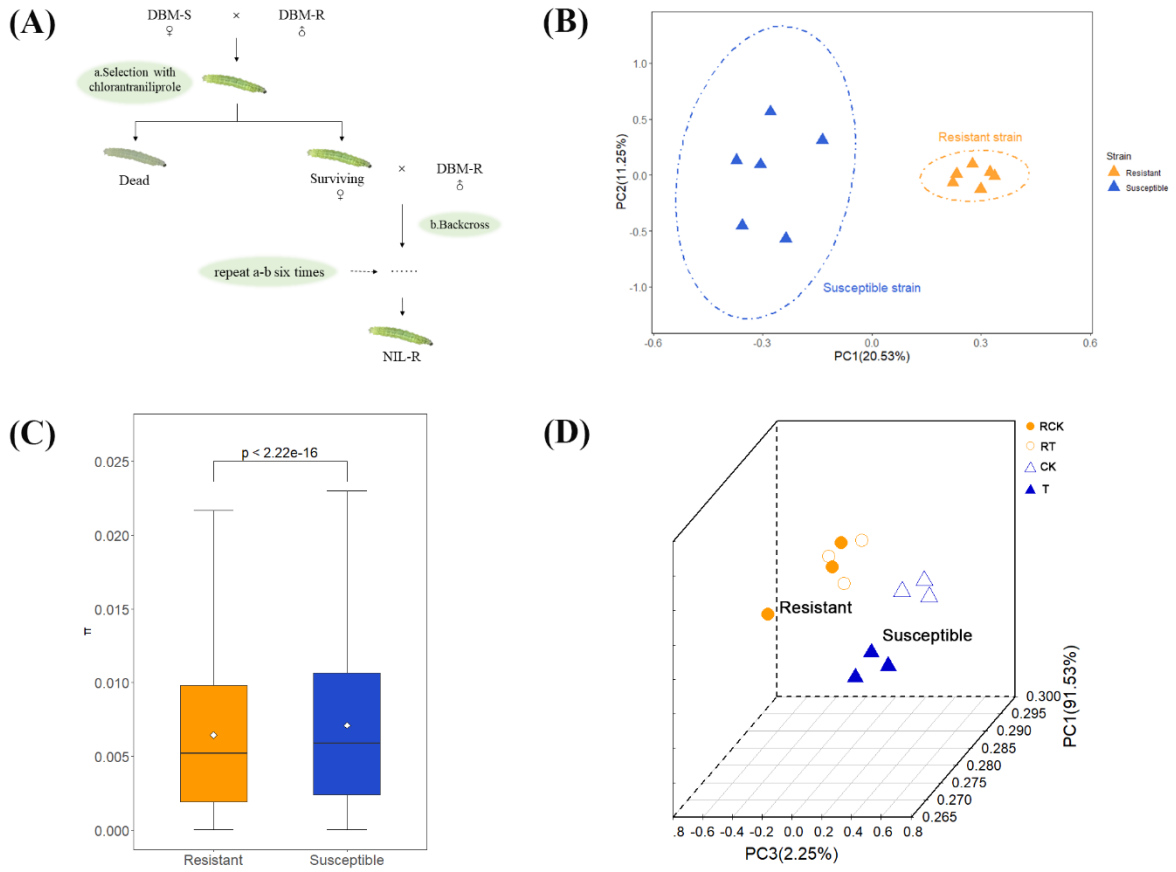
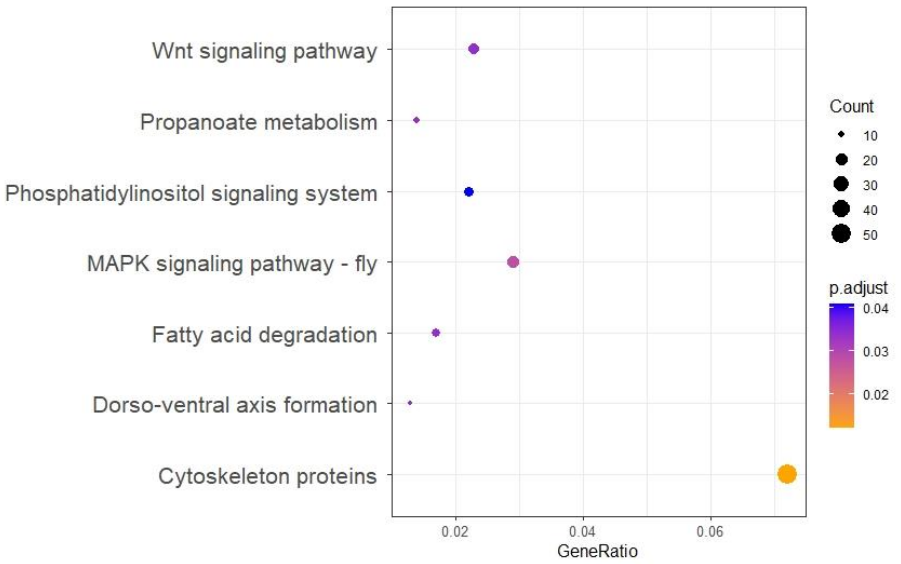
