Implication of antioxidant and detoxifying enzymes in the resistance of Holotrichia parallela larvae to EPN-Bt infection

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Abstract

Combining the entomopathogenic nematodes (EPNs), *Heterorhabditis beicherriana* LF strain and *Bacillus thuringiensis* (Bt) HBF-18 strain is a practical strategy to manage the larvae of *Holotrichia parallela* Motschulsky. However, the intrinsic resistance mechanism between *H. parallela* larvae and the EPN-Bt combination is unknown. Herein, antioxidant enzymes [superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)] and detoxifying enzymes [carboxylesterase (CarE), glutathione S-transferase (GST), and acetylcholinesterase (AChE)] of *H. parallela* larvae showed an activation-inhibition trend throughout the EPN-Bt exposure time course. Eight potentially key antioxidant and detoxifying enzyme genes in response to EPN-Bt infection were identified from the midgut of *H. parallela* larvae through RNA-seq. Spatiotemporal analysis showed they were ubiquitously expressed in all development stages and tissues. After silencing CAT, CarE, and GST1, the enzyme activities were significantly decreased by 30.29%, 68.80%, and 34.63%, respectively. Meanwhile, the mortality of grubs was increased by 18.40%, 46.30%, and 42.59% after exposure to EPN-Bt for 1 day. The expression level change trends of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), cap 'n' collar isoform-C (CncC), kelch-like ECH-associated protein 1 (Keap1), and CarE were consistent when exposed to EPN-Bt. Furtherly, RNAi-mediated PI3K silencing showed a similar downregulated trend between PI3K/Akt/CncC and CarE. Furthermore, silencing PI3K rendered grubs more susceptible to EPN-Bt and accelerated symbiotic bacteria multiplication in grubs. Overall, these results suggest that PI3K/Akt/CncC pathway mediates the expression of CarE and participates in the resistance of *H. parallela* larvae to EPN-Bt infection.

1. Introduction

Holotrichia parallela Motschulsky is one of the notorious soil-dwelling insect pest species in China (Toepfer et al. 2014). The larvae (white grubs) primarily feed on roots or stems of potatoes, peanuts, soybeans, and maize, leading to yield losses ranging from 10 to 20% (Ju et al. 2017; Zhang et al. 2019). The impact of grubs on potato yield and quality has hampered the growth of potatoes as China's major food strategy (Xu et al. 2019). Seed coating and spraying with chemical insecticides such as chlorpyrifos are the most common methods for controlling white grubs (Wang et al. 2020c). However, the excessive use of chemical pesticides leads to environmental pollution, food safety issues, and pest resistance (Liu et al. 2008). In addition, the global tendency to organic agriculture relying on ecological intensification has been promoted as one of the approaches to control pests (Crowder et al. 2010; Muneret et al. 2018). However, the practice may lead to different results for belowground pests. A recent study by Nyamwasa et al. (2020) indicated that returning or leaving decomposing creates a favorable microclimate for soil-dwelling insects, which cause severe damage to crops. Therefore, it is essential to develop environmentally friendly and effective strategies to control *H. parallela* larvae (Wang et al. 2020c; Li et al. 2021a).

Controlling insect pests using the synergy of bioagents is a potential pest management method. The combined effect is likely to reduce the application rate of bioagents and the host killing time, thereby increasing their efficacy against insect pests (Jia et al. 2016; Devi 2019). For example, synergistic action

between the combination of entomopathogenic nematodes (EPNs) and *Bacillus popilliae* Dutky, *Bacillus thuringiensis* (Bt) Berliner Buibui strain, *Beauveria bassiana* (Balsamo) Vuillemin GHA strain as well as *Metarhizium anisopliae* (Metsch.) Sorokin F-52 strain was successful against *Cyclocephala hirta*, *Anomala orientalis*, and *Cyclocephala lurida* Bland (Thurston et al. 1994; Koppenhöfer et al. 1999; Wu et al. 2014). Moreover, we previously detected the synergistic and additive action between the combination of EPN *H. beicherriana* LF and Bt (HBF-18) against *H. parallela* larvae (Li et al. 2021b). However, the additive action was less effective. The primary reason for this could be because the white grub's strong immune system effectively resists EPN and Bt's ability to establish infection, preventing the successful implementation of this combined strategy (Castillo et al. 2011; Bang et al. 2015; Garriga et al. 2020).

Insects are known to cope with pathogen threats using antioxidants and detoxifying enzymes in their immune systems (Dubovskiy et al. 2012). These enzymes play an important role in maintaining normal physiological and biochemical metabolism, decomposing toxic substances, and resisting pathogens (Zhu-Salzman and Zeng 2015). Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are three important antioxidant enzymes in insects. These enzymes possess the function of scavenging excessive reactive oxygen species (ROS), degrading hydrogen peroxide (H_2O_2), and preventing free-radical-associated damage (Felton and Summers 1995; Dubovskiy et al. 2008; Bi et al. 2010; Zhou et al. 2019). It is well documented that EPN or Bt threat can induce significant activity changes in antioxidant enzymes. For instance, the enzyme activities (SOD, POD, and CAT) of *Cnaphalocrocis medinalis* and *Anoplophora glabripennis* showed an upward-down trend after exposure to Cry1Ab/Cry1Ac and *Heterorhabditis sp.* ZH, respectively (Zhang et al. 2008; Ren et al. 2013). The antioxidant enzyme activities (SOD, POD, and CAT) of *Antheraea pernyi* larvae were increased after exposure to *Meronithidae* (Jia et al. 2020).

Insects will produce a variety of detoxifying enzymes in response to adversity stress induced by chemicals or invading pathogens to digest exogenous poisons and enhance their adaptability to adversity stress (Zhu et al. 2011; Jia et al. 2016). Among these enzymes, cytochrome P450 (P-450) is a large family of multifunctional enzymes that metabolizes plant allelochemicals and insecticides in insects (Schuler 2011; Zhou et al. 2012; Wang et al. 2015). Carboxylesterase (CarE) is a polyprotein that hydrolyzes various compounds, including thioester, aliesterase, and amides, thus reducing the toxicity of exogenous toxicants (Montella et al. 2012; Hatfield et al. 2016). Glutathione S-transferase (GST) plays a pivotal role in detoxification and cellular antioxidant defenses against oxidative insults (Hayes et al. 2005; Lumjuan et al. 2005). The acetylcholinesterase (AChE) is a key enzyme in the insect central nervous system, degrading the neurotransmitter acetylcholine and ensuring nerve transmission (Oehmichen and Besserer 1982). Previous studies showed that P-450, CarE, and GST in the midgut of *Popillia japonica* were involved in the physiological response to quisqualic acid intoxication (Adesanya et al. 2017). In addition, the activities of CarE, GST, and AChE in *Tenebrio molitor* were induced by *H. beicherriana* (Li et al. 2016). The activities of AChE and GST in *Holotrichia oblita* larvae were increased after feeding on Cry8Gal (Tan et al. 2013). It is generally known that EPN or Bt infection affects the

activities of antioxidant and detoxifying enzymes in insects. However, no detailed investigations have been performed to explore the complex enzymatic consequences of EPN and Bt jointly attacking insects.

In insects, cap ‘n’ collar isoform-C (CncC) induces the expression of various cytoprotective enzymes by binding to antioxidant responsive elements (ARE) on the 5’-flanking regions of many detoxifying genes to respond to electrophilic xenobiotics and oxidative stress (Casalino et al. 2007; Leiser and Miller 2010; Xue et al. 2016; Hu et al. 2018). Kelch-like ECH-associated protein 1 (Keap1) is the specific repressor of CncC (Sykiotis and Bohmann 2008). The CncC can be isolated from Keap1 under oxidative stress (Wilding 2018). In addition, the expressions of P-450, GST, and CarE are coordinately regulated by the CncC/Keap1-ARE signaling pathway (Wilding 2018). Furthermore, the upstream phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway can also regulate the CncC/Keap1 signaling pathway, which can induce the expression of detoxifying enzymes, scavenge reactive oxygen species, and inhibit apoptosis (Hao et al. 2015). Previous studies have shown that phoxim, pyriproxyfen, or chlorantraniliprole exposure can activate the PI3K/Akt/CncC signaling pathway and increase the transcription of various detoxifying enzymes in *Bombyx mori* (Cheng et al. 2018; Mao et al. 2019; Zhao et al. 2020). Several other studies have reported the involvement of CncC in the regulation of multiple P-450s in *Tribolium Castaneum* and *Leptinotarsa decemlineata* following exposure to tefluthrin and imidacloprid, respectively (Kalsi and Palli 2017; Gaddelapati et al. 2018). However, in-depth knowledge of transcriptional regulation of detoxification sites in pests is still limited. Moreover, the detoxification mechanism in *H. parallela* has not been documented yet.

