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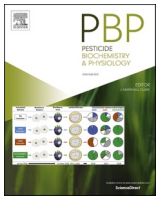

Transcriptomic and physiological analysis of the effect of octanoic acid on *Meloidogyne incognita*

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ABSTRACT

Root knot nematodes are the most devastating root pathogens, causing severe damage and serious economic losses to agriculture worldwide. Octanoic acid has been reported as one of the nematicides, and its mode of action is not fully understood. The main objective of this study was to elucidate the effect of octanoic acid on *Meloidogyne incognita* by transcriptomic analysis combined with physiological and biochemical assays. In the toxicity assays with octanoic acid, the threshold concentration with nematicidal activity and the maximum concentration to which nematodes could respond were 0.03 μL/mL and 0.08 μL/mL respectively. Microscopic observation combined with protein and carbohydrates assays confirmed that the structure of the second-stage juveniles (J2s) was severely disrupted after 72 h of immersion in octanoic acid. Transcriptome analysis has shown that octanoic acid can interfere with the nematode energy metabolism, lifespan and signaling. Although the effects are multifaceted, the findings strongly point to the cuticle, lysosomes, and extracellular regions and spaces as the primary targets for octanoic acid. In addition, nematodes can withstand the negative effects of low concentration of octanoic acid to some extent by up-regulating the defense enzyme system and heterologous metabolic pathways. These findings will help us to explore the nematicidal mechanism of octanoic acid and provide important target genes for the development of new nematicides in the future.

1. Introduction

Root knot nematodes (RKNs) of the genus *Meloidogyne* are devastating pests affecting agricultural production worldwide and are known to attack >3000 species of plants, causing economic losses of up to $118 billion annually (Naz et al., 2021; Jagdale et al., 2021). This group of nematodes include >100 species, with the most devastating species being *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne arenaria*, and *Meloidogyne hapla* (Li et al., 2015; El Aimani et al., 2022). Although many attempts have been made to control root-knot nematode disease, designing sustainable control management methods remains a challenge (Collange et al., 2011). Biological control, which refers to the use of organisms or their bioactive metabolites to control plant pathogens, has received more attention in the development of bionematicides for root-knot nematodes control (Chelinho et al., 2017; Engelbrecht et al., 2018). Since the introduction of nematode biocontrol by Duddington in 1951, researchers have developed various commercial biocontrol products containing live microorganisms, such as Biocon (*Paecilomyces lilacinus*), Xianchongbike (*Pochonia chlamydosporium*), Bio-Nemax (*Bacillus firmus*) (Lamovšek et al., 2013; Li et al., 2015; Gao et al., 2016). However, large-scale commercialization of these products are still difficult due to their unstable performance in field applications.

Based on these issues, bioactive metabolites of microbial origin have attracted greater attention in the control of root knot nematodes, and some have facilitated the development of novel nematicides or the discovery of new modes of action (Hüter, 2011; Kim et al., 2016). Several

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new nematode control models have been reported, such as the "Trojan horse" and"honey-trap" mechanism (Niu et al., 2010; Cheng et al., 2017). These findings have practical implications for nematode control, as it provides valuable information for the development of the most appropriate drug delivery methods (Seo et al., 2014).

In our previous studies, we have isolated a bacteria strain *Bacillus altitudinis* AMCC 1040 which showed significant nematicidal activity against root-knot nematodes in vitro, in pot and in field experiments (Wang et al., 2021a, 2021b). Subsequently, we confirmed that *Bacillus altitudinis* AMCC 1040 could produce six nematicidal substances, of which octanoic acid had the highest nematicidal activity (Ye et al., 2022). Octanoic acid, also known as caprylic acid, can be produced by various microorganisms such as *Megasphaera hexanoica* (Jeon et al., 2017). Many previous studies have reported strong nematicidal activity of octanoic acid against *Meloidogyne incognita* hatching and mortality in in vitro assays (Bansal and Bajaj, 2003; Zhang et al., 2012). However, its mode of action has not been evaluated, hindering further application in the control of root knot nematodes. The main objective of this study was to investigate the effect of octanoic acid and the response of *Meloidogyne incognita* J2s. To achieve this goal, we assessed the differential gene expression of *Meloidogyne incognita* J2s cultured with octanoic acid by RNA-Sequencing (RNA-Seq), as transcriptomic investigation can provide information about transcript abundance and its variation, as well as clues about the modes of action (Griffith et al., 2015; Kumarasingha et al., 2019).