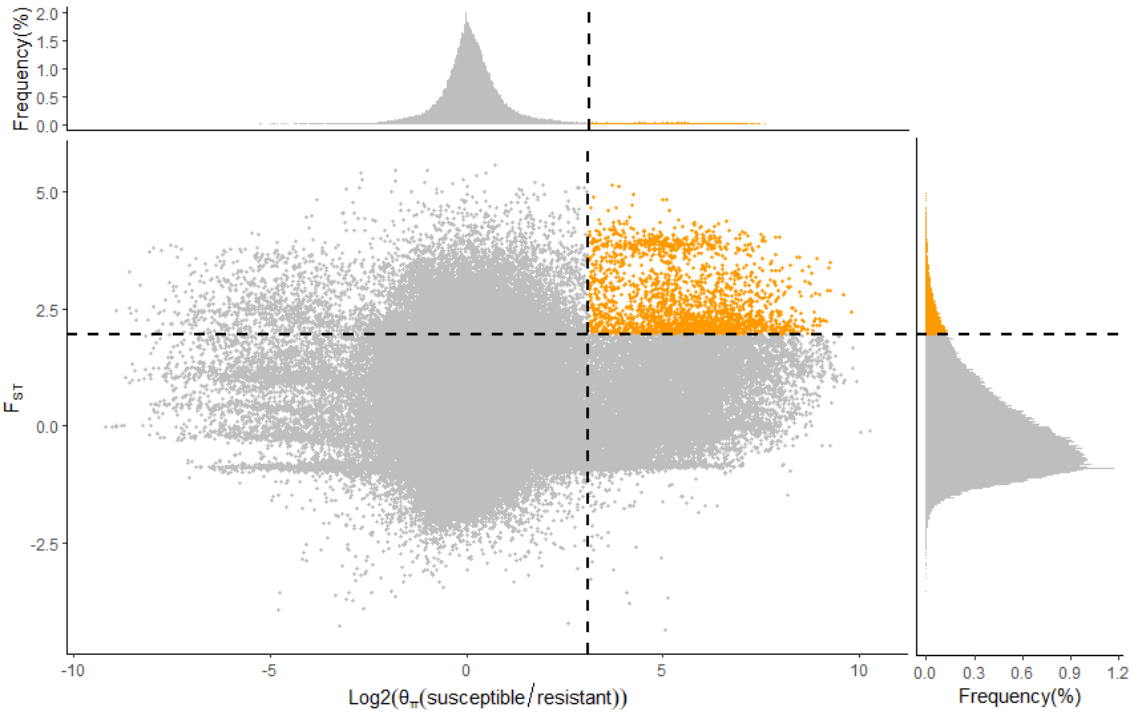