In this study, we investigated the impact of *H. beicherriana* LF and Bt (HBF-18) joint infection on multiple enzyme systems of *H. parallela*. Moreover, we studied the associated detoxification mechanisms following the joint infection in *H. parallela.*

2. Materials And Methods

2.1 Insect, entomopathogenic nematode, and *Bacillus thuringiensis*

Adults of *H. parallela* were collected from Shijiazhuang, Hebei Province, China. These insects were reared on fresh elm leaves in a plastic box under laboratory conditions at 25°C with a 12/12 h light/dark cycle and 70% relative humidity (RH) (Qu et al. 2021). The eggs were incubated at 18% soil moisture (w/w). The larvae of *H. parallela* were fed potatoes and kept individually in the holes of six-well bioassay trays (Corning Inc., NY, USA) filled with 15 g and 18% soil moisture of high temperature sterilized sandy loam soil. The 13 days old larvae were used for enzyme activity determination, RNA-seq, and qRT-PCR validation. The 18 days old larvae were used primarily for signaling pathway regulation and RNAi-related studies.

The EPN (*H. beicherriana* LF strain) and Bt (HBF-18) were prepared as previously described (Malan et al. 2011; Li et al. 2021b). Infective juveniles (IJs) of the EPN with survival rates greater than 95% were used

for relevant experiments. The cultured Bt (HBF-18) was suspended with sterile water and then quantified by counting colony-forming units (CFU) (Wang et al. 2020b).

2.2 Sample preparation and enzyme activity determination

The activity changes of antioxidant and detoxifying enzymes were measured following treatment with EPN, Bt, or EPN-Bt combination to investigate the effect of *H. beicherriana* LF and Bt (HBF-18) combination on the immune defense system of *H. parallela* larvae. As for EPN treatment, the IJs of *H. beicherriana* LF were prepared at 25 IJs/grub and then added to the soil surface of six-well bioassay trays. For Bt treatment, grubs were exposed to soil mixed with Bt (HBF-18) at a concentration of 0.79×10^7 CFU/g soil. The EPNs (25 IJs/grub) were equally added for the EPN-Bt combination treatment after the grubs had been transferred to the soil mixed with Bt (0.79×10^7 CFU/g soil) for 2 days. The six-well bioassay trays were then transferred into a dark incubator under the following conditions: 25°C and 80% RH. Sterile water treatment was used as the negative control. The whole body of treated grubs was continuously sampled ($n = 3$ for each treatment) for 4 days. Fresh samples were immediately determined for enzyme activity, and triplicate biological replicates were performed. SOD, POD, CAT, AChE, CarE, and GST enzyme assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The enzyme activities were determined according to the manufacturer's instructions, and the total protein levels were checked by the Bradford method (Bradford 1976).

2.3 RNA-seq, *de novo* assembly, and differential gene expression analysis

RNA-seq was performed to mine the key antioxidant and detoxifying enzyme genes involved in the defense response of white grubs against EPN, Bt, or EPN-Bt combination. The grubs were pretreated with the above methods (2.2). The grub's midgut samples ($n = 3$ for each treatment) were disintegrated and collected in RNase-free tubes after treatment with EPN for 1 day and 3 days, Bt for 3 and 5 days, and EPN-Bt combination for 1 day and 3 days. Sterile water treatment was used as the negative control. The RNA isolation of grub midgut and cDNA library preparation were all carried out according to the previous studies (Li et al. 2021a).

Paired-end sequencing was conducted on an Illumina HiSeq 2000 (Illumina Inc., USA) at Anoroad Genomics Co., Ltd. (Beijing, China). After sequencing, the raw reads were filtered to generate clean reads. Then, the clean reads of all treatments were loaded to Trinity (Trinity Release v2.4.0) (Grabherr et al. 2011) for *de novo* assembly under the paired-end mode with default parameters. The longest transcript sequence was recorded as 'Unigene'. In addition, Bowtie (v2.2.3) was performed to quantify the proportion of the clean reads that mapped to assembly sequences (Langmead et al. 2009). TransDecoder (TransDecoder Release v3.0.1) was used to identify the open reading frame (ORF) within transcript sequences. To annotate the Unigenes, we searched reference sequences against nucleotide sequence database (NT), non-redundant protein sequences (NR), and Uniprot databases, using Blast with a cut-off *E*-value of 10^{-5}. Trinotate (Trinotate Release V3.0.2) was used to integrate the functional annotation results of Unigenes.

The clean reads from different treatments were mapped separately to the assembled Unigenes. RPKMs (Reads Per Kilobase Million Mapped Reads) were used in DESeq2 to compare the difference of gene expression between the different treatments (Mortazavi et al. 2008; Anders and Huber 2010). Genes with |log2 ratio| ≥ 1 and q < 0.05 (adjusted *p*-value) were selected as significantly differentially expressed genes (DEGs). Then, all DEGs were further subjected to GO functional annotation using Blast2GO and mapped to terms in the KEGG pathway database using KOBAS software. Enrichment analysis was used to identify the GO terms and significantly regulated KEGG pathways. We selected the adjusted *p*-value < 0.05 as the threshold to determine significant enrichment of the gene sets.

2.4 Identification of key antioxidant and detoxifying enzyme genes and qRT-PCR validation

All candidate genes (i.e., SOD, POD, CAT, AchE, P-450, CarE, GST1, and GST2) were obtained by querying keywords of antioxidant and detoxifying enzyme genes and then manually verified with the BLASTX program at the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/). The eight significant DEGs from the (EPN-Bt) 1d-vs-EPN 1d comparison were selected for qRT-PCR validation. The primers used for qRT-PCR (Table S1) were designed using Primer Premier 6.0 software. The qRT-PCR experiment was performed with GOTaq® qPCR Master Mix (Promega, USA) on an ABI Prism 7500 Fast Detection System (Thermo Fisher, USA). qRT-PCR reactions were performed as described in the previous study (Li et al. 2021a). *H. parallela* GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal standard to normalize the expression level. Each qRT-PCR experiment was performed with three biological replicates and three technical replicates. The relative expression levels of target genes were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

2.5 Spatiotemporal analysis of antioxidant and detoxifying enzyme genes

To determine the expression profiles of these genes in different development stages and tissues, we collected eggs (5 days old, ~60 mg), first instar larvae (10 days old, ~60 mg), second instar larvae (18 days old), third instar larvae (28 days old) and various tissues (hemolymph, malpighian tubule, midgut, and fat body were taken from the second instar larvae) for RNA isolation. qRT-PCR was performed as described above (2.4).

2.6 RNA interference to *H. parallela* larvae

RNAi was used to study the functions of CAT, CarE, and GST1 involved in the antioxidant or detoxifying system of *H. parallela* against EPN, Bt, or EPN-Bt infection. Specific primers (Table S1) with T7 promoter sequences were designed to synthesize double-stranded RNA (dsRNA) of CAT, CarE, GST1, and GFP using the T7 RiboMAX™ Express RNAi kit (Promega, WI, USA) according to the manufacturer's instructions. The dsRNA product sizes were confirmed by electrophoresis on 1.2% agarose gel and adjusted to a final concentration of 1 μg/μL.

For *in vivo* RNAi, 1.5 μg of each dsRNA, dsGFP, and the same volume of RNase-free water were injected into the hemocoel of *H. parallela* second instar larvae (18 days old) through the abdomen (WPI microinjection system, USA). After injection, the larvae were put into an insectarium for normal feeding. The midgut samples (n = 3 for each treatment) from treated or control groups (dsGFP-injected and water-injected) were collected after treatment for 24, 48, and 72 h, respectively. The experiment was performed with three biological replicates. The qRT-PCR process was performed as described above (2.4).

2.7 Enzyme activity and susceptibility changes of *H. parallela* larvae after RNAi

The enzyme activities of CAT, CarE, and GST were detected after injecting corresponding dsRNA, dsGFP, and RNase-free water following our previous procedures for 24 h (2.6). The whole body (18 days old larvae, n = 3 for each treatment) was used to determine the enzyme activity changes, and triplicate biological replicates were performed.

