

## Article

# Comparative Transcriptome Analysis of Bt Resistant and Susceptible Strains in *Ostrinia furnacalis* (Guenée) (Lepidoptera: Crambidae)

Yaling Lin <sup>1,2</sup>, Qing Gao <sup>2,3</sup> , Yueqin Wang <sup>2</sup>, Zhenying Wang <sup>2</sup> , Kanglai He <sup>2</sup> , Suqin Shang <sup>1,\*</sup>  and Tiantao Zhang <sup>2,\*</sup> 

<sup>1</sup> Biocontrol Engineering Laboratory of Crop Diseases and Pests of Gansu Province, College of Plant Protection, Gansu Agricultural University, Lanzhou 730070, China; linyaling95@163.com

<sup>2</sup> State Key Laboratory for the Biology of the Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; gaoqing3399@163.com (Q.G.); wangyueqin\_fly@126.com (Y.W.); zywang@ippcaas.cn (Z.W.); klhe@ippcaas.cn (K.H.)

<sup>3</sup> College of Agronomy and Biotechnology, Hebei Normal University of Science and Technology, Qinhuangdao 066000, China

\* Correspondence: shangsq@gsau.edu.cn (S.S.); zhangtiantao@caas.cn (T.Z.)

**Simple Summary:** The Asian corn borer can reduce corn production or even lead to no harvest, causing huge economic losses. *Bacillus thuringiensis* (Bt) produces insecticidal crystal proteins that have specific insecticidal effects on Lepidoptera, Coleoptera, Diptera and other pests during the sporulation process. The use of transgenic Bt crops to replace pesticides to control the Asian corn borer has become a common trend. However, long-term planting of a single Bt transgenic corn will inevitably produce resistant strains. This study confirmed that the Asian corn borer can develop resistance Bt toxin through long-term exposure in the laboratory. In addition, the gene expression of Cry1F- and Cry1Ie-resistant strains in the transcriptome is different from that of susceptible strains, and a larger proportion of differentially expressed genes (DEGs) in both resistant strains were significantly down-regulated, several of these Bt-resistance candidate genes were differentially expressed in both resistant strains, Most of the glutathione S-transferases in ACB-FR showed an up-regulation trend, but down-regulated in ACB-IeR. These differentially expressed genes can be further studied to understand the mechanism of Bt resistance.

**Abstract:** The evolution of target pest population resistance to Bt toxins is the most relevant threat to the sustainability of Bt technology, thus it is necessary to clarify insect resistance mechanisms. Firstly, the resistance level of Asian corn borer was determined by bioassay. After 28 generations selection in the lab, the Cry1Ie-resistant strain (ACB-IeR) developed more than 862-fold resistance to Cry1Ie, and the Cry1F-resistant strain (ACB-FR) developed 961-fold resistance to Cry1F. The results show that long-term exposure to Bt toxins can lead to resistance. Then, we compared the differential expression genes (DEGs) of ACB-FR and ACB-IeR with susceptible strain (ACB-BtS), and analyzed GO function and KEGG pathway through transcriptome sequencing. The comparison showed that in Bt-resistant strains, many genes have a significant down-regulated trend. Several Bt-resistance candidate genes were differentially expressed in both resistant strains. Furthermore, the DEGs were verified by RT-qPCR and showed similar trend. These results provide candidate genes for further research on the Bt resistance mechanism.

**Keywords:** *Ostrinia furnacalis*; *Bacillus thuringiensis*; Cry1F; Cry1Ie; transcriptome; resistance



**Citation:** Lin, Y.; Gao, Q.; Wang, Y.; Wang, Z.; He, K.; Shang, S.; Zhang, T. Comparative Transcriptome Analysis of Bt Resistant and Susceptible Strains in *Ostrinia furnacalis* (Guenée) (Lepidoptera: Crambidae). *Agriculture* **2022**, *12*, 298. <https://doi.org/10.3390/agriculture12020298>

Academic Editors: Filippo De Curtis and Roy Kennedy

Received: 16 December 2021

Accepted: 17 February 2022

Published: 19 February 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Corn (*Zea mays* L.), as one of the most important food crops, is a high-quality feed crop and a high-yield cash crop, as well as an industrial raw material [1]. Asian corn borer (ACB),

*Ostrinia furnacalis* (Guenée) (Lepidoptera: Crambidae) is an important boring insect of corn, which damages mainly in the whorl leaves, stalks and ears. In addition, the wound allows plant pathogens to invade crops after the Asian corn borer infestation [2]. Therefore, the Asian corn borer can seriously affect the yield and quality of corn in direct or indirect ways, leading to a substantial reduction in corn production or causing huge economic damage. It has become an important factor restricting the healthy and high-quality development of the corn industry. Previously, chemical pesticides and natural enemies were used to control pests [3], but these methods have shown limited effects. Pesticides can effectively and quickly kill the pest, but they have the effects of teratogenicity, carcinogenesis and mutagenesis [4] and will remain in the agricultural produce; they are not only harmful to humans and animals, but also affect the quality and safety of the agricultural produce. Furthermore, the Asian corn borer can develop resistance to pesticides [5]. Additionally, natural enemies do not produce harmful substances but are not immediately effective.

The gram-positive bacterium *Bacillus thuringiensis* (Bt) is by far the most economically successful entomopathogen [6,7]. The insecticidal crystal (Cry) protein, produced during the sporulation process, has specific insecticidal effects on Lepidoptera, Coleoptera, Diptera and other pests, but is harmless to humans and other non-target insects [8,9]. The resistance evolution of pests is the main threat to their long-term use of genetically modified crops with Bt toxins [10]. Bt transgenic crops are promising for controlling the Asian corn borer, but people are still worried about the development of resistance [11]. It has been reported that the resistance of the Asian corn borer to Bt toxins such as Cry1F, Cry1Ie and Cry1Ac has been observed in laboratory selection [12,13]. Most of the current research suggests that the change in specific receptor sites on the insect midgut BBMV is the main reason why the target pests are resistant to Bt toxins [6,14]. Thus, the formation of membrane pores is the main reason for the cytotoxicity of Cry toxin in insects [15]. Mutations in the ABC transporter or cadherin-like protein (CAD) [16] are currently identified as potential resistance mechanisms. Most mutations could influence the proteins, but some of them can confer resistance with only minor structural changes. It has been reported that specific binding between Bt and some receptor proteins such as aminopeptidase (APN) [17] and alkaline phosphatases (ALP) [18] is responsible for the lethal effect of the Bt toxin. Additionally, V-type ATPase and heat shock 70 kDa proteins have also been shown to be Bt-binding proteins in the Asian corn borer [8]. However, the research on the mechanism of Bt resistance of the Asian corn borer is still limited, and the definite mechanism of the interaction between the Bt toxin and the receptor is still poorly understood.

In recent years, transcriptome sequencing has played an increasingly important role in the discovery and identification of functional genes [19]. In addition to the study of differential gene expression, it provides a powerful research tool for studying insect resistance mechanisms. In previous studies, Zhang et al. [20] analyzed transcripts to determine that the previously known genes associated with Bt resistance, aminopeptidase N1 (*apn1*) and *apn3*, as well as the ATP-binding cassette transporter group G (*abcg*) gene were significantly down-regulated in ACB-AbR and ACB-AcR. Shabbir et al. [21] studied the transcriptome and proteome of the susceptible Asian corn borer strain and the Cry1Ah-resistant strain, and identified 7,007 differential expressions genes (DEGs) between ACB-AhR and ACB-BtS strains and 182 differentially expressed proteins (DEPs). Resistance-related genes include aminopeptidase N, alkaline phosphatase, ABCC3, cadherin-like protein and glutathione S-transferase (GSTs). From the perspective of molecular biology, this study compared the midgut tissues of Cry1F-resistant strain (ACB-FR), Cry1Ie-resistant strain (ACB-IeR) and susceptible strain (ACB-BtS) at the transcriptome (RNA-seq) level. The analysis of DEGs was further verified by RT-qPCR. Therefore, by comparing ACB-FR and ACB-IeR with ACB-BtS simultaneously, we can have a more comprehensive understanding of the genes related to resistance. This is expected to provide new ideas for delaying the resistance of Asian corn borer to transgenic corn, and to discovering new genes related to the Bt resistance. These studies are valuable for understanding the systematic difference between Bt susceptible and resistant strains.