Fig. 1. Genetic differentiation between susceptible and resistant strains of *plutella xylostella*. A) Construction of near-isogenic *Plutella xylostella* strains resistant to chlorantraniliprole. B) Principle component (PC) analysis of SNPs. C) Significant difference in population nucleotide diversity (θπ), as evaluated using the t-test (P<2.22e-16). D) Principle component (PC) analysis of gene counts. Two colors represent two strains of DBM, the orange for resistant strain and the blue for susceptible strain. Different graphs represent samples, RCK, Resistant stain without treatment by chlorantraniliprole (LC_{50}); RT, Resistant stain after treated with chlorantraniliprole (LC_{50}); CK, Susceptible strain without exposure to any insecticide; and T, Susceptible strain exposed to chlorantraniliprole (LC_{50}).



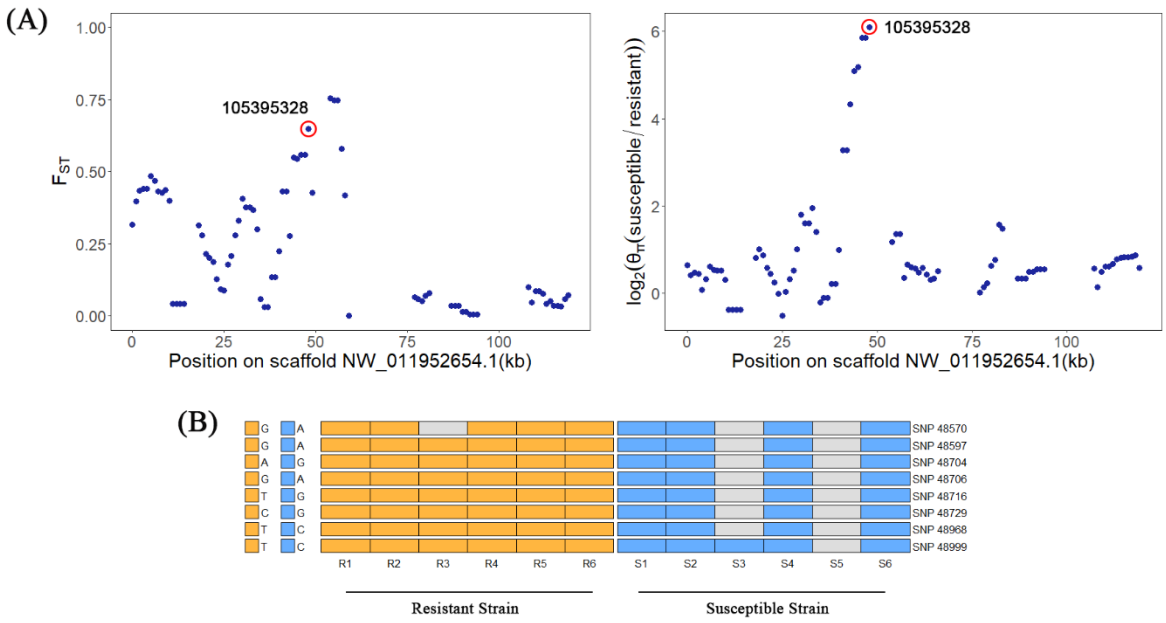
723

724 Fig. 2. KEGG pathway annotation classification of genes located in FST>0.5 region of the DBM
725 genome of strains differing in susceptibility to chlorantraniliprole. The ordinate left is the KEGG
726 classification, and the abscissa is the ratio of gene enrichment in the pathway. The size of the round
727 spot represents the number of gene enriched in the pathway, and the color represents the size of
728 p.adjust value.



729

730 **Fig. 3.** Distribution of F_{ST} and θπ ratio (θπ(susceptible/resistant)) across, respectively, the X and Y
731 axes with associated frequency plots. The top-right corner where orange points are located
732 represents the genome region under selection pressure for insecticide. The horizontal and vertical
733 gray dashed lines represent the top 5% value of standardized F_{ST} (2.00, F_{ST}=0.44) and
734 log2(θπ(susceptible/resistant)) (3.81, θπ(susceptible/resistant) = 14.03).



735

736 **Fig. 4.** *CYP6B6-like* (geneID: 105395328) shows different genetic signatures between resistant
737 strain and susceptible strain DBM. A) F_{ST} and log2 (θπ (susceptible/resistant)) plot around the
738 *CYP6B6-like* locus. The F_{ST} and log2 (θπ (susceptible/resistant)) value of *CYP6B6-like* is almost
739 highest for scaffold NW_011952654.1, circled in red. B) The 8 SNPs were identified in resistant
740 strains of DBM and absent in susceptible strains of DBM. SNPs were named according to their
741 position on the scaffold. The gray cell means data missing.