Susceptibility changes of grubs against EPN, Bt, or EPN-Bt combination were also monitored after RNA interference with CAT, CarE, and GST1, respectively. Twenty-four hours after injection, the grubs were treated as described above (2.2). Each treatment contained 36 individuals (grub) with three replicates. The grub mortality was monitored after applying EPN, Bt, or EPN-Bt combination.

2.8 PI3K/Akt/CncC signaling pathway regulates the expression of CarE

To investigate whether the PI3K/Akt pathway mediated the expression of CarE by regulating the CncC/Keap1 pathway, we screened these key genes (PI3K, Akt, CncC, and Keap1) from the transcriptome data and manually verified them with the BLASTX program at the NCBI. The comparison of CncC and Keap1 sequences with other species were analyzed by DNAMAN 6. The phylogenetic tree of PI3K and Akt was constructed by MEGA7.0 using the neighbor-joining method with p-distance under the default parameters. Bootstrap values were obtained by the bootstrap method using 1,000 repetitions.

The relative expression levels of pathway-related genes were measured after the grubs (18 days old) were exposed to EPN-Bt combination for 1 day and 3 days. The grubs exposed to sterile water for 1 day and 3 days were used as control. After that, RNAi-mediated PI3K gene silencing was performed. Then, the relative expression levels of pathway-related genes were detected at 24 h after dsPI3K injection. The grubs were injected with the same volume of RNase-free water as the control treatment. In addition, the enzyme activity of CarE was also determined after injecting dsPI3K, dsGFP, and the same volume of RNase-free water for 24 h, respectively. dsPI3K synthesis, injection, RNA extraction from the midgut, qRT-PCR procedure, and the determination of CarE enzyme activity were performed as previously described (2.2, 2.3, 2.4, and 2.6). The primers used for qRT-PCR and RNAi are summarized in Table S1.

2.9 PI3K silencing affects *H. parallela* larvae survival and symbiotic bacteria multiplication

To further test whether the silencing of PI3K affected the susceptibility of grubs against EPN or Bt, the percent survival of grubs (18 days old) against EPN, Bt, or EPN-Bt combination was monitored respectively, after RNA interference with PI3K for 24 h. The grubs were treated with EPN, Bt, or EPN-Bt combination according to the above method (2.2) after injection, and the grubs injected with dsGFP and the same volume of RNase-free water were used as controls, respectively. Each treatment contained 18 individuals (grub) with three replicates. The percent survival of grubs was assessed daily.

The number of symbiotic bacteria in the hemolymph of grubs was also measured by counting CFU after the injected grubs were exposed to EPN and EPN-Bt combination for 1 day, respectively. Specifically, 5 μL hemolymph of exposed grubs was individually collected with a pipettor and suspended in a 1.5 mL centrifuge tube with 1 mL PBS (138 mM NaCl, 2.7 mM KCl and 10 mM Na_2HPO_4, pH 7.4). After that, 20 μL bacteria suspension was coated on nutrient-bromothymol blue agar plates (NBTA, nutrient agar, 0.0025% bromothymol blue, and 0.004% triphenyl tetrazolium chloride medium), and then the plates were incubated at 28°C for 48 h. Thirty grubs were used for each treatment, and three technical replicates (plate) were performed.

2.10 Statistical analysis

Data analysis was performed using GraphPad Prism software version 8.0. All proportions were transformed by arcsine square root transformation in Microsoft Excel 5.0 before analysis (Abbott 1987). The significance levels of enzyme activity determination and spatiotemporal analysis were analyzed using one-way analysis of variance (ANOVA) followed by Tukey significant difference test ($P < 0.05$). The significance levels of *in vivo* RNAi, enzyme activity and susceptibility change after RNAi, signaling pathway regulation, and the count of symbiotic bacteria were analyzed using multiple *t*-test or *t*-test at the significance levels set at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns, not significant. The survival curves were compared with Kaplan-Meier analysis, and *P*-value < 0.05 was regarded as statistically significant (log-rank test). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

3. Results

3.1 Determination of enzyme activity

The effects of EPN-Bt treatment on antioxidant enzymes (SOD, POD, CAT) and detoxifying enzymes (CarE, GST, AchE) in *H. parallela* larvae are shown in Fig. 1. Within 3 days of treatment with EPN, Bt, or EPN-Bt combination, the activities of these enzymes were all almost enhanced compared with the water control. In addition, the activities of these enzymes reached the peak on the second day after treatment with EPN-Bt combination, and was significantly higher than that of other treatments (SOD: $F = 9.11$, $df = 3, 8$, $P = 0.006$; POD: $F = 10.45$, $df = 3, 8$, $P = 0.027$; CAT: $F = 6.186$, $df = 3, 8$, $P = 0.018$; CarE: $F = 12.74$, $df = 3, 8$, $P = 0.002$; GST: $F = 9.39$, $df = 3, 8$, $P = 0.049$; AchE: $F = 50.91$, $df = 3, 8$, $P < 0.001$). It was worth noting that the activities of these enzymes were inhibited to varying degrees after treatment with EPN-Bt combination for 4 days.

3.2 Sequence assembly and functional annotation

Eight midgut cDNA libraries of *H. parallela* larvae exposed to EPN, Bt, EPN-Bt combination and sterile water (CK) at two time-points were sequenced and generated 38,845,132 – 45,527,760 clean raw reads (Table S2). Subsequently, the clean reads from the eight samples were combined to do *de novo* assembly, resulting in 70,976 Unigenes with a mean length of 771 bp and an N50 of 1,200 bp (Fig. S1a), and 76.5% aligned reads mapped to the clean reads (Fig. S1b). From these Unigenes, a total of 16,016 predicted ORFs was obtained, and the lengths ranged from 297 bp to 2,577 bp, with a mean length of 1,130 and an N50 of 1,557 (Fig. S1c). 27,752 (39.10%) Unigenes were annotated at least in one database, including 5,932 in NT, 27,752 in NR, 10,557 in BLASTP and 15,167 in BLASTX against Uniprot (Fig. S1d).

3.3 Analysis of DEGs and qRT-PCR validation

The DEGs between the EPN-Bt group and Bt groups, EPN groups as well as the CK group were identified using DEseq2. Similar numbers of genes were upregulated or downregulated in (EPN-Bt) 1d-vs-CK 1d comparison, (EPN-Bt) 1d-vs-EPN 1d comparison and (EPN-Bt) 1d-vs-Bt 3d comparison (Fig. 2a). The majority of DEGs (82.58%, 4,428 genes) were downregulated in the (EPN-Bt) 3d-vs-CK 3d comparison, while vast DEGs (61.42%, 2,124 genes) were upregulated in the (EPN-Bt) 3d-vs-EPN 3d comparison. Venn diagrams showed that the co-expressed DEGs for the three groups of (EPN-Bt) 1d-vs-CK 1d/EPN 1d/Bt 3d were 4,263 (15.53%, 662 upregulated and 62.28%, 2,655 downregulated) (Fig. 2b). After treatment with EPN-Bt combination for 3 days, the co-expressed DEGs for the groups of (EPN-Bt) 3d-vs-CK 3d/EPN 3d/Bt 5d were significant reduced, only 1,312 (23.32%, 306 upregulated and 61.28%, 804 downregulated) (Fig. 2c).

Some antioxidants and detoxifying genes were regulated, indicating that they were involved in protecting *H. parallela* larvae against EPN and Bt infection. The annotations of these genes in transcriptome data are summarized in Table 1. To confirm the reliability of RNA-seq, these eight antioxidant and detoxifying enzyme genes from the (EPN-Bt) 1d-vs-EPN 1d comparison were selected for qRT-PCR validation. The volcano plot for the (EPN-Bt) 1d-vs-EPN 1d comparison is shown in Fig. 2d. The expression levels of the selected eight genes in the (EPN-Bt) 1d group were normalized to the EPN 1d group. The qRT-PCR data showed a positive linear relationship with the RNA-seq (R^2 = 0.8429, P = 0.0013 at 95% confidence interval) (Fig. 2e).