2. Materials and methods

2.1. Preparation of nematodes and octanoic acid solutions

This study targeted a representative species, *Meloidogyne incognita*. Nematodes were maintained on tomato plants and incubated in a greenhouse at 25 ± 5 °C. Sixty days after inoculation, infected plants were uprooted and washed gently washed to remove adhering soil. The formed egg masses (each containing 300–500 eggs) were carefully picked out from the roots with forceps and surface sterilized with 1.5% NaClO solution (Laquale et al., 2020). Second stage juveniles (J2s) were obtained by incubating egg masses in 24-well culture plates filled with 2 mL distilled water for 5 days at 28 °C and collected using an 3 mL plastic transfer pipet to an 50 mL sterile glass bottle and counted by microscope (Murungi et al., 2018).

A stock solution (0.2 μL/mL) was prepared by adding 2 μL of octanoic acid (Aladdin, 99% purity) to 9998 μL of distilled water.

2.2. Nematicidal properties of octanoic acid

A 1 mL suspension of *M. incognita* J2s (containing about 500 J2s) was placed in a 2 mL sterile centrifuge tube, and then the volumes of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mL of octanoic acid stock solutions (0.2 μL/mL) and a final volume of 2 mL was reached with sterile ddH_2O water to obtain 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10 μL/mL final test concentrations. After incubated at 28 °C in the dark, J2s were periodically examined for viability under an Olympus BX51 optical microscope until 12 h. J2s were considered dead if they were linear shapes or insensitive to mechanical contact, and worms were judged to be alive if they were able to move or curved naturally (Ye et al., 2022). J2s mortality (%) = dead juveniles / total juveniles × 100 (Kaur et al., 2016). Corrected mortality (%) = [(mortality percentage in treatment − mortality percentage in untreated control) / (100 − mortality percentage in untreated control)] × 100 (Bi et al., 2018). The sterile ddH_2O was used as a negative control, all the nematicidal bioassays were performed with three replicates per treatment.

2.3. Effects of octanoic acid on morphology and structure of J2s

For morphological observation and tissue integrity measurements, nematodes were treated with octanoic acid at two characteristic concentrations of 0.03 μL/mL and 0.08 μL/mL and incubated for 12 h, 24 h, and 72 h. J2s were then spread uniformly on glass slides, observed under Olympus BX51 optical microscope and photographed. To study the effect of octanoic acid on the tissue integrity of *M. incognita*, the variability of proteins, carbohydrates were tested. Both substances were detected by spectrophotometry, and the details of the methods were given in supporting information 1. In this experiment, complete smash of nematodes was important for subsequent analysis. To meet the requirements, approximately 8000 J2s from the bioassay experiments were centrifuged at 12,000 rpm for 1 min at 4 °C, and the supernatant was carefully aspirated and discarded. The centrifuge tubes were filled with 50 μL of distilled water (when making crude enzyme solution, distilled water was replaced with extracting solutions provided by Solarbio Kit) and 100 sterile glass beads (Solarbio, China, Φ 600–800 μm) and immediately placed in liquid nitrogen for 1 min for quick-freezing. The pretreated centrifuge tubes containing J2s were placed on ice and thoroughly ground with an electric tissue grinder OSE-Y10 (TIANGEN, Beijing, China, no-load speed: 8000 rpm) until all fragments were observed in the field of view under the microscope. The sterile ddH_2O was used as a negative control, all the bioassays were performed with three replicates per treatment.