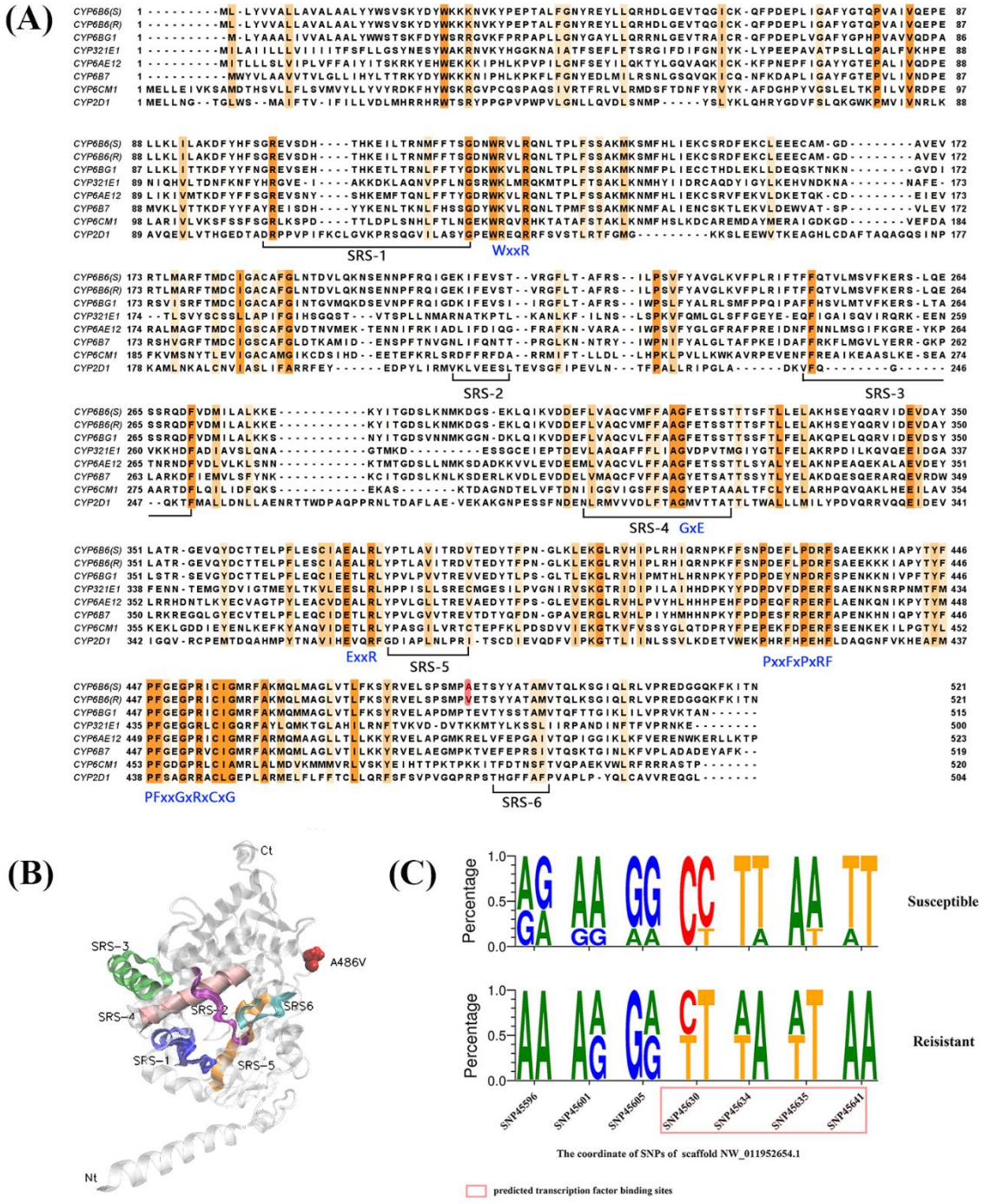


Fig. 5. Sequence alignment, 3D structure of CYP6B6-like and diploid genotypes' frequency of potential transcriptional regulatory region of *CYP6B6-like*. A) Sequence alignment of amino acid sequences from insect CYP6 and mammalian CYP2. The sequences are colored orange according to their level of conservation. Five conserved motifs of the protein in insect and six SRSs were labeled, and the mutation (A486V) was highlighted in red. The alignment of the sequences was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and Jalview 2.11.1.0. B) Three-dimensional structure of cytochrome P450 6B6-like. Six putative SRSs are colored and predicted based to mammalian CYP2 protein structure. SRSs 1 to 6 are shown in blue, purple, green, pink, orange and cyan, respectively. The mutation (A486V) is marked in red. C) The

SeqLogo plots show the diploid genotypes' frequency of potential transcriptional regulatory region of *CYP6B6-like* (2000nt regions upstream of gene), which were significantly different between resistant strains and the susceptible strain.