## 2. Materials and Methods

### 2.1. Bioassay and Resistance Ratio

Three *O. furnacalis* laboratory strains were raised in State Key Laboratory for the Biology of the Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. Cry1Ie-resistant strains (ACB-IeR) are exposed to Cry1Ie toxins with a concentration of 16.0 µg/g and Cry1F-resistant strains (ACB-FR) are exposed to Cry1F toxins with a concentration of 3.0 µg/g to maintain the intensity of selection, and susceptible strain (ACB-BtS) is kept on an artificial diet without any toxins. The Cry1F toxin protein used in this experiment was produced by Marianne P. Carey, Case Western Reserve University, USA. Cry1Ie toxin protein was purchased from ABZYM Biosciences, Beijing, China.

The protein-food feeding method was used to determine the toxicological reaction of each strain to Bt toxin [22]. ACB-BtS was exposed to six concentrations of Cry1Ie toxin (0, 0.2, 0.4, 1, 2, 5 µg/g) and seven concentrations of Cry1F toxin (0, 0.04, 0.08, 0.20, 0.40, 1.00, 2.00, 5.00 µg/g); ACB-IeR was exposed to six concentrations of Cry1Ie toxin (0, 40, 80, 200, 400, 1000 µg/g); ACB-FR was exposed to six concentrations of Cry1F toxin (0, 10, 20, 50, 100, 250, 500 µg/g), and the corresponding concentration of artificial feed was distributed into 48-well plates. A single starvation-treated 24-h-old neonate was put into each hole; a total of 144 larvae were tested at each concentration. ddH<sub>2</sub>O replaced the toxins to mix with an artificial diet that was used as a control. The larvae were reared at 27 ± 1 °C, 70–80% relative humidity (RH) and a photoperiod of 16:8 h (L: D). Investigating the results after seven days, the number of survivals and total body weight were recorded, and larvae were considered dead if they had died or weighed less than 0.1 mg. The Probit statistical model in SPSS 19.0 was used to calculate the lethal concentration of each strain and each toxin that causes 50% of the individual's mortality (LC<sub>50</sub>), as well as the LC<sub>50</sub> ratio of the susceptible strain and the resistant strain when exposed to the same toxin as the resistance ratio (RR).

### 2.2. Midgut Dissection and RNA Extraction

Fourth instar larvae were put on ice to make them comatose, the midgut was dissected and put into a PBS buffer to remove the food content, then transferred into water to wash it again, the water absorbed with filter paper and then put into 200 µL Trizol. The midguts of five individuals were collected as one biological replicate, and three biological replicates were collected from ACB-BtS, ACB-FR and ACB-IeR strains.

Total RNA was extracted from the midgut of ACB-BtS, ACB-FR and ACB-IeR strains following the manufacturer's instructions for Trizol reagent. The quantity and quality of RNA were evaluated by 1% agarose gel electrophoresis and spectrophotometry on Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). All samples were stored at −80 °C until they were used for first-strand cDNA synthesis.

### 2.3. Library Preparation for Transcriptome Sequencing

Nine single-ended cDNA libraries were constructed using total RNA samples from the midgut of ACB-BtS, ACB-FR and ACB-IeR. Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase-free agarose gel electrophoresis. After total RNA was extracted, mRNA of each sample was enriched by Oligo (dT) magnetic beads. Then, the enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse transcription into cDNA with random primers. Second-strand cDNA was synthesized by DNA polymerase I, RNase H, dNTP and buffer. Then, the cDNA fragments were purified with QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end-repaired, single A base added, and ligated to Illumina sequencing adapters. The ligation products were size-selected by agarose gel electrophoresis, PCR amplified and sequenced using Illumina Novaseq 6000 by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China).

#### 2.4. Assembly and Functional Gene Annotation

By filtering raw data, lower-quality reads were filtered. The linker sequence was removed to obtain high-quality clean data by fastp (version 0.18.0) [23]. After screening the raw data, the Trinity program was used to assemble high-quality clean data [24]. To minimize the sequence's redundancy, the CD-HIT program was used to obtain the longest transcript contigs [25]. After the library quality has been evaluated to meet the requirements of bioinformatics analysis, expression level and gene structure analysis can be carried out. DESeq2 was used to calculate the expression level of candidate genes in different treatments [26]. BLASTX was used to compare unigene sequences with NCBI protein databases: set e-value  $\leq 10^{-5}$ , and using the annotation information with the highest similarity in the comparison as the final comment information. DEGs were calculated as FPKM (fragments per kilobase pair of exon model per million fragments mapped), which were used to compare the expression of up-regulated or down-regulated transcripts with ACB-BtS strain.

#### 2.5. Selection of Differentially Expressed Genes among ACB-BtS, ACB-FR and ACB-IeR

Blast2GO was used to predict the classification of the function of unigenes in different treatments by EuKaryotic of orthologous groups (KOG) database [27]. KEGG pathway enrichment was conducted to analysis by the online KEGG Automatic Annotation Server (KAAS) [28,29]. In order to understand the differential gene, we not only performed GO function and KEGG pathway enrichment analysis for all the differential genes in the transcriptome, but also used the false discovery rate (FDR) method to determine the threshold. The  $FDR \leq 0.05$  and the absolute value of  $\log_2FC \geq 1$  were used as evaluation criteria to compare the significance level of gene expression between different ACB strains. In addition, the genes annotated as candidate Bt resistance genes were verified by RT-qPCR in *O. furnacalis* midgut.

#### 2.6. RT-qPCR Analysis

The genes associated with Bt resistance were screened out from the transcriptome and summarized pathway annotation candidate genes in the KEGG pathway with  $p$ -value  $< 0.05$ , and the part of the screened resistance-related genes that was duplicated with genes involved in the KEGG pathway was deleted, then RT-qPCR was used to verify these genes. Total RNA was prepared from the midgut of ACB-FR, ACB-IeR and ACB-BtS strains with three biological replicates. The TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) was used to synthesize cDNAs. The primers used for RT-qPCR were designed with Primer 3PLUS (Supplementary Materials: Table S1).

Reactions were set up in triplicate using 20  $\mu$ L reaction volumes containing 10  $\mu$ L of  $2\times$  PerfectStart Green qPCR SuperMix (TransGen Biotech, China), 0.8  $\mu$ L of PCR forward primer (10  $\mu$ M), 0.8  $\mu$ L of PCR reverse primer (10  $\mu$ M), 0.4  $\mu$ L of passive reference dye (50 $\times$ ), 1.0  $\mu$ L of cDNA as template and 7.0  $\mu$ L sterilized ultra-pure grade water. The ABI 7500 fast real-time PCR system was used for amplification at 95  $^{\circ}$ C (30 s), followed by 40 cycles at 95  $^{\circ}$ C (10 s) and 60  $^{\circ}$ C (20 s). Then, melting curve analysis was conducted to evaluate the specificity of amplification. Before RT-qPCR, the amplification efficiency of all the primers was verified. The actin gene of ACB was used to normalize the RT-qPCR data. Relative expression levels for target genes in different strains were calculated via the  $2^{-\Delta\Delta CT}$  method [30]. The expression difference among ACB-FR, ACB-IeR and ACB-BtS strains was analyzed by one-way analyses of variance (ANOVAs) statistical method. Differences in the three strains were compared using Turkey test.

### 3. Results

#### 3.1. Insect Resistance Levels

After 28 generations of selection in the lab, ACB-IeR had developed more than 862-fold resistance to Cry1Ie (Table 1). Analogously, ACB-FR had developed 961-fold resistance to Cry1F (Table 1).

**Table 1.** The larvae lethal concentration (LC) and resistance ratio (RR) of ACB-IeR and ACB-FR strains compared with the susceptible ACB-BtS strains on Cry1Ie and Cry1F toxins.

Bt Toxin	Insect Strain	LC <sub>50</sub> (95% CI) <sup>1</sup> µg/g	Resistance Ratio
Cry1F	ACB-BtS	0.52 (0.36–0.70)	/
	ACB-FR	>500.00	>961.00
Cry1Ie	ACB-BtS	1.16 (0.84–1.66)	/
	ACB-IeR	>1000.00	>862.07

<sup>1</sup> 95% confidence interval.