Table 1
The annotations of genes related to antioxidant and detoxification

Unigene ID	Description	Log2Fold Change					
		(EPN-Bt) 1d-vs-			(EPN-Bt) 3d-vs-		
		CK 1d	Bt 3d	EPN 1d	CK 3d	Bt 5d	EPN 3d
TRINITY_DN14704_c0_g1	Superoxide dismutase (SOD)	2.88	=	4.32	2.25	=	8.30
TRINITY_DN18932_c2_g1	Peroxidase (POD)	10.39	5.73	11.60	6.30	=	=
TRINITY_DN9842_c0_g1	Catalase (CAT)	1.85	=	2.01	1.64	=	=
TRINITY_DN17760_c6_g1	Acetylcholinesterase (AchE)	-2.94	=	1.21	-1.59	2.56	=
TRINITY_DN8208_c0_g1	Cytochrome P-450 (P-450)	8.64	10.57	11.83	1.89	8.04	=
TRINITY_DN16841_c1_g1	Carboxylesterase (CarE)	4.11	=	4.74	-2.55	=	=
TRINITY_DN10439_c1_g1	Glutathione S-transferase 1 (GST1)	1.54	=	4.10	1.05	=	2.37
TRINITY_DN14506_c0_g4	Glutathione S-transferase 2 (GST2)	4.40	=	3.47	4.99	4.14	=

3.4 GO analysis and KEGG pathways

To explore the potential functions of these DEGs, GO terms were used to classify the functions of the *H. parallela* DEGs. (Fig. S2). In this case, the totals of 5,817, 5,698, 5,679, 6,440, 6,528 and 6,369 DEGs respectively for the comparisons of the (EPN-Bt) 1d-vs-CK 1d, (EPN-Bt) 1d-vs-EPN 1d, (EPN-Bt) 1d-vs-Bt 3d, (EPN-Bt) 3d-vs-CK 3d, (EPN-Bt) 3d-vs-EPN 3d and (EPN-Bt) 3d-vs-Bt 5d were annotated in 56 GO terms. Genes related to cell parts, cellular process, binding and metabolic process were dominant. The top ten most significantly enriched pathways for each comparison are shown in Table S3. The enriched pathways were almost consistent and mainly included the lysine degradation, huntington disease, signalling pathways regulating pluripotency of stem cells.

3.5 Spatiotemporal analysis of antioxidant and detoxifying enzyme genes

To better understand the functions of these eight antioxidant and detoxifying enzyme genes, their relative expressions in different developmental stages and tissues were further determine by qRT-PCR (Fig. 3). These eight antioxidant and detoxifying enzyme genes ubiquitously expressed in all stages and tissues.

Stage distribution analysis showed that SOD, CAT, AchE, P-450, CarE and GST1 were mainly expressed in the second instar larvae (SOD: $F = 11.05$, $df = 3, 8$, $P = 0.003$; CAT: $F = 10.15$, $df = 3, 8$, $P = 0.004$; AchE: $F = 18.02$, $df = 3, 8$, $P < 0.001$; P-450: $F = 15.57$, $df = 3, 8$, $P = 0.001$; CarE: $F = 11.38$, $df = 3, 8$, $P = 0.003$; GST1: $F = 23.14$, $df = 3, 8$, $P < 0.001$), while POD and GST2 were dominantly expressed in the third instar larvae (POD: $F = 5.96$, $df = 3, 8$, $P = 0.019$; GST2: $F = 100.23$, $df = 3, 8$, $P < 0.001$) (Fig. 3a). Tissue distribution analysis showed that SOD, CAT, P-450, CarE and GST2 possessed the higher abundance in the midgut (SOD: $F = 4.15$, $df = 3, 8$, $P = 0.048$; CAT: $F = 24.90$, $df = 3, 8$, $P < 0.001$; P-450: $F = 46.18$, $df = 3, 8$, $P < 0.001$; CarE: $F = 64.58$, $df = 3, 8$, $P < 0.001$; GST2: $F = 17.74$, $df = 3, 8$, $P = 0.001$), while POD possessed the higher abundance in hemolymph ($F = 10.82$, $df = 3, 8$, $P = 0.001$), AchE in malpighian tube ($F = 43.32$, $df = 3, 8$, $P < 0.001$), GST1 in the fat body ($F = 5.50$, $df = 3, 8$, $P = 0.024$) (Fig. 3b).

3.6 RNAi, and enzyme activity and susceptibility changes after RNAi

Double-stranded RNA of CAT, CarE, and GST1 was injected into the hemocoel of *H. parallela* second instar larvae, respectively. The RNAi efficiency at 24, 48, and 72 h after injection was determined by qRT-PCR (Fig. S3; Fig. 4a). The results revealed that the significant knockdown was achieved at 24 h after dsRNA injection, and the knockdown efficiency could maintain for at least 72 h (Fig. S3). The expression difference between the control groups (dsGFP-injected and water-injected, $P > 0.05$) was not significant after injecting the corresponding dsRNA for 24 hours (Fig. 4A). In addition, the expression of CAT, CarE, and GST1 was significantly depressed by 99.17%, 93.06%, and 96.81%, respectively, compared with water injection ($P < 0.001$).

Twenty-four hours after the corresponding dsRNA injection, the enzyme activities of CAT, CarE, and GST were significantly depressed by 30.29%, 68.80%, and 34.63%, respectively, compared to the water-injected grubs ($P < 0.01$), and no significant difference was detected between the two controls ($P > 0.05$) (Fig. 4b). The larval susceptibility was subsequently investigated after silencing CAT, CarE, and GST1, respectively (Fig. 4c). Twenty-four hours after injection, the grubs were exposed to EPN for 1 day, Bt for 3 days, and EPN-Bt combination for 1 day incorporated diets. While dsGFP did not show significant differences to water control ($P > 0.05$), the grubs injected with dsRNA exhibited higher mortality than the water control. Silencing of CarE resulted in a significant increase in grub mortality. The mortality was increased by 21.41% (EPN 1d, $P < 0.05$), 20.37% (Bt 3d, $P < 0.05$), and 46.30% (EPN-Bt 1d, $P < 0.01$), respectively, compared with water control. In addition, silencing of GST1 also significantly increased grub mortality by 42.59% (EPN-Bt 1d, $P < 0.01$).

3.7 PI3K/Akt/CncC signalling pathway regulates the expression of CarE

The annotations of PI3K, Akt, CncC, and Keap1 are summarized in Table S4. Through sequence alignment and phylogenetic tree analysis, the key genes of PI3K/Akt and CncC/Keap1 pathway of *H. parallela* were successfully identified (Fig. S4). The relative expression of PI3K, Akt, CncC, Keap1, and CarE at the mRNA level was significantly upregulated by 170.67%, 21.77%, 367.15%, 262.85%, and

85.66% ($P < 0.05$), respectively, after exposure to EPN-Bt combination for 1 day compared to the water-injected control group (Fig. 5a). However, the relative expression of these genes was significantly downregulated by 92.32%, 87.08%, 96.25%, 56.93%, and 52.01% ($P < 0.05$), respectively, after exposure for 3 days (Fig. 5b). Twenty-four hours after dsPI3K injection, the relative expression of PI3K was significantly depressed by 60.25% compared with water control ($P < 0.05$) (Fig. S5). Meanwhile, Akt, CncC, and CarE at the mRNA level were significantly downregulated by 87.08%, 36.18%, and 42.65% ($P < 0.05$), respectively. Besides, dsPI3K injection had no significant effect on Keap1 expression ($P > 0.05$) (Fig. 5c). In addition, the activity of CarE was significantly depressed by 46.18% in comparison to the water-injected grubs after silencing of PI3K for 24 h ($P < 0.05$) (Fig. 5d).

3.8 PI3K silencing affects *H. parallela* larvae survival and symbiotic bacteria multiplication

Results showed that silencing of PI3K rendered grubs more susceptible to EPN, Bt, and EPN-Bt combination than water-injected control, and there was no significant difference between the two control groups (dsGFP-injected and water-injected, $P > 0.05$) (Fig. 6a). Furthermore, silencing of PI3K resulted in a significant increase in the number of symbiotic bacteria in the hemolymph of grubs when subjected to EPN and EPN-Bt combination for 1 day (Fig. 6b). The number of symbiotic bacteria between the two control groups (dsGFP-injected and water-injected) was not significant ($P > 0.05$).

4. Discussion

In recent years, the increase in potato insect pest damage seriously influenced the development of the potato industry in China (Gao 2021). Grubs are the most common subterranean pests of potatoes, and they are extremely harmful (Xu et al. 2019). A recent study indicated the combined application of EPN and Bt had synergistic or additive mortality effects against white grubs, and the additive action might be due to some resistance mechanism in grubs (Li et al. 2021b). In addition, previous research had elucidated the biochemical basis of synergism between *Metarhizium anisopliae* and chlorantraniliprole against *Locusta migratoria*, synergism between *Lecanicillium muscarium* and matrine against *Bemisia tabaci*, and both studies showed that the antioxidant and detoxifying enzymes were involved in insect resistance (Jia et al. 2016; Ali et al. 2017). Therefore, it is of great significance to study the antioxidant and detoxification mechanisms of white grubs to improve the EPN-Bt combination's control effect.