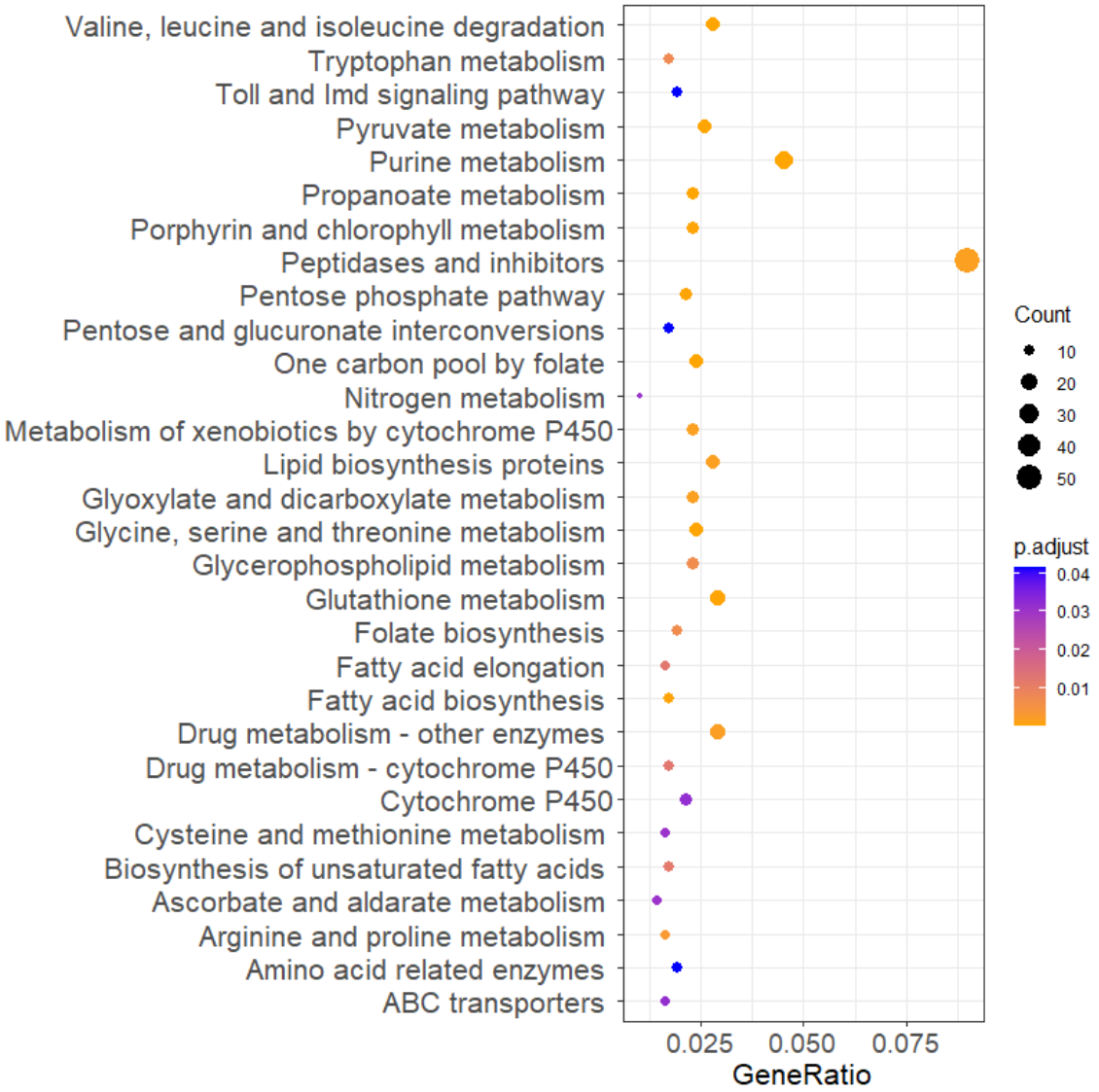


Fig. 6. KEGG pathway annotation classification of DEGs between strains. The ordinate left is the KEGG pathway, and the abscissa is the ratio of gene enriched in the pathway. The size of the round spot represents the number of gene enriched in the pathway, and the color represents the size of p.adjust value.