### 3.2. RNA-Seq and Sequence Assembly

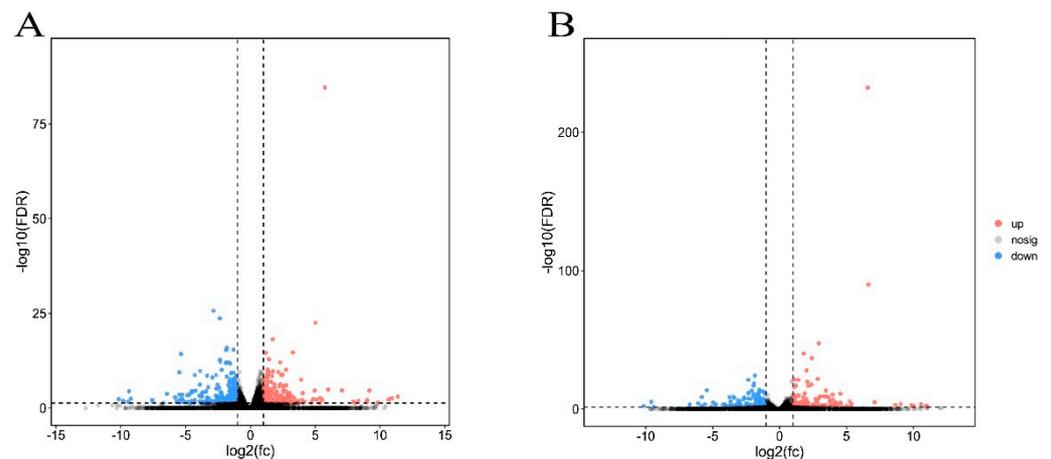
Quality control of data was obtained from transcriptome sequencing, and the RNA sequencing results of ACB-FR, ACB-IeR and ACB-BtS ranged from 39,687,801 to 50,484,709 (Table 2). The clean sequence of each library ranges from 39,127,741 to 49,893,262 reads. In addition, the GC content ranges from 48.99% to 49.95%. The total number of reads that can be mapped to the genome ranges from 31,285,151 to 41,890,268 and the proportion of effective reads was from 79.96% to 85.34%. The raw reads of ACB-BtS, ACB-FR and ACB-IeR strain were submitted to the SRA database with accession number SRR16556445, SRR16571663 and SRR16572026.

**Table 2.** Summary of average reads in transcriptomes of ACB-FR, ACB-IeR and susceptible (ACB-BtS) strains.

Samples	Raw Reads	Clean Reads	Q20 (%)	Q30 (%)	GC (%)	Total Mapped
ACB-BtS	39,687,801	39,127,741	97.63	93.31	48.99	31,285,151 (79.96%)
ACB-FR	47,222,879	46,637,357	97.89	93.90	49.72	39,798,320 (85.34%)
ACB-IeR	50,484,709	49,893,262	98.06	94.30	49.95	41,890,268 (83.96%)

### 3.3. Differentially Expressed Genes between Cry1F and Cry1Ie-Resistant Strains Compared with Susceptible Strains of *O. furnacalis*

Differentially expression genes (DEGs) were screened with  $FDR \leq 0.05$  and  $\log_2FC$  absolute value  $> 1$  as a significantly different gene (Supplementary Materials: Table S5). The results showed that a total of 283 down-regulated and 172 up-regulated genes were differentially expressed (Figure 1A) in ACB-FR compared with ACB-BtS strains. Moreover, a total of 229 down-regulated and 133 up-regulated genes were selected in ACB-IeR compared with ACB-BtS strains (Figure 1B). This comparison showed that a larger proportion of genes were significantly down-regulated in the Bt-resistant strain. Based on former reports and DEGs in the transcriptome analysis, some well-known Bt receptors such as cadherin, ABC transporter, ALP and APN were screened out and listed in Supplementary Table S2. Other genes include UDP-glucuronosyltransferase gene, trypsin, heat shock protein (HSP) 68-like gene, glutathione S-transferase 1-like gene (GSTs), etc. Several of these Bt-resistance candidate genes were differentially regulated in both of the resistant strains. Most of the heat shock proteins (Supplementary Materials: Table S6) were down-regulated in ACB-FR compared with ACB-BtS strains, but it was expressed higher in ACB-IeR. UDP-glucuronosyltransferase (Supplementary Materials: Table S7) and trypsin, especially alkaline C-like (Supplementary Materials: Table S8), were up-regulated in ACB-IeR and ACB-FR compared with ACB-BtS strains. The differential expression of GSTs in Cry1F- and Cry1Ie-resistant strains was shown in Table S9. From the list, five GSTs were up-regulated in ACB-FR and ACB-IeR, only ncbi\_114360773 significantly up-regulated with  $\log_2FC$  value of 2.75 in ACB-FR strain and MSTRG.22972 up-regulated with  $\log_2FC$  value of 1.91 in the ACB-IeR strain. Three GSTs were down-regulated in ACB-FR and ACB-IeR strains, but only ncbi\_114365341 was significantly down-regulated in ACB-IeR strains with  $\log_2FC$  value  $-1.66$ .



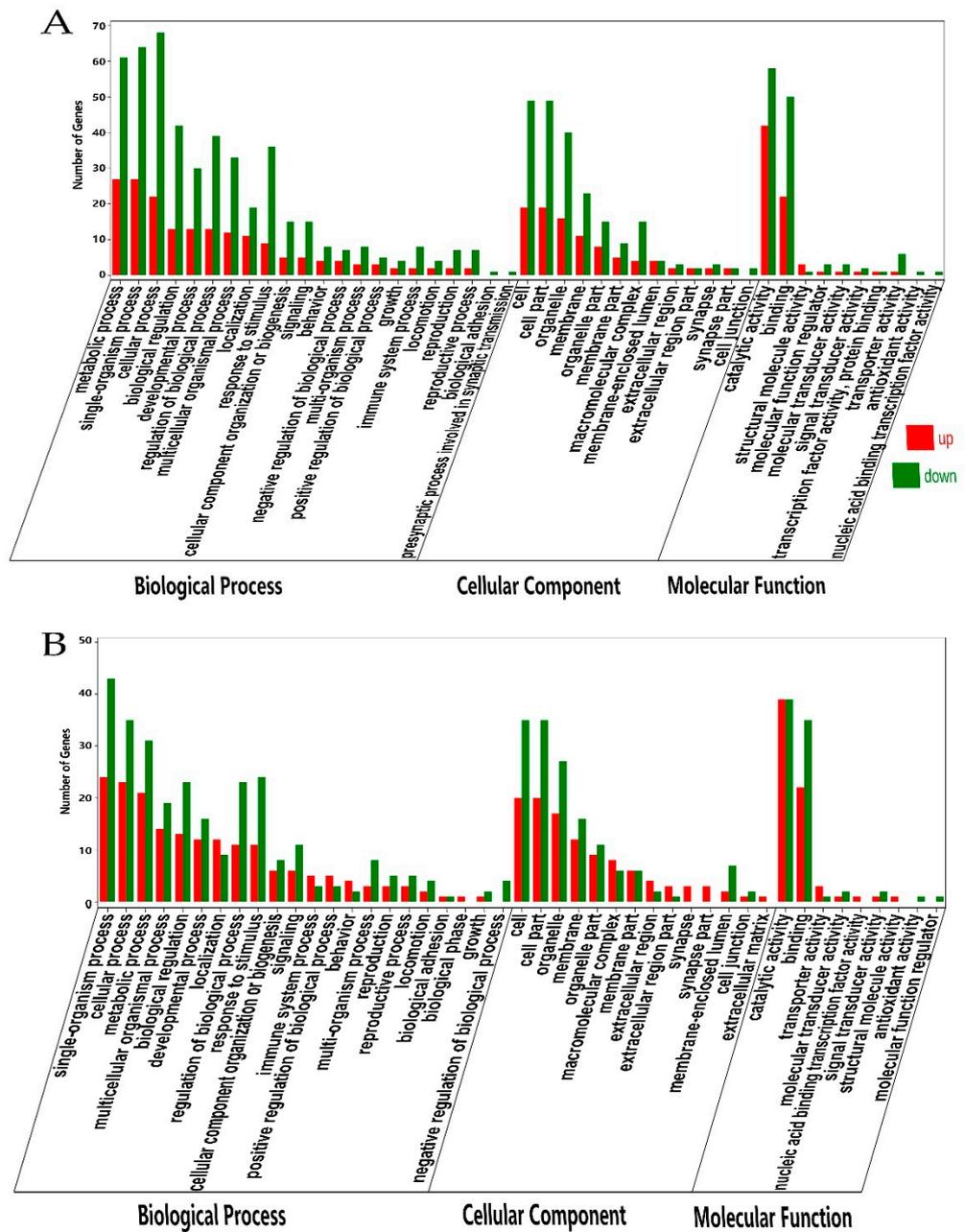
**Figure 1.** Volcano plots of differentially expressed genes (DEG). (A) Cry1F-resistant vs. susceptible *O. furnacalis*. (B) Cry1Ie-resistant vs. susceptible *O. furnacalis*. The scattered dots in the figure indicate each gene: red indicates that the gene is up-regulated, blue indicates that the gene is down-regulated and gray genes indicate no differential expression.