2.4. Transcriptome analysis

To elucidate the mode of action of the lethal effect of octanoic acid on *M. incognita*, nematodes were treated with low and high concentrations of octanoic acid both long and short treatment times. RNA extracted from four treatment groups were used to construct cDNA libraries, including HC1 (0.08 μL/mL treated for 5 min), HC2 (0.08 μL/mL treated for 10 min), LC1 (0.03 μL/mL treated for 5 h) and LC2 (0.03 μL/mL treated for 10 h), as well as their controls (CKH: sterilized ddH_2O treated for 10 min; CKL: sterilized ddH_2O treated for 10 h), with three biological replicates in each group. Total RNA was isolated by using *mir*Vana™ miRNA Isolation Kit (Thermo Fisher Scientific, New York, USA) according to the manufacturer's protocol. Assessment of the obtained RNA integrity was performed by 1% agarosegel electrophoresis and on the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA), meanwhile, RNA concentration and purity was measured on a NanoDrop2000 (Thermo Fisher Scientific) (Neupane et al., 2019). The cDNA libraries were constructed according to the Illumina's library construction protocol and sequenced using Illumina HiSeq. 2500.

Transcriptome sequencing and analysis were conducted by OE biotech Co., Ltd. (Shanghai, China). Raw data were processed with Trimmomatic (Bolger et al., 2014). To obtain clean reads, reads containing ploy-N and low quality reads were removed. Trinity was used to assemble high-quality clean reads into expressed sequence tag clusters and assembled into transcript (Grabherr et al., 2011). Clean reads were then mapped to the reference genome using hisat2 (PRJEB8714, https://parasite.wormbase.org) (Kim et al., 2015). FPKM values were calculated for each gene using cufflinks (Trapnell et al., 2010). The functions estimateSizeFactors and nbinomTest of the DESeq (2012) R package were used to identify differentially expressed single genes (DEGs) (Anders and Huber, 2013). DEGs were identified at $P < 0.05$ and foldChange >2 or foldChange <0.5. Based on hypergeometric distribution, GO enrichment and KEGG pathway enrichment analyses were performed separately for DEGs using R language (Kanehisa et al., 2007).

2.5. qPCR analysis of selected DEGs

Real time quantitative PCR (qPCR) was performed to assess the transcript levels encoded by the selected genes. The treatments and group divisions used for qPCR analysis were the same as described in 2.4. In the HC treatment group (Both in HC1 and HC2), genes involved in the biosynthesis process of very long-chain fatty acids, which are structural components of the cuticle and lysosomes, were selected.

Genes related to the lysosome and the cuticle were also detected in the LC treatment group (Both in LC1 and LC2), in addition to which we evaluated the transcript levels of the genes involved in xenobiotic metabolic process and innate immune response. The primers used in this study were listed in supporting information 2, Table S1. For qPCR analysis, RNA was reverse transcribed to cDNA by using TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR Kit. The reaction mix contained total RNA 0.5 μg, 5 × TransScript All-in-one SuperMix for qPCR 5 μL, gDNA Remover 0.5 μL, and nuclease-free ddH_2O up to 10 μL. The amplification program was: 42 °C, 15 min; 85 °C, after reverse transcription, 90 μL nuclease-free ddH_2O was added and stored in a −20 °C refrigerator for later use. qPCR was performed in LightCycler®480II PCR thermal cycler (Roche, Swiss) using a PerfectStart™ Green qPCR SuperMix under the following conditions: 94 °C for 30 s, followed by 45 cycles at 94 °C for 5 s and 60 °C for 30 s. At least three independent replicates were used to quantify the transcription of each gene. Relative ploidy changes in gene expression were normalized to 18S rRNA. Experiments were performed with three replicates per treatment.

2.6. Association analysis between nematode enzyme activity determination and related gene expression

Carboxylesterase (CarE), catalase (CAT), glutathione S-transferase (GST) and acetylcholinesterase (AchE) activities were measured according to the manufacturer's protocol (Supporting information 3) using a commercial assay kit (Solarbio, China) to illustrate the physiological response of nematodes to octanoic acid. The crude enzyme solution was prepared in the same way as described in 2.3. The extracts were used as a negative control and all bioassays were performed in three replicates per treatment. Meanwhile, relevant genes were selected and the relative expression was displayed by heat map.

2.7. Statistical analysis

All statistical analyses were performed using analysis of variance (ANOVA) with SAS statistical software. Pie and bar charts were produced by GraphPad Prism 6. The relative expression of selected genes was calculated using $2^{-\Delta\Delta Ct}$. The ComplexHeatmap R package was used to create heat map.