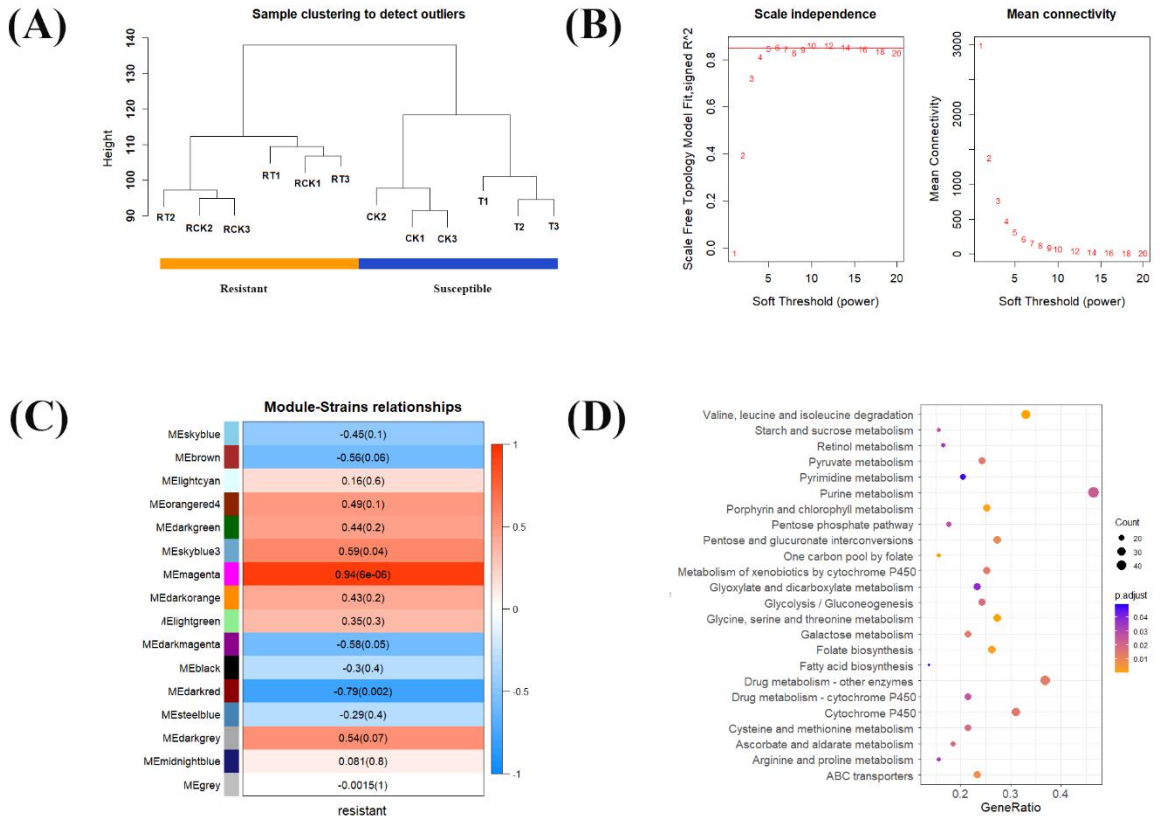


Fig. 7. Weighted gene co-expression network analysis of 12 samples from two strains of DBM. A) Clustering dendrogram of 12 samples and the trait (resistance). RCK, Resistant stain without treatment by chlorantraniliprole (LC_{50}); RT, Resistant stain after treated with chlorantraniliprole (LC_{50}); CK, Susceptible strain without exposure to any insecticide; and T, Susceptible strain exposed to chlorantraniliprole (LC_{50}). B) Analysis of the scale-free fit index for various soft-thresholding powers (β). C) Heatmap of the correlation between module eigengenes and chlorantraniliprole-resistance (the color from blue → red (-1→1) represents the relationship between module and trait. Each row corresponds to a module eigengene, column to a trait. Each cell contains the corresponding correlation and p-value. D) KEGG pathway annotation classification of genes in magenta module. The ordinate left is the KEGG pathway, and the abscissa is the ratio of gene enriched in the pathway. The size of the round spot represents the number of gene enriched in the pathway, and the color represents the size of p.adjust value.