### 3.4. Gene Ontology and Pathway Enrichment

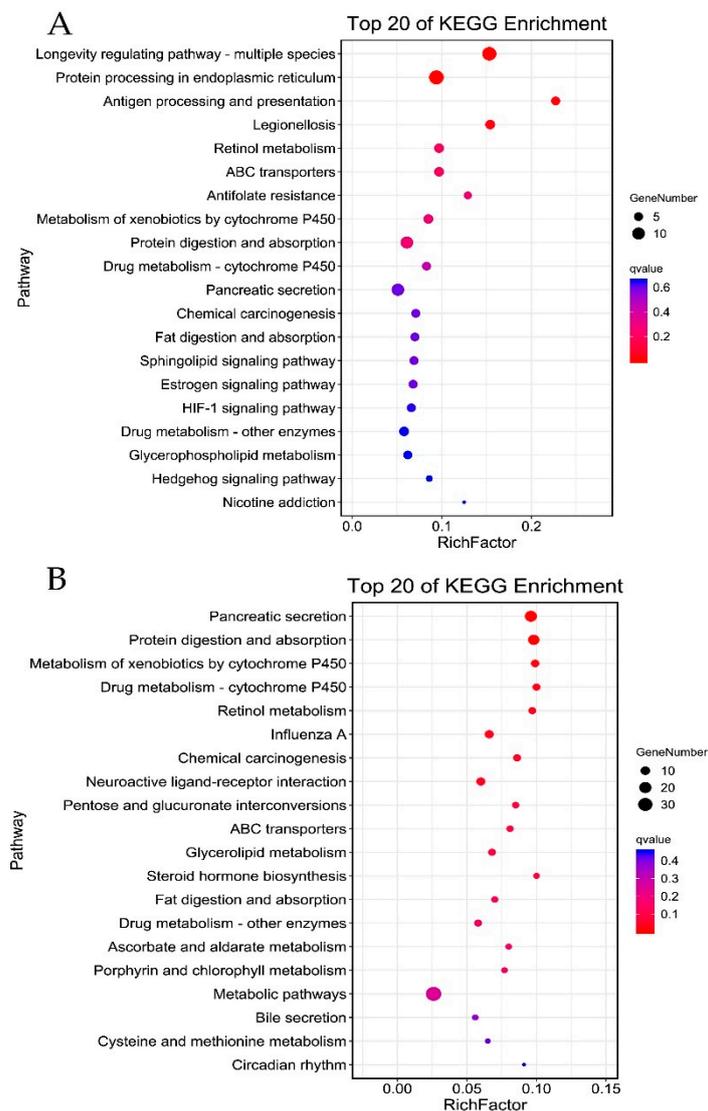
In order to enrich the identified differential genes into specific functional categories and explore the biological significance behind transcriptomics data, ACB-FR or ACB-IeR was compared with ACB-BtS unigene for GO analysis, according to the Biological Process, Cellular Component and Molecular Function for classification and annotation. The three classification categories were subdivided into forty-five secondary GO-terms (Figure 2).

In terms of Biological Process, unigenes were allocated to 22 aspects such as cellular process, single-organism process and metabolic process. Concerning cellular components, the cell part and cell and organelle represent most of the genes. In the Molecular Function category, catalytic activity and binding were the most abundant. ACB-FR and ACB-IeR, compared with ACB-BtS, have the largest number of genes annotated in the secondary classification of catalytic activity, single-organism process and cellular process. Comparing ACB-FR with ACB-BtS (Figure 2A), in the case of catalytic activity, 100 DEGs were linked, and there are 58 DEGs down-regulated and 42 DEGs up-regulated in ACB-FR. There are 91 related DEGs in the single-organism process, of which 64 DEGs were down-regulated and 27 DEGs were up-regulated. In the cellular process, there are 90 related DEGs; 68 DEGs were down-regulated in ACB-FR, and 22 DEGs were up-regulated (Supplementary Materials: Table S3). Comparing ACB-IeR with ACB-BtS (Figure 2B), in the case of catalytic activity, there are 78 related DEGs, including 39 down-regulated DEGs and 39 up-regulated DEGs in ACB-FR strains. In the single-organism process, there are 67 related DEGs; 43 DEGs were down-regulated and 24 DEGs were up-regulated, and 58 DEGs were related to the cellular process; 35 DEGs were down-regulated and 23 DEGs were up-regulated (Supplementary Materials: Table S3).

A total of 130 candidate genes with pathway annotation were allocated to reference pathways in KEGG when ACB-FR was compared with ACB-BtS. In the result, 15 pathways with  $p \leq 0.05$  (Supplementary Materials: Table S4) were enriched, including “Longevity regulating pathway-multiple species” and “Protein processing in endoplasmic reticulum”, which had the lowest  $p$ -value. The top 20 most-enriched pathways were shown in Figure 3A. However, comparing ACB-IeR with ACB-BtS, 94 candidate genes with pathway annotations were assigned to the reference pathways in KEGG, and 18 pathways were enriched with  $p \leq 0.05$  (Supplementary Materials: Table S4), including those with the lowest  $p$ -value “Pancreatic secretion” and “Protein digestion and absorption”. The top 20 most-enriched pathways were shown in Figure 3B.



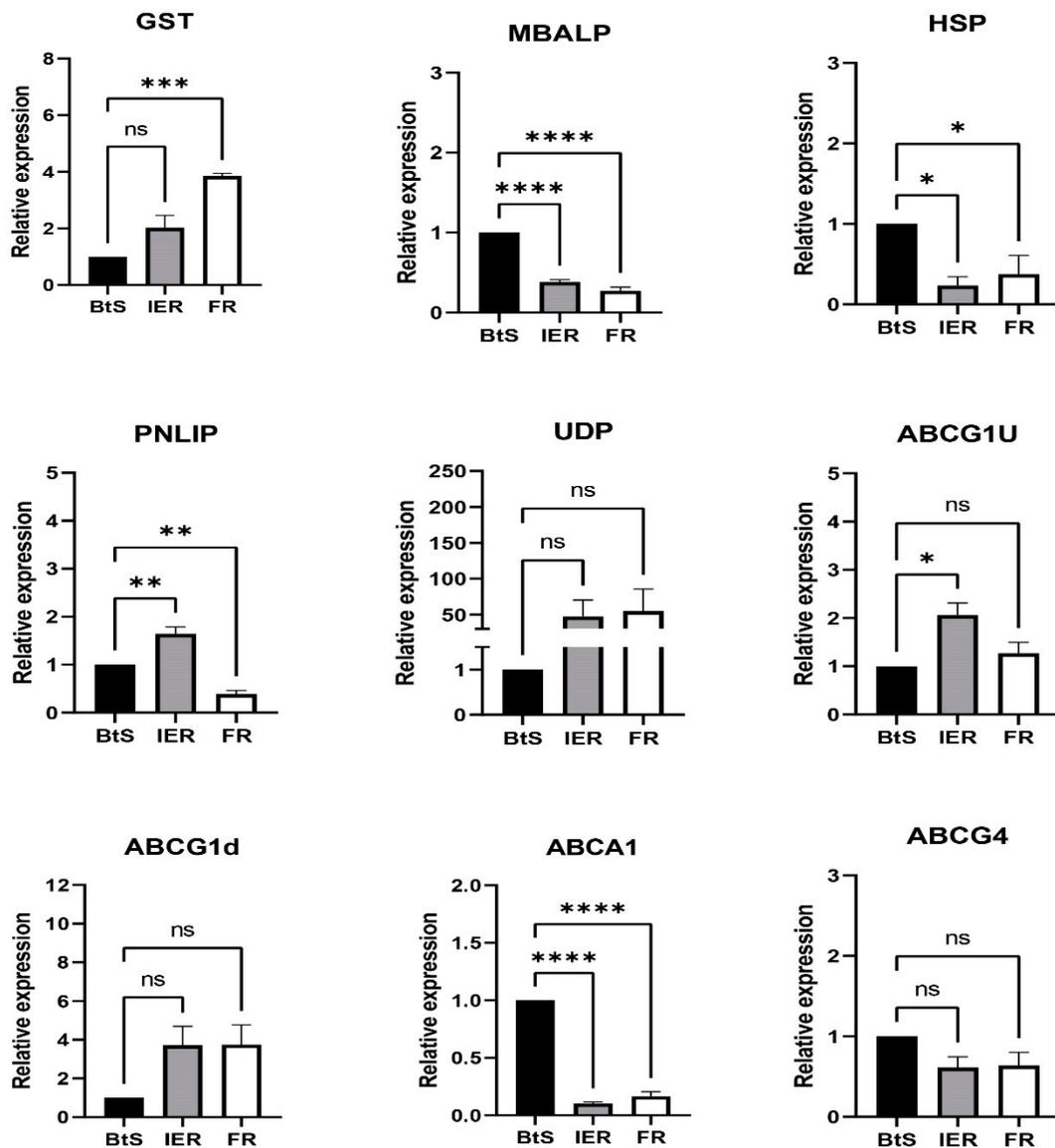
**Figure 2.** Gene ontology (GO) classification of the differentially expression genes in different ACB strains. (A) ACB-FR vs. ACB-BtS; (B) ACB-IeR vs. ACB-BtS. The abscissa is the second level GO term, the ordinate is the number of genes in the term, red column indicates up-regulation, and green column indicates down-regulation.