3. Results

3.1. The nematicidal activity of octanoic acid acts in a dose- and time-dependent manner

As shown in Fig. 1(a), octanoic acid had no effect on the death of J2s at low concentrations (0.01 μL/mL and 0.02 μL/mL), and after incubation for 12 h, when octanoic acid was increased to 0.03 μL/mL, 100% death of J2s began to be observed. Upon further investigation, we found that 0.03 μL/mL may be the critical concentration, because at concentration below 0.03 μL/mL, such as 0.028 μL/mL, there was no nematicidal activity even after treatment for 48 h (data not shown). In addition, we studied the time required to reach complete nematode death at each effective dose and found that it took approximately 30 min to 10 h when the concentration was between 0.04 μL/mL–0.07 μL/mL. Notably, 100% mortality occurred after 15 min of treatment with 0.08 μL/mL, however, at concentrations greater than this, the nematodes died immediately within 1 min. Therefore, we hypothesized that 0.08 μL/mL may be the closest to the highest concentration to which nematodes can respond. Therefore, we mainly analyzed the nematicidal properties of 0.03 μL/mL and 0.08 μL/mL (Fig. 1b and Fig. 1c). It is noteworthy that complete nematodes death occured in a short period of time rather than in a gradual manner under either 0.03 μL/mL or 0.08 μL/mL octanoic acid treatment. When octanoic acid was supplemented at 0.03 μL/mL, J2s were active within 10 h after treatment, however, complete death of J2s occurred within 60 min after 10 h (Fig. 1b). A similar trend was observed at a dose of 0.08 μL/mL, with instantaneous death of J2s within 5 min after 10 min treatment with octanoic acid (Fig. 1c). Based on these results, subsequent studies focused on these two key concentrations.

3.2. Effects of octanoic acid on the structure of J2s

Morphological changes of *M. incognita* were observed by light microscopy after incubation with octanoic acid at concentrations of 0.03 μL/mL and 0.08 μL/mL. In the water control, J2s were active, healthy and normal in shape. Under 0.03 μL/mL treatment, J2s were killed, but the structures of J2s was not significantly changed even after 72 h of treatment. However, under 0.08 μL/mL treatment, after 24 h treatment, J2s were completely killed and accompanied by the formation of multiple giant vacuoles, and subsequently, the body of J2s became incomplete and the structures of intestine, pharynx and other organs were became disorganized after 72 h of treatment (Fig. 2).

To better investigate the effects of octanoic acid on the tissue integrity of J2s, protein and carbohydrates were detected spectrophotometrically between the different treatments. The results showed that the changes in protein and carbohydrates were basically the same, and both substances showed a decreasing trend in nematodes during the octanoic acid treatment. After 12 h of treatment, carbohydrates decreased significantly ($P < 0.01$) under low and high octanoic acid compared with CK, but there was no significant difference in protein content index (Fig. 3b and Fig. 3d). At 24 h post-treatment, the changes in these two indicators were quite prominent compared to the control, and subsequently, at 72 h of exposure, significant differences were achieved between 0.03 μL/mL and 0.08 μL/mL (Fig. 3b and Fig. 3d).