Antioxidant enzymes (SOD, POD, and CAT) are also known as enzymes that protect the cellular system because SOD can reduce O_2 to H_2O_2, while POD and CAT can catalyze H_2O_2 to H_2O (Felton and Summers 1995). These reactions can eliminate the damage of reactive oxygen species (ROS) to biofilm (Yamamoto et al. 2005). Insects also harbour detoxifying enzymes, such as CarE, GST, and AchE, which can metabolize exogenous toxins (Xu et al. 2006) and have been the target of insecticide synergists (Wang et al. 2016; Ali et al. 2017). Our data showed that the activities of antioxidant enzymes (SOD, POD, CAT) and detoxifying enzymes (CarE, GST, AchE) were almost activated during the early period after EPN,

Bt, or EPN-Bt combination exposure. Moreover, these enzyme activities of EPN-Bt treatment reached a peak earlier and higher than EPN or Bt treatment alone (Fig. 1). This could be explained that under the dual pressure of EPN and Bt, the white grubs suffered a stronger stimulus signal than the single dose (Koppenhöfer et al. 1999). Then, the antioxidant and detoxifying enzymes were significantly activated for scavenging the ROS and metabolizing exogenous toxins (Foyer and Noctor 2013; Cheng et al. 2018). However, the enzyme activities were suppressed after treatment with the EPN-Bt combination for 4 days (Fig. 1). The possible reason may be associated with the persistent infection of nematodes, the rapid propagation of symbiotic bacteria, and the increase of Bt intake, which destroyed the cellular structure of the midgut and fat body of grubs and hindered the normal physiological metabolism (Vachon et al. 2012; Wu et al. 2015). Our results are consistent with the findings reported by Wu et al. (2018) in *Bradysia odoriphaga* after exposure to *Steinernema feltiae* SF-SN and thiamethoxam combination.

RNA-seq has been widely applied to identify candidate genes that potentially participated in insects' antioxidant and detoxification process (Shang et al. 2020; Wu et al. 2020). Here, we screened some white grubs' antioxidant and detoxification enzyme genes after exposure to EPN, Bt, or EPN-Bt combination (Table 1). Results showed that the variation trends of these enzymes in the transcriptional level were consistent with the enzyme activity determination (Table 1 and Fig. 1), indicating that these genes play an important role in the defense response of grubs. Subsequent spatiotemporal analysis results showed that these genes were widely distributed in all development stages and tissues (Fig. 3). Considering that the midgut and fat body of insects possess the function of defending against foreign invaders and metabolizing chemical pesticides (Zhang et al. 2011; Mao et al. 2019); we thereby selected CAT and CarE genes with significantly high expression in the midgut as well as GST1 gene possessing the greatest abundance in the fat body for further *in vivo* functional studies (The P-450 and GST2 genes were not silenced successfully, the data are not shown). Our results showed that the mRNA level and enzyme activities of CAT, CarE, and GST1 were significantly decreased after RNAi, and meanwhile the grub mortality was increased (Fig. 4). Interestingly, silencing of CarE could significantly increase the mortality of white grubs whether treatment with EPN and Bt alone or EPN-Bt combination (Fig. 4c). CarE has been proved to participate in detoxifying numerous endogenous and exogenous agrochemicals through hydrolysis (Wang et al. 2020a). The CarE in *H. parallela* may be related to drug resistance (Zhao et al. 2014). Besides, the activity of *H. parallela* CarE was significantly activated after infection with the *H. bacteriophora* Cangzhou strain, which was higher than that of *Holotrichia oblita* and *Anomala corpulenta* (Sun et al. 2014). Therefore, we suggested that *H. parallela* CarE played a major role in detoxification against EPN and Bt.

The PI3K/Akt signaling pathway participates in proliferation, differentiation, apoptosis, glucose transport, and other cellular processes (Hietakangas and Cohen 2009). In addition, PI3K/Akt can regulate the downstream CncC/Keap1 signalling pathway, which is involved in the regulation of detoxifying genes such as GST (Walsh et al. 2014), CarE (Chen et al. 2012), and P-450 (Nakajima 2014). In our study, the KEGG pathway analysis indicated that the PI3K/Akt signalling pathway was significantly enriched and participated in all the comparison groups (Table S3). Therefore, we hypothesized that PI3K/Akt might mediate the expression of CarE by regulating the CncC/Keap1 pathway and thus enhance the resistance

of *H. parallela* to EPN and Bt. The key genes, including PI3K, Akt, CncC, and Keap1 have been identified in *T. castaneum*, *B. mori*, and *Drosophila melanogaster* (Hochmuth et al. 2011; Kalsi and Palli 2017; Hu et al. 2018), while no available information for *H. parallela*. Here, these key regulatory genes of *H. parallela* were successfully identified for the first time through sequence alignment and phylogenetic tree analysis (Fig. S4). The gene expression of PI3K/Akt/CncC signalling pathway was evaluated after exposure to the EPN-Bt combination and RNAi mediated PI3K gene silencing. These results confirmed our hypothesis, which indicated the same change trends between PI3K/Akt/CncC and CarE (Fig. 5a-c). Moreover, the mRNA level of CarE was significantly increased after exposure to the EPN-Bt combination for 1 day, while significantly reduced for 3 days (Fig. 5a-b). Similar changes in the mRNA level of CarE were detected in *B. mori* after chlorantraniliprole exposure (Mao et al. 2019). Therefore, we suggested that CarE was involved in detoxification at 1 day. The inhibition of CarE was the main cause of grub death when challenged by the EPN-Bt combination. Furthermore, CarE was the target site for insecticide inhibition in *Aphis gossypii* and *Sitobion avenae* (Gong et al. 2014; Xu et al. 2014). Thus, *H. parallela* CarE would be a promising potential target for EPN-Bt resistance management and grub control.

As an actin-binding protein, Keap1 inhibits CncC activity by sequestering it in the cytoplasm and targeting it for proteasomal degradation (Sykiotis and Bohmann 2008). Our results indicated that the change trends of PI3K, Akt, CncC, and Keap1 were the same when grubs were challenged by the EPN-Bt combination (Fig. 5a-b). These results are consistent with the findings of *B. mori*, in which their changes were synchronous after exposure to pyriproxyfen for 24 or 48 h (Zhao et al. 2020). However, the expression level of Keap1 did not change significantly after PI3K silencing (Fig. 5c). We hypothesized that grubs might regulate Keap1 through another pathway when exposed to the EPN-Bt combination. This pathway may serve as a protective mechanism against too low or too high detoxification levels.

In addition, silencing of PI3K significantly depressed the activity of CarE (Fig. 5d) and rendered grubs more susceptible to EPN, Bt, and EPN-Bt combination (Fig. 6a). Furthermore, the silencing of PI3K resulted in a significant increase in symbiotic bacteria number in the hemolymph of grubs (Fig. 6b). The increased quantity of symbiotic bacteria can be attributed to grubs' lower CarE activity due to PI3K interference, which diminished the degradation ability of symbiotic bacterial toxins (Li et al. 2016). Since there is no suitable method to count the number of Bt in the midgut of grubs and ingestion would seriously affect the number of Bt, we failed to count.

Based on the above studies, we summarized a detoxification pathway of grubs against EPN-Bt combination infection (Fig. 7). After treatment of grubs with EPN-Bt combination for 1 day, the transcriptional level of PI3K and Akt gene was upregulated, and the CncC/keap1 signaling pathway was significantly activated. Meanwhile, CncC dissociated from Keap1 and translocated to the nucleus under oxidative stress of EPN, Bt, or EPN-Bt combination exposure. The CncC was linked to the ARE upstream of the CarE gene, causing downstream CarE mRNA expression. Then the increase of CarE activity inhibited the multiplication of symbiotic bacteria. On the contrary, the transcriptional level of the PI3K/Akt/CncC/CarE pathway was significantly suppressed after treatment with the EPN-Bt combination for 3 days.