**Figure 3.** Top 20 KEGG pathways enrichment bubble chart, (A) ACB-FR vs. ACB-BtS; (B) ACB-IeR vs. ACB-BtS. The vertical axis is the pathway name, and the horizontal axis is the rich factor (the ratio of differentially expressed genes to all annotated genes in the pathway). The size of  $q$ -value is represented by the color of the point. Additionally, the smaller the  $q$ -value, the deeper of the red color. The size of the bubble indicates the number of differential genes contained in each pathway.

### 3.5. RT-qPCR Verification of Differentially Expressed Genes

According to the fold change of RT-qPCR analysis, the results support differential expression at the gene level. Compared with susceptible strains, some differentially expressed genes have the same trend in resistant strains, but several of these Bt-resistance candidate genes were differentially regulated in both resistant strains. Additionally, the expression levels of glutathione S-transferase 1-like, UDP-glucuronosyltransferase 2B14-like and ABCG1 were all up-regulated in both resistant strains (Figure 4). However, membrane-bound alkaline phosphatase-like (MBALP), heat shock protein 68-like and ABCA1 were down-regulated in both resistant strains (Figure 4). Moreover, pancreatic triacylglycerol lipase-like (PNLIP) was up-regulated in ACB-IeR and down-regulated in ACB-FR.



**Figure 4.** RT-qPCR results of differentially expressed genes. Three biological replicates of each strain of ACB-BtS, ACB-FR and ACB-IeR were performed in three technical replicates. The height of the bar graph represents the average of  $2^{-\Delta\Delta CT}$  values. The significance threshold is set to  $p \leq 0.05$ , “ns” indicates that the difference is not significant, “\*” indicates the difference is significant, and the number of “\*” indicates the degree of significance.

#### 4. Discussion

The evolution of resistance of target pest populations to Bt toxins is the most relevant threat to the sustainability of Bt technology in the future [7], and clarifying insect resistance mechanisms will help improve Bt technology [31]. In the present study, the ACB-IeR strain has a higher estimated Cry1Ie resistance ratio, more than 862-fold increase compared to ACB-BtS, after 28 generations of selection (Table 1). In another study, the ACB-IeR strain also had more than 850-fold resistance to Cry1Ie, and Cry1F-resistant strains have not shown cross-resistance to Cry1Ie [32]. When the target pest is resistant to one Bt toxin, it will produce cross-resistance to other Bt toxins with the same or similar action sites and binding receptors, while it has no or low cross-resistance to Bt toxins with different types of action sites [33,34]. As previously reported, the high cross-resistance of European corn borer Cry1Ab-resistant strains to Cry1Ac is due to a common binding site [35,36]. In addition, Cry1F-resistant European corn borer does not have cross-resistance

with Cry1Ab and Cry9C, because Cry1F has no common binding site with Cry1Ab and Cry9C [34,35]. To understand the mechanism of Bt resistance, it is necessary to study the binding sites of Bt toxins in *O. furnacalis*. This has guiding significance for the development and utilization of bivalent transgenic insect-resistant maize and different transgenic insect-resistant maize rotations.

Furthermore, a comparative analysis of transcriptome data shows that more genes in ACB-FR and ACB-IeR strains are down-regulated compared to susceptible strains (Figure 1). In the current study, the transcriptome analysis revealed 62–63% of genes were down-regulated in the Bt-resistant strains compared to the susceptible strain. Similarly, an analysis of chlorantraniliprole-susceptible and -resistant strains of *Plutella xylostella* revealed similar global gene down-regulation, where including many receptors and transporters [37]. Conversely, up-regulated genes related to immunity and detoxification genes were found in the current study and in Cry1A-resistant *P. xylostella* [38]. Thus, the down-regulation of Bt targets and transporters and simultaneous induction of detoxification mechanisms could lead to increased resistance to insecticides. In our transcriptome analysis, most genes within the ABCG family were down-regulated in ACB-IeR and ACB-FR compared with ACB-BtS; a similar result was reported in the ACB-AcR strain [20], which indicates that ABCG genes are putative receptors of Cry1Ac, Cry1Ie and Cry1F, and need more data to support this conclusion. Similarly, trypsin-like genes were up-regulated in ACB-IeR and ACB-FR compared with ACB-BtS (Table S2), but the functional relationship with Bt resistance is still unclear.

As the well-known receptor of Bt toxins, cadherin, APN and ALP was listed in Table S2. In total, 45 cadherins, 9 ALPs and 30 APNs were differentially expressed in ACB-IeR or -FR strains. Cadherins acted as receptors of Cry proteins are reported in many insect orders [39,40]. Binding with Cry1 toxins can form critical toxic oligomer that can effectively kill the insect [41]. Much research indicates that mutation of cadherin can generally link with Cry1A proteins resistance in *Pectinophora gossypiella*, *Heliothis virescens* and *Helicoverpa armigera* [14,42,43]. Knocking down by RNAi and knocking out by CRISPR/Cas 9 of cadherin can decrease the insect's susceptibility to Bt proteins [44]. Synergism between cadherin and ABCC2 were reported by in vitro and in vivo test [45,46].

In particular, Bt-resistance mechanisms have involved several ABC transporters from subgroups A [47], C [14,48] and G [49]; as reported previously [20], members of the three subgroups are down-regulated in Cry1Ab- and Cry1Ac-resistant *O. furnacalis*. The results of RT-qPCR showed that the abundance of *Ofabca2* was reduced in the midgut of Cry1F- and Cry1Ie-resistant larvae (Figure 4), and interestingly, *Ofabcg1* increased both in Cry1F- and Cry1Ie-resistant larval midgut transcripts and RT-qPCR results (Figure 4). This may indicate that although Cry toxins have some of the same receptors, the molecular mechanisms involved in developing resistance characteristics of different resistant *O. furnacalis* strains may be different.

Less differential expression ALPs (nine ALPs) were found in the two resistant strains. GPI-tethered or soluble ALPs involve dephosphorylation. Decreasing expression of ALPs were found in resistant strains of *Ostrinia furnacalis*, *Plutella xylostella*, *Spodoptera frugiperda* and *Helicoverpa armigera* [50,51], but in a resistant strain of *Diatraea saccharalis*, ALP transcript levels were not reduced [52]. In our result, only ncbi\_114355045 was significantly up-regulated in ACB-FR and -IeR strains, ncbi\_114362710 was significantly down-regulated in ACB-IeR strains and ncbi\_114355386 was significantly down-regulated in ACB-FR strains. Further experiments could unearth the functions of these genes.