3.3. Transcriptomic profiling

3.3.1. General features of transcriptome assembly and annotation

A total of 850,697,340 clean reads from 18 samples were obtained

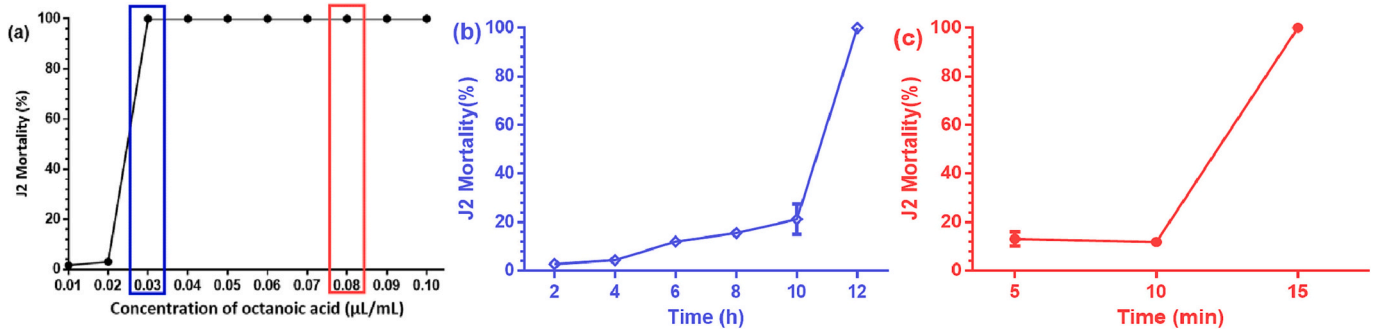

Fig. 1. The dynamic nematicidal activity of different concentrations of octanoic acid at a treatment time of 12 h (a), and the nematicidal characteristics of 0.03 μL/mL (b) and 0.08 μL/mL.

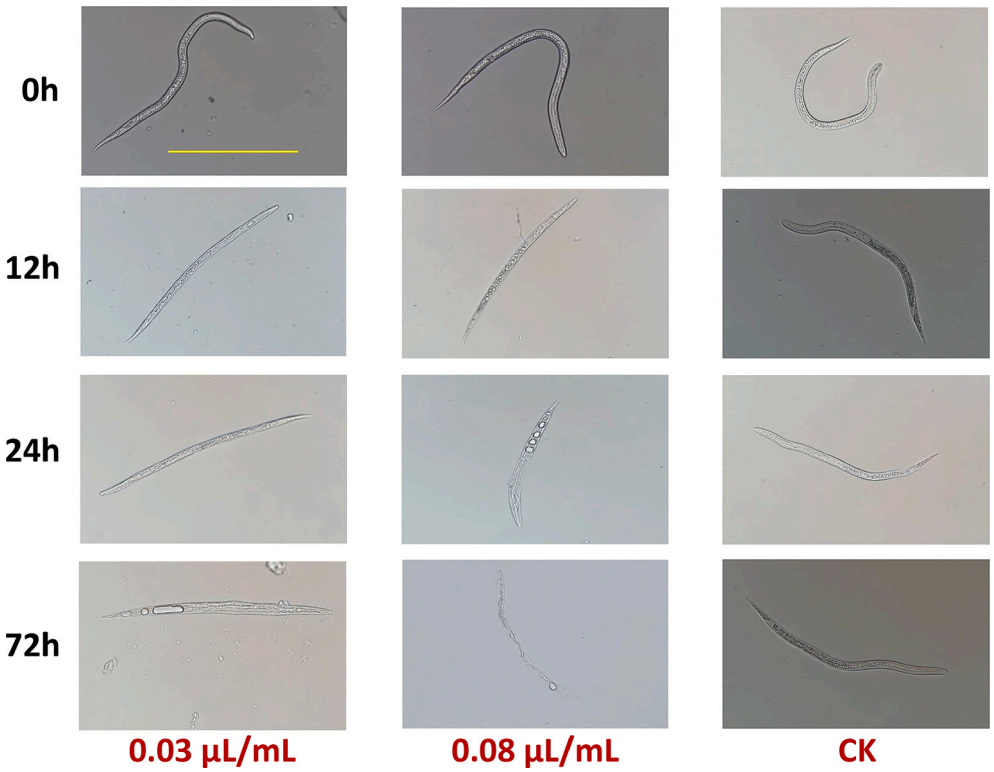

Fig. 2. The dynamic damage of different concentrations of octanoic acid on J2s. (a) 0.03 μL/mL; (b) 0.08 μL/mL; (c) CK. Scale bars = 200 μm.