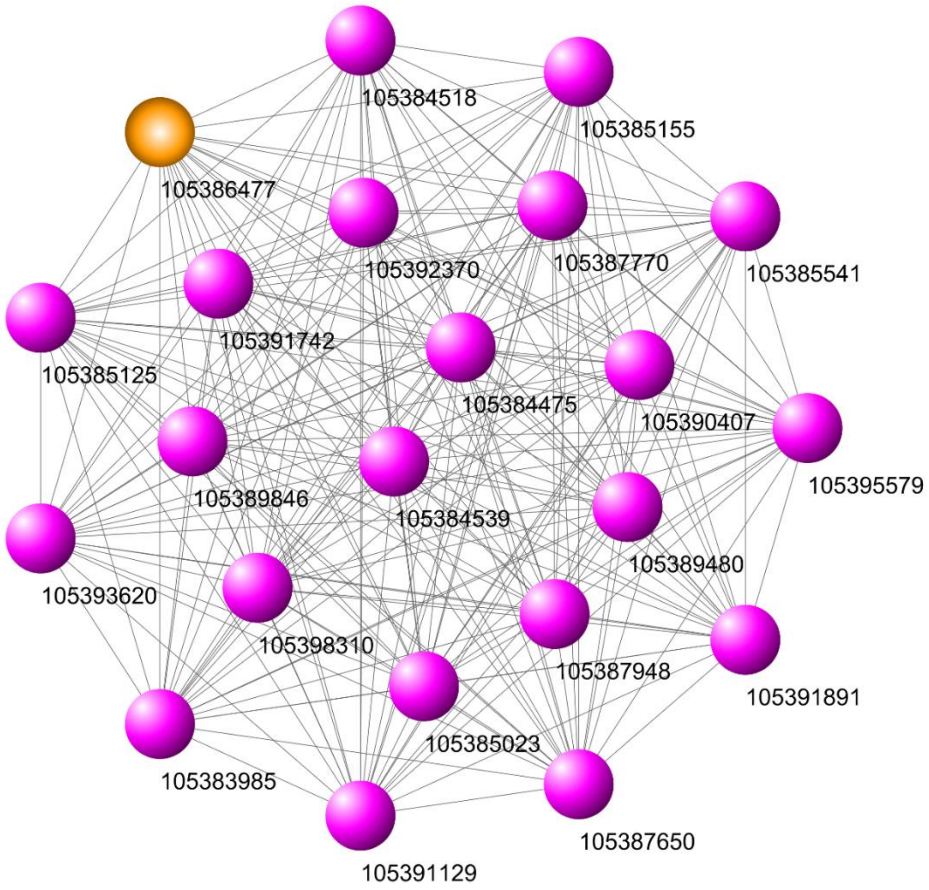


Fig. 8. The network of 22 hub genes (geneID) in the magenta module. A P450 gene is marked in orange. 105384539: electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial-like; 105384475: trifunctional purine biosynthetic protein adenosine-3; 105391891: adenylosuccinate lyase; 105395579: nucleoside-diphosphatase uda-1-like; 105391742: uncharacterized; 105385541: uncharacterized; 105391129: trifunctional purine biosynthetic protein adenosine-3-like; 105389846: lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial; 105384518: uncharacterized; 105387770: plasmatocyte-spreading peptide-like; 105385155: calmodulin; 105386477: cytochrome P450 6k1-like; 105393620: senecionine N-oxygenase-like; 105389480: peroxiredoxin 1; 105385125: serine/threonine-protein kinase MRCK beta-like; 105392370: pyrroline-5-carboxylate reductase-like; 105387948: probable methylcrotonoyl-CoA carboxylase beta chain, mitochondrial; 105387650: cytochrome b5-like;105390407: uncharacterized; 105398310: solute carrier family 22 member 13-like;105383985: uncharacterized; 105385023: la-related protein 1-like.