In addition, more differential expression APNs (30 APNs) were found in ACB-FR and -IeR strain. Not all the APNs could interaction with Cry toxins; some of them were reported associated with resistance [53]. In *H. armigera* Cry1Ac resistant strain, there was a 22 aa deletion of APN, and in *Spodoptera exigua*, APN1 was absent in Cry1Ca resistant strain midgut [54]. Further SNP or deletion can be analyzed between resistant and susceptible strains of *O. furnacalis*. The knocking down of APNs could decrease the susceptibility to

Cry1A toxins in *Chilo suppressalis* [55], the gene down-regulated in ACB-FR or ACB-IeR, probably the receptors of Cry1F or Cry1Ie.

Moreover, previous studies have shown that most of the genes annotated as GSTs and trypsin in ACB-AhR have low expression levels [21]. Interestingly, we screened data related to GSTs from the transcript; most of the GSTs in ACB-FR showed an up-regulation trend, but it was down-regulated in ACB-IeR (Supplementary Materials: Table S9). The different expression GSTs in ACB-FR may be involved in Bt resistance, and could be partially responsible for Cry1F-specific resistance. The difference was that the genes annotated as trypsin alkaline C-like are mostly up-regulated in Cry1F- and Cry1Ie-resistant strains (Supplementary Materials: Table S8). According to previous reports, GSTs were related to resistance and participated in the detoxification of exogenous substances [56]. Trypsin is also considered the main protease for detoxification [57]. Thus, further research is needed to clarify the role of genes in the mechanism of Bt resistance.

In addition, GO and KEGG pathway analysis was performed on the DEGs of ACB-FR, ACB-IeR and ACB-BtS to find more genes related to Bt resistance in ACB. It can be seen that a large part of genes is related to catalytic activity in terms of molecular function (Supplementary Materials: Table S3). In the previous report, the molecular function part of the resistant strain of *P. xylostella* involved the most genes in the category of catalytic activity. These findings indicate that the resistance mechanism may also be related to catalytic activity [38,58]. Furthermore, KEGG pathway enrichment analysis showed that ACB-FR and ACB-IeR was significantly enriched in a series of genes in the same KEGG pathways (Supplementary Materials: Table S4), such as retinol metabolism, ABC transporters, metabolism of xenobiotics by cytochrome P450, protein digestion and absorption, pancreatic secretion, chemical carcinogenesis, fat digestion and absorption and drug metabolism-cytochrome P450; these pathways may be related to Bt toxins resistance. ABC transporters play a key role in the mode of action of the Bt toxin. Previous reports have shown that mutations in the ABC protein can confer high levels of resistance [59]. The two KEGG pathways about P450 are very important in the metabolism of xenobiotics and drugs. CYP450 includes many subfamilies, but only six exist in insects, five are insect specific, CYP6/CYP9/CYP12/CYP18 and CYP28, and one, CYP4, includes sequences from vertebrates [60,61], especially for CYP4 and CYP6 which are reported to relate with insecticide resistance [62,63]. Bt toxins also act as xenobiotics or drugs for insects, and enrichment on the two pathways in the comparison of resistant and susceptible strains indicates P450s are related to Bt resistance.

## 5. Conclusions

Cry1Ie- and Cry1F-resistant strains were selected in our lab and compared with susceptible strains. ACB-IeR strains evolved more than 862.07-fold greater resistance to Cry1Ie than susceptible strain ACB-BtS, and ACB-FR strains evolved more than 961-fold greater resistance to Cry1F than ACB-BtS. Further analysis of the transcriptomes of the three strains showed that a larger proportion of genes were significantly down-regulated in the Bt-resistant strain. This study is based on the transcripts of Cry1Ie- and Cry1F-resistant strains, focusing on finding the differences between the two strains at the molecular level, which is believed to provide ideas for delaying Bt resistance.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture12020298/s1>, Table S1: Primers used for RT-qPCR experiment; Table S2: The transcripts significantly up- or down-regulated in ACB-IeR or -FR compared with ACB-BtS strain; Table S3: Gene ontology analysis of *Ostrinia furnacalis* transcriptome; Table S4: Significantly enriched KEGG pathways in *Ostrinia furnacalis*. Table S5: Differentially expressed genes among ACB-BtS, ACB-FR and ACB-IeR. Table S6: Transcript data about heat shock protein; Table S7: Transcript data about UDP-glucuronosyltransferase; Table S8: Transcript data about trypsin alkaline C-like genes; Table S9: Transcript data about glutathione S-transferase genes.

**Author Contributions:** Conceptualization, Y.L. and T.Z.; Data curation, Q.G. and T.Z.; Funding acquisition, Z.W.; Investigation, Y.W. and T.Z.; Methodology, T.Z.; Project administration, Y.L.; Resources, Y.W. and T.Z.; Supervision, K.H. and S.S.; Writing—original draft, Y.L.; Writing—review and editing, Z.W. and T.Z. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the China Agriculture Research System (CARS-02).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in the article and Supplementary Materials.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

Asian corn borer (ACB), *Bacillus thuringiensis* (Bt), Susceptible strain (ACB-BtS), Cry1Ie-resistant strain (ACB-IeR), Cry1F-resistant strain (ACB-FR), Differential expressions genes (DEGs), glutathione S-transferase (GSTs).

## References

1. Wang, Z.; Lu, X.; He, K.; Zhou, D.R. Review of history, present situation and prospect of the Asian maize borer research in China. *J. Shenyang Agric. Univ.* **2000**, *31*, 402–412.
2. Song, L.; Wei, L.; Wang, Z.; He, K.; Cong, B. Effect of infestation by the Asian corn borer together with *Fusarium verticillioides* on corn yield loss. *Acta Phytophy. Sin.* **2009**, *36*, 487–490.
3. Feng, C.; Huang, J.; Song, Q.; Stanley, D.; Lü, W.; Zhang, Y.; Huang, Y. Parasitization by *Macrocentrus cingulum* (Hymenoptera: Braconidae) influences expression of prophenoloxidase in Asian corn borer *Ostrinia furnacalis*. *Arch. Insect Biochem. Physiol.* **2011**, *77*, 99–117. [[CrossRef](#)]
4. Patel, S.; Sangeeta, S. Pesticides as the drivers of neuropsychotic diseases, cancers, and teratogenicity among agro-workers as well as general public. *Environ. Sci. Pollut. Res.* **2019**, *26*, 91–100. [[CrossRef](#)] [[PubMed](#)]
5. Yu, G.; Zheng, L.; Quan, Y.; Wei, H. Sublethal pesticide exposure improves resistance to infection in the Asian corn borer. *Ecol. Entomol.* **2018**, *43*, 326–331. [[CrossRef](#)]
6. Schnepf, E.; Crickmore, N.; Van Rie, J.; Lereclus, D.; Baum, J.; Feitelson, J.; Zeigler, D.; Dean, D. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 775–806. [[CrossRef](#)] [[PubMed](#)]
7. Jurat-Fuentes, J.L.; Heckel, D.G.; Ferré, J. Mechanisms of resistance to insecticidal proteins from *Bacillus thuringiensis*. *Annu. Rev. Entomol.* **2021**, *66*, 121–140. [[CrossRef](#)] [[PubMed](#)]
8. Xu, L.N.; Ferry, N.; Wang, Z.Y.; Zhang, J.; Edwards, M.; Gatehouse, A.; He, K.L. A proteomic approach to study the mechanism of tolerance to Bt toxins in *Ostrinia furnacalis* larvae selected for resistance to Cry1Ab. *Transgenic Res.* **2013**, *22*, 1155–1166. [[CrossRef](#)] [[PubMed](#)]
9. Comas, C.; Lumbierres, B.; Pons, X.; Albajes, R. No effects of *Bacillus thuringiensis* maize on nontarget organisms in the field in southern Europe: A meta-analysis of 26 arthropod taxa. *Transgenic Res.* **2014**, *23*, 135–143. [[CrossRef](#)]
10. Morin, S.; Biggs, R.; Sisterson, M.; Shriver, L.; Eilers-Kirk, C.; Higginson, D.; Holley, D.; Gahan, L.; Heckel, D.; Carriere, Y.; et al. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 5004–5009. [[CrossRef](#)]
11. Gould, F. Sustainability of Transgenic Insecticidal Cultivars: Integrating Pest Genetics and Ecology. *Annu. Rev. Entomol.* **1998**, *43*, 701–726. [[CrossRef](#)] [[PubMed](#)]
12. Wang, Y.Q.; Wang, Y.D.; Wang, Z.Y.; Bravo, A.; Soberón, M.; He, K.L. Genetic Basis of Cry1F-Resistance in a Laboratory Selected Asian Corn Borer Strain and Its Cross-Resistance to Other *Bacillus thuringiensis* Toxins. *PLoS ONE* **2016**, *11*, e0161189. [[CrossRef](#)] [[PubMed](#)]
13. He, K.L.; Wang, Z.Y.; Zhou, D.R.; Wen, L.P.; Song, Y.Y.; Yao, Z.Y. Evaluation of transgenic Bt corn for resistance to the Asian corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* **2003**, *96*, 935–940. [[CrossRef](#)] [[PubMed](#)]
14. Gahan, L.; Gould, F.; Heckel, D. Identification of a Gene Associated with Bt Resistance in *Heliothis virescens*. *Science* **2001**, *293*, 857–860. [[CrossRef](#)] [[PubMed](#)]
15. Heckel, D.G. How do toxins from *Bacillus thuringiensis* kill insects? An evolutionary perspective. *Arch. Insect Biochem. Physiol.* **2020**, *104*, e21673. [[CrossRef](#)] [[PubMed](#)]
16. Vadlamudi, R.; Weber, E.; Ji, I.; Ji, T.; Bulla, L. Cloning and Expression of a Receptor for an Insecticidal Toxin of *Bacillus thuringiensis*. *J. Biol. Chem.* **1995**, *270*, 5490–5494. [[CrossRef](#)] [[PubMed](#)]