In conclusion, the antioxidant and detoxifying enzymes of *H. parallela* played an important role in the immune system against EPN-Bt infection. Furthermore, CarE regulated by PI3K/Akt/CncC pathway participate in the resistance of *H. parallela* to EPN-Bt infection. More studies are needed to elucidate the detoxification mechanism of the fat body of white grubs. In addition, whether other detoxification enzymes such as P-450, GST, and AchE are involved in the PI3K/Akt/CncC signaling pathway or other signalling pathways still needs further exploration.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

JY and EL conceived and designed the experiments. EL, HW, and ZW performed the experiments. EL analyzed the data and wrote the manuscript text. SZ, YC, and KL participated in the data analysis. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The subjects used in the current study are important agricultural pests and therefore do not require approval by any ethics committee or institutional review board.

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Figures

Figure 1

Activity changes of antioxidant enzymes (SOD, POD, CAT) and detoxifying enzymes (CarE, GST, AchE) of *Holotrichia parallela* larvae after exposure to EPN (*Heterorhabditis beicherriana* LF), Bt (HBF-18) or EPN-Bt combination

The EPNs were added after the application of Bt for 2 days in the combination treatments. At the same time point, the enzyme activity data of Bt treatment alone corresponded to Bt treatment in combination. Data represent the means ± SEM of three replicates with 3 larvae per replicate (One-way ANOVA, Tukey's test, $P < 0.05$). (a) SOD stands for superoxide dismutase. (b) POD, peroxidase. (c) CAT, catalase. (d) CarE, carboxylesterase. (e) GST, glutathione S-transferase. (f) AchE, acetylcholinesterase

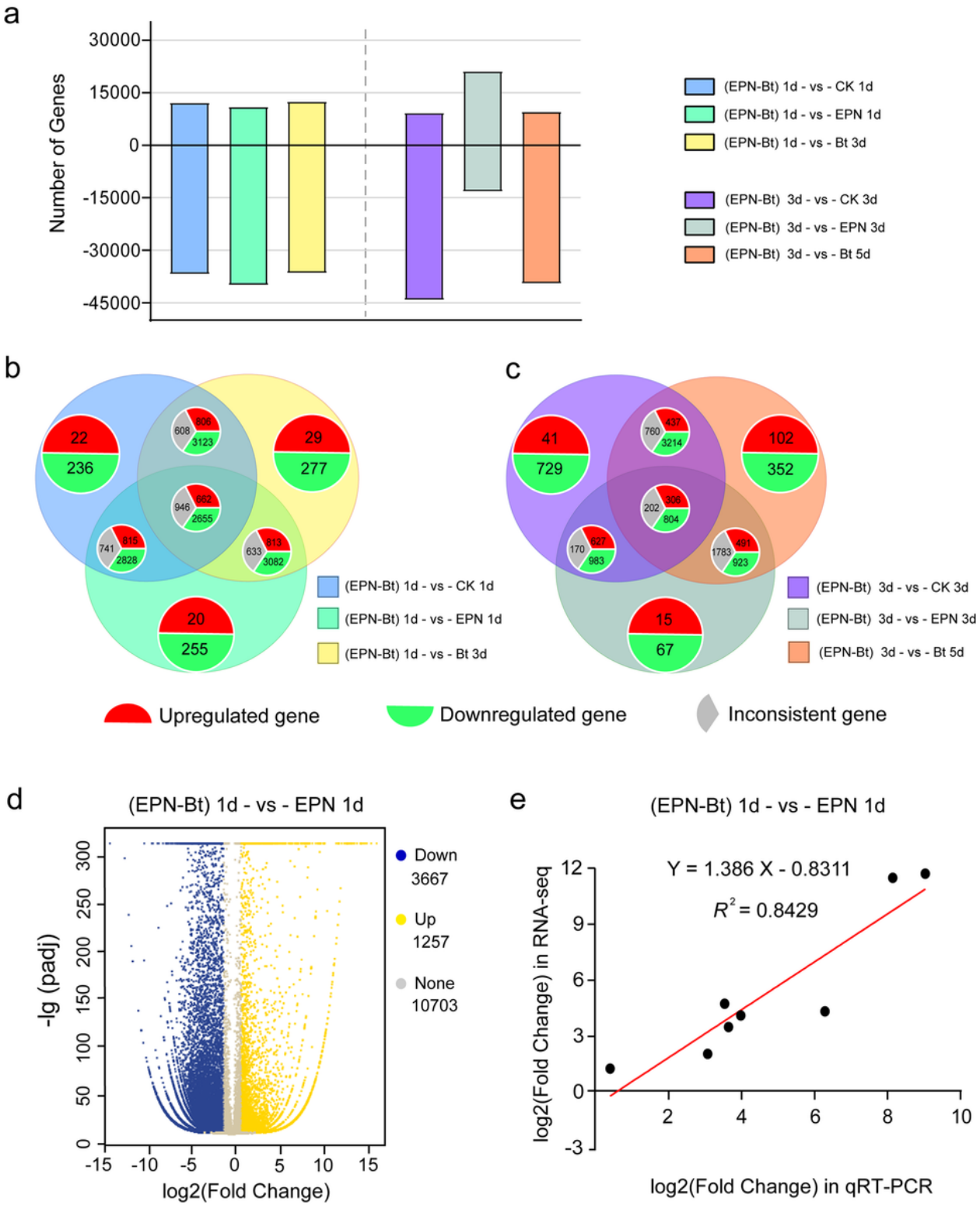

Figure 2

Infection of *Holotrichia parallela* larvae with EPN (*Heterorhabditis beicherriana* LF), Bt (HBF-18), or EPN-Bt combination elicits distinct transcriptomic profiles

(a) The number of DEGs (upregulated or downregulated) in six comparison groups, including (EPN-Bt) 1d-vs-CK 1d, (EPN-Bt) 1d-vs-EPN 1d, (EPN-Bt) 1d-vs-Bt 3d, (EPN-Bt) 3d-vs-CK 3d, (EPN-Bt) 3d-vs-EPN 3d and

(EPN-Bt) 3d-vs-Bt 5d.

(b) Venn diagram of unique and common DEGs (upregulated, downregulated or inconsistent) among (EPN-Bt) 1d-vs-CK 1d, (EPN-Bt) 1d-vs-EPN 1d and (EPN-Bt) 1d-vs-Bt 3d.

(c) Venn diagram of unique and common DEGs (upregulated, downregulated or inconsistent) among (EPN-Bt) 3d-vs-CK 3d, (EPN-Bt) 3d-vs-EPN 3d and (EPN-Bt) 3d-vs-Bt 5d.

(d) Volcano map of DEGs in (EPN-Bt) 1d-vs-EPN 1d group. The X-axis represents the fold change value after log2 conversion, and Y-axis represents the adjusted *p*-value after -log10 conversion. Yellow dots represent the upregulated DEGs, blue dots represent the downregulated DEGs, and gray dots represent the non-DEGs.

(e) Comparison results between RNA-seq and qRT-PCR data in (EPN-Bt) 1d-vs-EPN 1d comparison. The relative expression of selected genes was calculated with the $2^{-\Delta\Delta Ct}$ method based on the value of the EPN 1d group that served as the calibrator. *H. parallela* GAPDH was used as an internal standard to normalize the expression level. R^2 means the square of the correlation coefficient