17. Knight, P.; Crickmore, N.; Ellar, D. The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Mol. Microbiol.* **1994**, *11*, 429–436. [[CrossRef](#)]
18. Bravo, A.; Gill, S.S.; Soberon, M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* **2007**, *49*, 423–435. [[CrossRef](#)]
19. Zhang, Y.; Dong, X.; Liu, J.; Hu, M.; Zhong, G.; Geng, P.; Yi, X. Molecular Cloning, Expression and Molecular Modeling of Chemosensory Protein from *Spodoptera litura* and Its Binding Properties with Rhodojaponin III. *PLoS ONE* **2012**, *7*, e47611. [[CrossRef](#)]
20. Zhang, T.T.; Coates, B.; Wang, Y.Q.; Wang, Y.D.; Bai, S.X.; Wang, Z.Y.; He, K.L. Down-regulation of aminopeptidase N and ABC transporter subfamily G transcripts in Cry1Ab and Cry1Ac resistant Asian corn borer, *Ostrinia furnacalis* (Lepidoptera: Crambidae). *Int. J. Biol. Sci.* **2017**, *13*, 835–851. [[CrossRef](#)]
21. Shabbir, M.Z.; Zhang, T.T.; Wang, Z.Y.; He, K.L. Transcriptome and proteome alternation with resistance to *Bacillus thuringiensis* Cry1Ah toxin in *Ostrinia furnacalis*. *Front. Physiol.* **2019**, *10*, 27. [[CrossRef](#)] [[PubMed](#)]
22. Zhang, T.T.; He, M.X.; Gatehouse, A.; Wang, Z.Y.; Edwards, M.; He, K.L. Inheritance Patterns, Dominance and Cross-Resistance of Cry1Ab- and Cry1Ac-Selected *Ostrinia furnacalis* (Guenée). *Toxins* **2014**, *6*, 2694–2707. [[CrossRef](#)] [[PubMed](#)]
23. Chen, S.; Zhou, Y.; Chen, Y.; Gu, J. fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **2018**, *34*, i884–i890. [[CrossRef](#)]
24. Grabherr, M.G.; Haas, B.J.; Yassour, M.; Levin, J.Z.; Thompson, D.A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **2011**, *29*, 644–652. [[CrossRef](#)]
25. Li, W.; Godzik, A. Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **2006**, *22*, 1658–1659. [[CrossRef](#)]
26. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 1–21. [[CrossRef](#)] [[PubMed](#)]
27. Conesa, A.; Götz, S.; García-Gómez, J.M.; Terol, J.; Talón, M.; Robles, M. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **2005**, *21*, 3674–3676. [[CrossRef](#)]
28. Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T. Gene ontology: Tool for the unification of biology. *Nat. Genet.* **2000**, *25*, 25–29. [[CrossRef](#)]
29. Kanehisa, M.; Araki, M.; Goto, S.; Hattori, M.; Hirakawa, M.; Itoh, M.; Katayama, T.; Kawashima, S.; Okuda, S.; Tokimatsu, T. KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* **2007**, *36*, D480–D484. [[CrossRef](#)]
30. Livak, K.; Schmittgen, T. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]
31. Zhang, D.D.; Jin, M.H.; Yang, Y.C.; Zhang, J.F.; Yang, Y.B.; Liu, K.Y.; Soberón, M.; Bravo, A.; Xiao, Y.T.; Wu, K.M. Synergistic resistance of *Helicoverpa armigera* to Bt toxins linked to cadherin and ABC transporters mutations. *Insect Biochem. Mol. Biol.* **2021**, *137*, 103635. [[CrossRef](#)]
32. Wang, Y.Q.; Yang, J.; Quan, Y.D.; Wang, Z.Y.; Cai, W.Z.; He, K.L. Characterization of Asian corn borer resistance to Bt toxin Cry1Ie. *Toxins* **2017**, *9*, 186. [[CrossRef](#)] [[PubMed](#)]
33. He, M.X.; He, K.L.; Wang, Z.Y.; Wang, X.Y.; Li, Q. Selection for Cry1Ie resistance and cross-resistance of the selected strain to other Cry toxins in the Asian corn borer, *Ostrinia furnacalis* (Lepidoptera: Crambidae). *Acta Entomol. Sin.* **2013**, *56*, 1135–1142.
34. Pereira, E.J.G.; Lang, B.A.; Storer, N.P.; Siegfried, B.D. Selection for Cry1F resistance in the European corn borer and cross-resistance to other Cry toxins. *Entomol. Exp. Appl.* **2008**, *126*. [[CrossRef](#)]
35. Hua, G.; Masson, L.; Jurat-Fuentes, J.L.; Schwab, G.; Adang, M.J. Binding analyses of *Bacillus thuringiensis* Cry  $\delta$ -endotoxins using brush border membrane vesicles of *Ostrinia nubilalis*. *Appl. Environ. Microbiol.* **2001**, *67*, 872–879. [[CrossRef](#)] [[PubMed](#)]
36. Siqueira, H.A.; Moellenbeck, D.; Spencer, T.; Siegfried, B.D. Cross-resistance of Cry1Ab-selected *Ostrinia nubilalis* (Lepidoptera: Crambidae) to *Bacillus thuringiensis*  $\delta$ -endotoxins. *J. Econ. Entomol.* **2004**, *97*, 1049–1057. [[CrossRef](#)]
37. Lin, Q.; Jin, F.; Hu, Z.; Chen, H.; Yin, F.; Li, Z.; Dong, X.; Zhang, D.; Ren, S.; Zhang, Z. Transcriptome Analysis of Chlorantraniliprole Resistance Development in the Diamondback Moth *Plutella xylostella*. *PLoS ONE* **2013**, *8*, e72314. [[CrossRef](#)]
38. Lei, Y.Y.; Zhu, X.; Xie, W.; Wu, Q.J.; Wang, S.L.; Guo, Z.J.; Xu, B.Y.; Li, X.C.; Zhou, X.G.; Zhang, Y.J. Midgut transcriptome response to a Cry toxin in the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *Gene* **2014**, *533*, 180–187. [[CrossRef](#)]
39. Wu, Y.D. Detection and Mechanisms of Resistance Evolved in Insects to Cry Toxins from *Bacillus thuringiensis*. *Adv. Insect Physiol.* **2014**, *47*, 297–342.
40. Sayed, A.; Nekl, E.; Siqueira, H.; Wang, H.; Ffrench-Constant, R.; Bagley, M.; Siegfried, B. A novel cadherin-like gene from western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), larval midgut tissue. *Insect Mol. Biol.* **2007**, *16*, 591–600. [[CrossRef](#)]
41. Bravo, A.; Likitvatanavong, S.; Gill, S.; Soberón, M. *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochem. Mol. Biol.* **2011**, *41*, 423–431. [[CrossRef](#)] [[PubMed](#)]
42. Fabrick, J.; Ponnuraj, J.; Singh, A.; Tanwar, R.; Unnithan, G.; Yelich, A.; Li, X.C.; Carriere, Y.; Tabashnik, B. Alternative Splicing and Highly Variable Cadherin Transcripts Associated with Field-Evolved Resistance of Pink Bollworm to Bt Cotton in India. *PLoS ONE* **2014**, *9*, e97900. [[CrossRef](#)]
43. Zhang, H.N.; Tian, W.; Zhao, J.; Jin, L.; Yang, J.; Liu, C.H.; Yang, Y.H.; Wu, S.W.; Wu, K.M.; Cui, J.J.; et al. Diverse genetic basis of field-evolved resistance to Bt cotton in cotton bollworm from China. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 10275–10280. [[CrossRef](#)]