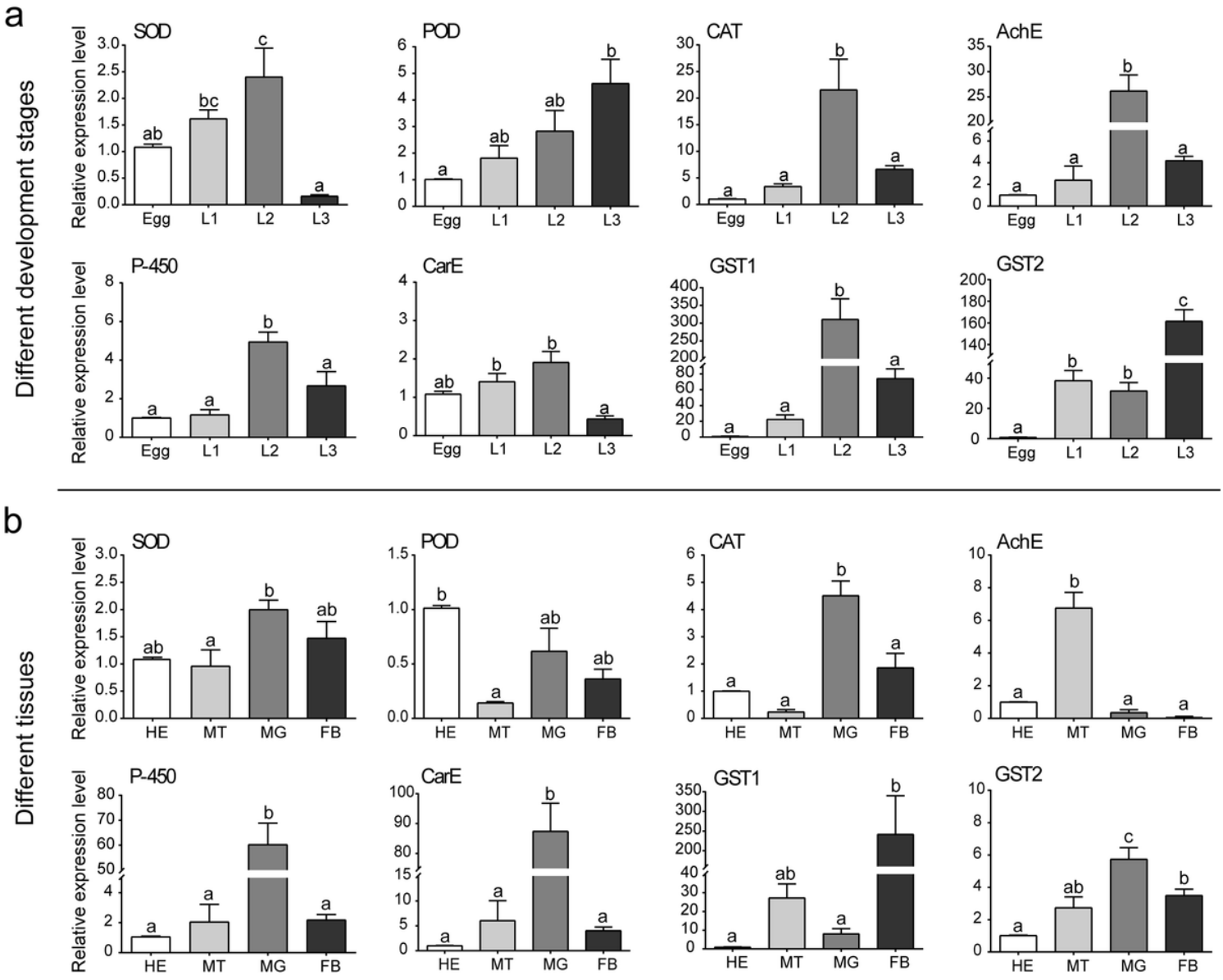

Figure 3

Spatiotemporal expression of SOD, POD, CAT, AchE, P-450, CarE, GST1, and GST2 in *Holotrichia parallela* larvae

(a) The developmental expression patterns. The relative expression level of target transcripts in the egg served as the calibrator for developmental expression profiling. Egg (5 days old), L1 (first instar larvae, 10 days old), L2 (second instar larvae, 18 days old), L3 (third instar larvae, 28 days old).

(b) The tissue-specific expression patterns in hemolymph (HE), malpighian tubule (MT), midgut (MG), and fat body (FB), respectively, and hemolymph were employed as the calibrator.

As for (a) and (b), data are means ± SEM of three biological replicates, and the relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method. *H. parallela* GAPDH was used as an internal standard to

normalize the expression level. Different letters above the bars indicate significant differences at $P < 0.05$ (One-way ANOVA, Tukey's test)

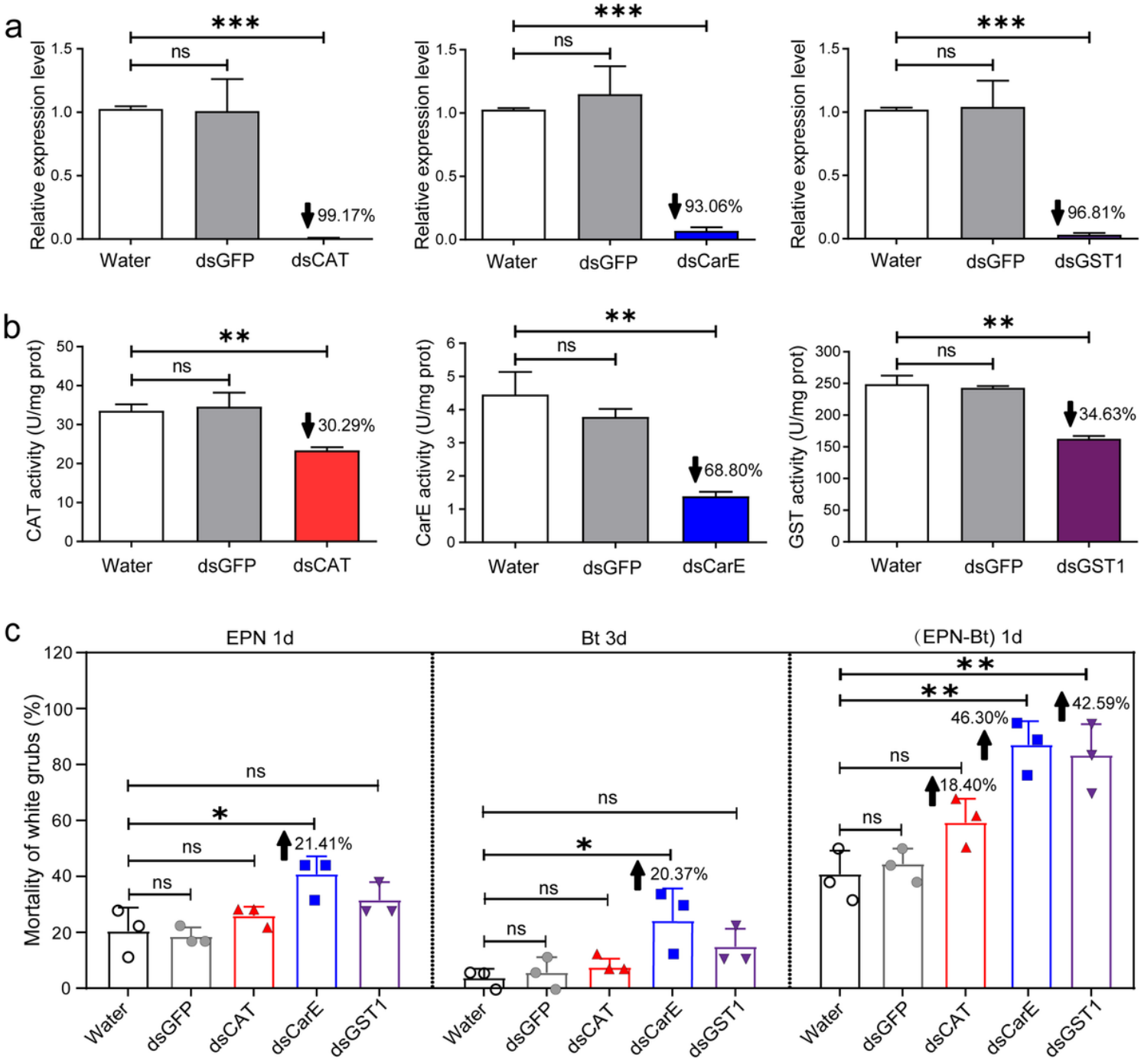

Figure 4

The functions of CAT, CarE, and GST1 in *Holotrichia parallela* larvae

(a) Relative expression levels of CAT, CarE, and GST1 after injecting the corresponding dsRNA for 24 h. Data are means ± SEM of three biological replicates, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method based on the value of water-injected (control) that served as the calibrator. *H. parallela* GAPDH was used as an internal standard to normalize the expression level.

(b) The enzyme activities of CAT, CarE, and GST after injecting the corresponding dsRNA for 24 h. Data represent the means ± SEM of three replicates with 3 larvae per replicate.

(c) The mortality of white grubs exposed to EPN (*H. beicherriana* LF, 100 IJs/grub) for 1 day, Bt (HBF-18, 1.5×10^7 CFU/g soli) for 3 days, and EPN-Bt combination for 1 day, respectively, after dsRNA injection for 24 h. The EPNs were added after the application of Bt for 2 days in the combination treatments. Data represent the means ± SEM of three replicates with 36 larvae per replicate.