44. Wang, J.; Zhang, H.N.; Wang, H.D.; Zhao, S.; Zuo, Y.Y.; Yang, Y.H.; Wu, Y.D. Functional validation of cadherin as a receptor of Bt toxin Cry1Ac in *Helicoverpa armigera* utilizing the CRISPR/Cas9 system. *Insect Biochem. Mol. Biol.* **2016**, *76*, 11–17. [[CrossRef](#)] [[PubMed](#)]
45. Liu, Z.X.; Fu, S.; Ma, X.L.; Baxter, S.W.; Vasseur, L.; Xiong, L.; Huang, Y.P.; Yang, G.; You, S.J.; You, M.S. Resistance to *Bacillus thuringiensis* Cry1Ac toxin requires mutations in two *Plutella xylostella* ATP-binding cassette transporter paralogs. *PLoS Pathog.* **2020**, *16*, e1008697. [[CrossRef](#)]
46. Bretschneider, A.; Heckel, D.; Pauchet, Y. Three toxins, two receptors, one mechanism: Mode of action of Cry1A toxins from *Bacillus thuringiensis* in *Heliothis virescens*. *Insect Biochem. Mol. Biol.* **2016**, *76*, 109–117. [[CrossRef](#)] [[PubMed](#)]
47. Tay, W.; Mahon, R.; Heckel, D.; Walsh, T.; Downes, S.; James, W.; Lee, S.; Reineke, A.; Williams, A.; Gordon, K. Insect Resistance to *Bacillus thuringiensis* Toxin Cry2Ab Is Conferred by Mutations in an ABC Transporter Subfamily A Protein. *PLoS Genet.* **2015**, *11*, e1005534. [[CrossRef](#)]
48. Badenes-Pérez, F.; Morrison, A.; Vogel, H.; Crickmore, N.; Kain, W.; Wang, P.; Heckel, D.; Jiggins, C. Parallel Evolution of *Bacillus thuringiensis* Toxin Resistance in Lepidoptera. *Genetics* **2011**, *189*, 675–679.
49. Guo, Z.J.; Kang, S.; Zhu, X.; Xia, J.X.; Wu, Q.J.; Wang, S.; Xie, W.; Zhang, Y.J. Down-regulation of a novel ABC transporter gene (*Pxvwhite*) is associated with Cry1Ac resistance in the diamondback moth, *Plutella xylostella* (L.). *Insect Biochem. Mol. Biol.* **2015**, *59*, 30–40. [[CrossRef](#)]
50. Li, H.; Oppert, B.; Higgins, R.; Huang, F.; Buschman, L.; Gao, J.; Zhu, K. Characterization of cDNAs encoding three trypsin-like proteinases and mRNA quantitative analysis in Bt-resistant and-susceptible strains of *Ostrinia nubilalis*. *Insect Biochem. Mol. Biol.* **2005**, *35*, 847–860. [[CrossRef](#)] [[PubMed](#)]
51. Tabashnik, B.; Liu, Y.; Unnithan, D.; Carrière, Y.; Dennehy, T.; Morin, S. Shared genetic basis of resistance to Bt toxin Cry1Ac in independent strains of pink bollworm. *J. Econ. Entomol.* **2004**, *97*, 721–726. [[CrossRef](#)]
52. Zhang, M.; Wei, J.Z.; Ni, X.Z.; Zhang, J.; Jurat-Fuentes, J.; Fabrick, J.; Carrière, Y.; Tabashnik, B.; Li, X.C. Decreased Cry1Ac activation by midgut proteases associated with Cry1Ac resistance in *Helicoverpa zea*. *Pest Manag. Sci.* **2019**, *75*, 1099–1106. [[CrossRef](#)]
53. Tabashnik, B.; Finson, N.; Johnson, M.; Heckel, D. Cross-resistance to *Bacillus thuringiensis* toxin CryIF in the diamondback moth (*Plutella xylostella*). *Appl. Environ. Microbiol.* **1994**, *60*, 4627–4629. [[CrossRef](#)]
54. Jackson, R.; Gould, F.; Bradley, J.; Van Duyn, J. Genetic variation for resistance to *Bacillus thuringiensis* toxins in *Helicoverpa zea* (Lepidoptera: Noctuidae) in eastern North Carolina. *J. Econ. Entomol.* **2006**, *99*, 1790–1797. [[CrossRef](#)] [[PubMed](#)]
55. Wang, X.Y.; Du, L.X.; Liu, C.X.; Gong, L.; Han, L.Z.; Peng, Y.F. RNAi in the striped stem borer, *Chilo suppressalis*, establishes a functional role for aminopeptidase N in Cry1Ab intoxication. *J. Invertebr. Pathol.* **2017**, *143*, 1–10. [[CrossRef](#)]
56. Pavlidi, N.; Vontas, J.; Van Leeuwen, T. The role of glutathione S-transferases (GSTs) in insecticide resistance in crop pests and disease vectors. *Curr. Opin. Insect Sci.* **2018**, *27*, 97–102. [[CrossRef](#)] [[PubMed](#)]
57. Liu, C.X.; Xiao, Y.T.; Li, X.C.; Oppert, B.; Tabashnik, B.; Wu, K.M. Cis-mediated down-regulation of a trypsin gene associated with Bt resistance in cotton bollworm. *Sci. Rep.* **2014**, *4*, 7219. [[CrossRef](#)] [[PubMed](#)]
58. Ji, Z.; Wang, G.; Xie, Z.; Wang, J.; Zhang, C.; Dong, F.; Chen, C. Identification of novel and differentially expressed microRNAs of dairy goat mammary gland tissues using Solexa sequencing and bioinformatics. *PLoS ONE* **2012**, *7*, e49463. [[CrossRef](#)] [[PubMed](#)]
59. Gahan, L.J.; Pauchet, Y.; Vogel, H.; Heckel, D.G. An ABC Transporter Mutation Is Correlated with Insect Resistance to *Bacillus thuringiensis* Cry1Ac toxin. *PLoS Genet.* **2010**, *6*, e1001248. [[CrossRef](#)]
60. Feyereisen, R. Insect P450 enzymes. *Annu. Rev. Entomol.* **1999**, *44*, 507–533. [[CrossRef](#)]
61. Berge, J.B.; Feyereisen, R.; Amichot, M. Cytochrome P450 Monooxygenases and Insecticide Resistance in Insects. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **1998**, *353*, 1701–1705. [[CrossRef](#)] [[PubMed](#)]
62. Zou, F.; Guo, Q.; Shen, B.; Zhu, C. A cluster of CYP6 gene family associated with the major quantitative trait locus is responsible for the pyrethroid resistance in *Culex pipiens pallens*. *Insect Mol. Biol.* **2019**, *28*, 528–536. [[PubMed](#)]
63. Schama, R.; Pedrini, N.; Juárez, M.P.; Nelson, D.R.; Torres, A.Q.; Valle, D.; Mesquita, R.D. *Rhodnius prolixus* supergene families of enzymes potentially associated with insecticide resistance. *Insect Biochem. Mol. Biol.* **2016**, *69*, 91–104. [[CrossRef](#)] [[PubMed](#)]