As for (a-c), the grubs injected with dsGFP and the same volume of RNase-free water were used as controls, respectively. Statistical differences were determined by multiple *t*-test at the significance levels set at **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns, not significant

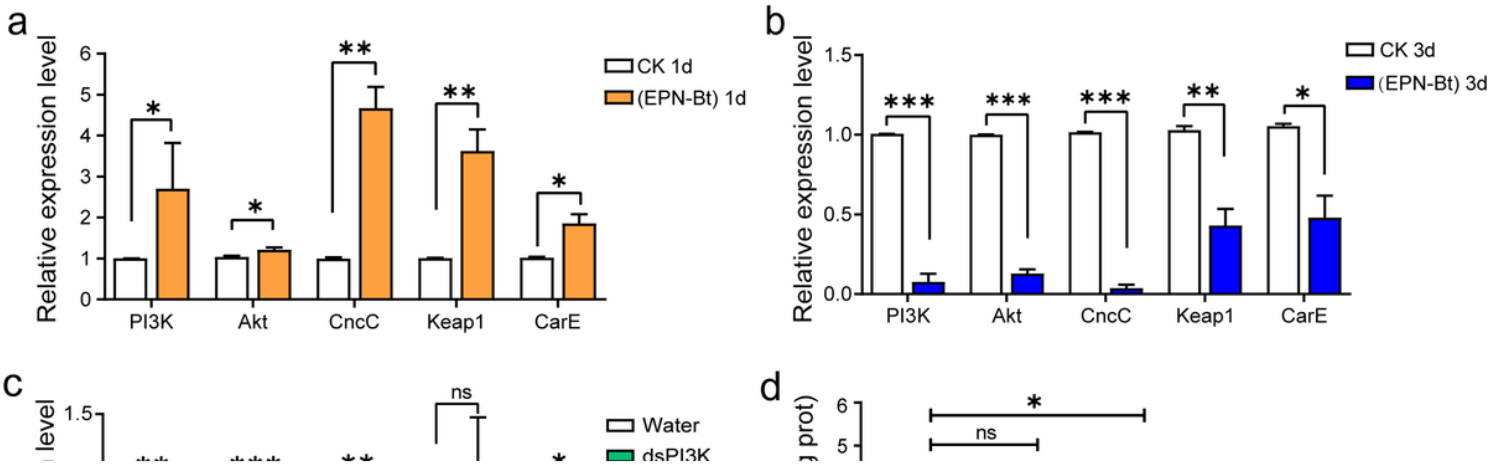

Figure 5

PI3K/Akt/CncC signalling pathway regulates the expression of CarE

(a-b) The relative expression of PI3K, Akt, CncC, and CarE in the midgut of *H. parallela* larvae after exposure to EPN-Bt combination for 1 day and 3 days, respectively.

(c) The relative expression of PI3K, Akt, CncC, and CarE at 24 h after dsPI3K injection.

(d) The enzyme activities of CarE after injecting dsPI3K for 24 h.

As for (a-c), data are means ± SEM of three biological replicates. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method based on the value of water-injected (control) that served as the calibrator. *H. parallela* GAPDH was used as an internal standard. Statistical differences were determined by *t*-test at the significance levels set at **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns, not significant. As for (d), the grubs

injected with dsGFP and the same volume of RNase-free water were used as controls, respectively. Statistical differences were determined by multiple *t*-test at the significance levels set at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant

Figure 6

PI3K silencing affects *Holotrichia parallela* larvae survival and symbiotic bacteria multiplication

(a) Effect of injection of dsPI3K on the survival of grubs when exposed to EPN (*H. beicherriana* LF, 100 IJs/grub), Bt (HBF-18, 1.5×10^7 CFU/g soli), and EPN-Bt combinations, respectively. The EPNs were added after the application of Bt for 2 days in the combination treatments. The experiments were repeated three times with similar results, and 18 larvae were tested per replicate. The survival curves were compared with Kaplan-Meier analysis, and *P*-value < 0.05 was regarded as statistically significant (log-rank test). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(b) Effect of injection of dsPI3K on the symbiotic bacteria multiplication in grubs when exposed to EPN and EPN-Bt combination for 1 day, respectively. Thirty grubs were used for each treatment. Each dot in the graph represented an individual grub. Data are means ± SEM, and statistical differences were determined by multiple *t*-test at the significance levels set at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant. As for (a-b), the grubs injected with dsGFP and the same volume of RNase-free water were used as control, respectively

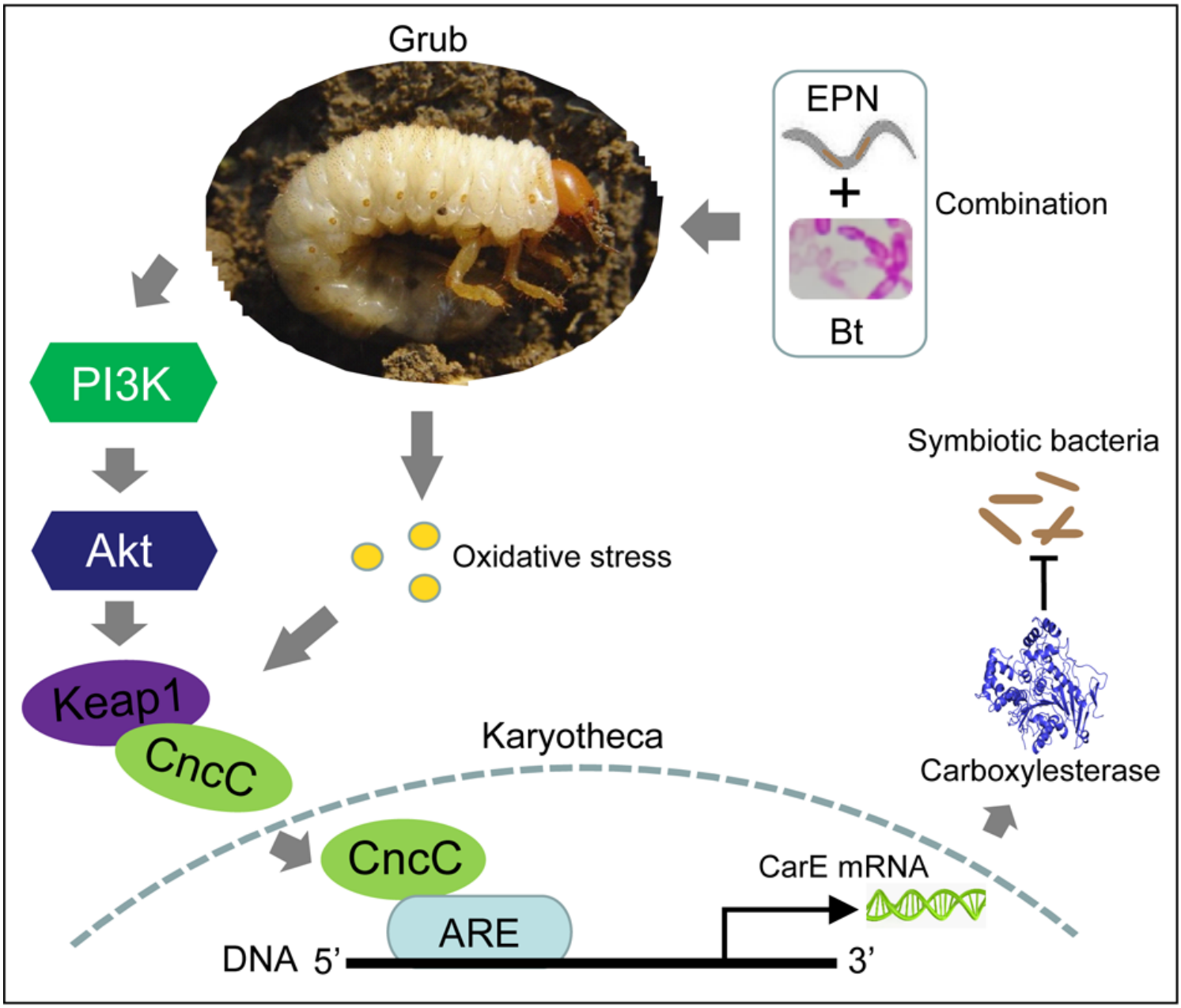

Figure 7

Model figure of key genes involved in the detoxification signalling pathway of *Holotrichia parallela* larvae

PI3K: Phosphatidylinositol 3-kinase; Akt: Protein kinase B; CncC: Cap 'n' collar isoform-C; Keap1: Kelch-like ECH-associated protein 1; ARE: Antioxidant responsive elements

Supplementary Files

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