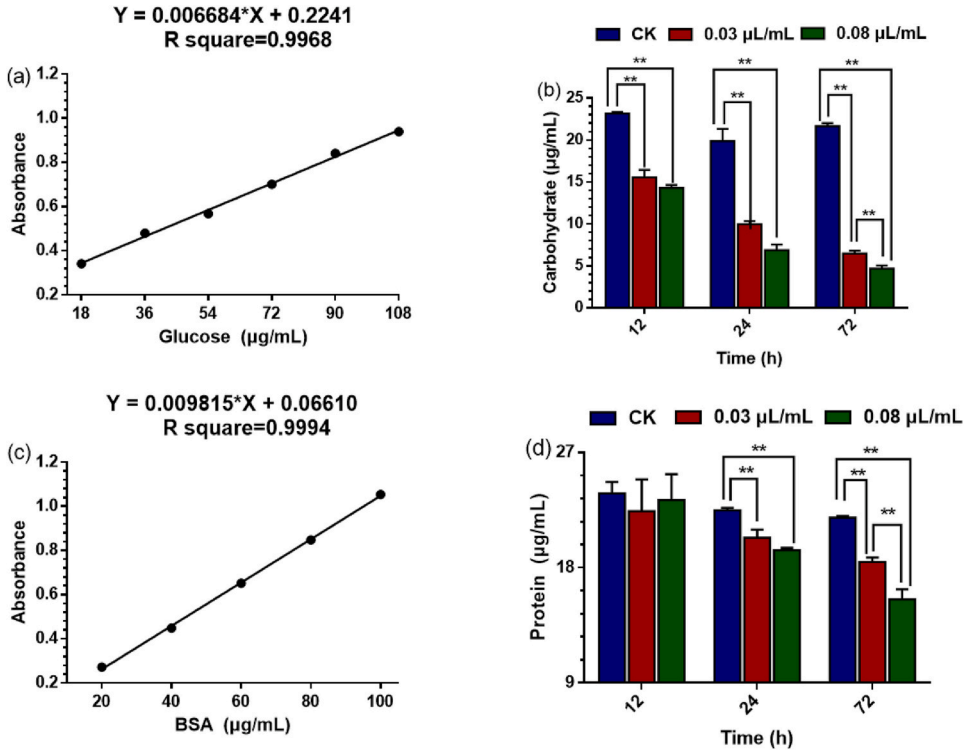

Fig. 3. The dynamic effects of octanoic acid on the carbohydrate and protein contents of J2s. (a) Standard curve of glucose standard solution; (b) Differences carbohydrate content between different treatments; (c) Standard curve of BSA standard solution; (d) Differences protein content between different treatments. ** indicates significant difference at $P < 0.01$ level.

after trimming of adaptor and low quality bases, while mapping to the reference genome *Meloidogyne incognita* (PRJEB8714) with an average of 90.26% (Supporting information 4, Table S5). All sequence reads were deposited in the National Center for Biotechnology Information (NCBI) under the accession numbers SUB11799498. Principal component analysis (PCA) was performed on the RNA-seq data set to reveal

similarities and dissimilarities in gene expression across treatments. As shown in supporting information 4, Fig. S1, samples from different groups were distinguished, while replicates within the same group were clustered closely together, indicating that the within-group differences were greater than the between-group differences and were significant.

In the high dosage treatment, 1151 (CKH), 878 (HC1), and 55 (HC2) genes were found to be specifically expressed between groups (Supporting information 4, Fig. S2a). In contrast, 433 (CKL), 598 (LC1), and 720 (LC2) genes were specifically expressed under low dosage treatment (Supporting information 4, Fig. S2b). There was more overlap of co-expressed genes in the LC group compared to the HC group. Interestingly, only 27,043 genes were expressed in HC2 compared to over 32,000 genes in all other groups, suggesting that the expression profile of HC2 was significantly disrupted by long-time high concentrations of octanoic acid.

3.3.2. *Identification of DEGs*

By comparison with CKH, the HC1 group, treated with high dose of octanoic acid for 5 min, possessed 1912 DEGs (1106 up-regulated and 806 down-regulated), while the treatment time was increased to 10 min and the HC2 group acquired 1735 DEGs (1079 up-regulated and 656 down-regulated). Compared to CKL, the LC1 group treated with low dose octanoic acid for 5 h had 1302 DEGs (599 up-regulated, 703 down-regulated) and the LC2 group treated with low dose for 10 h had 1790 DEGs (1058 up-regulated, 732 down-regulated), showing a significant increase in the effects of elevated dose treatment and extended

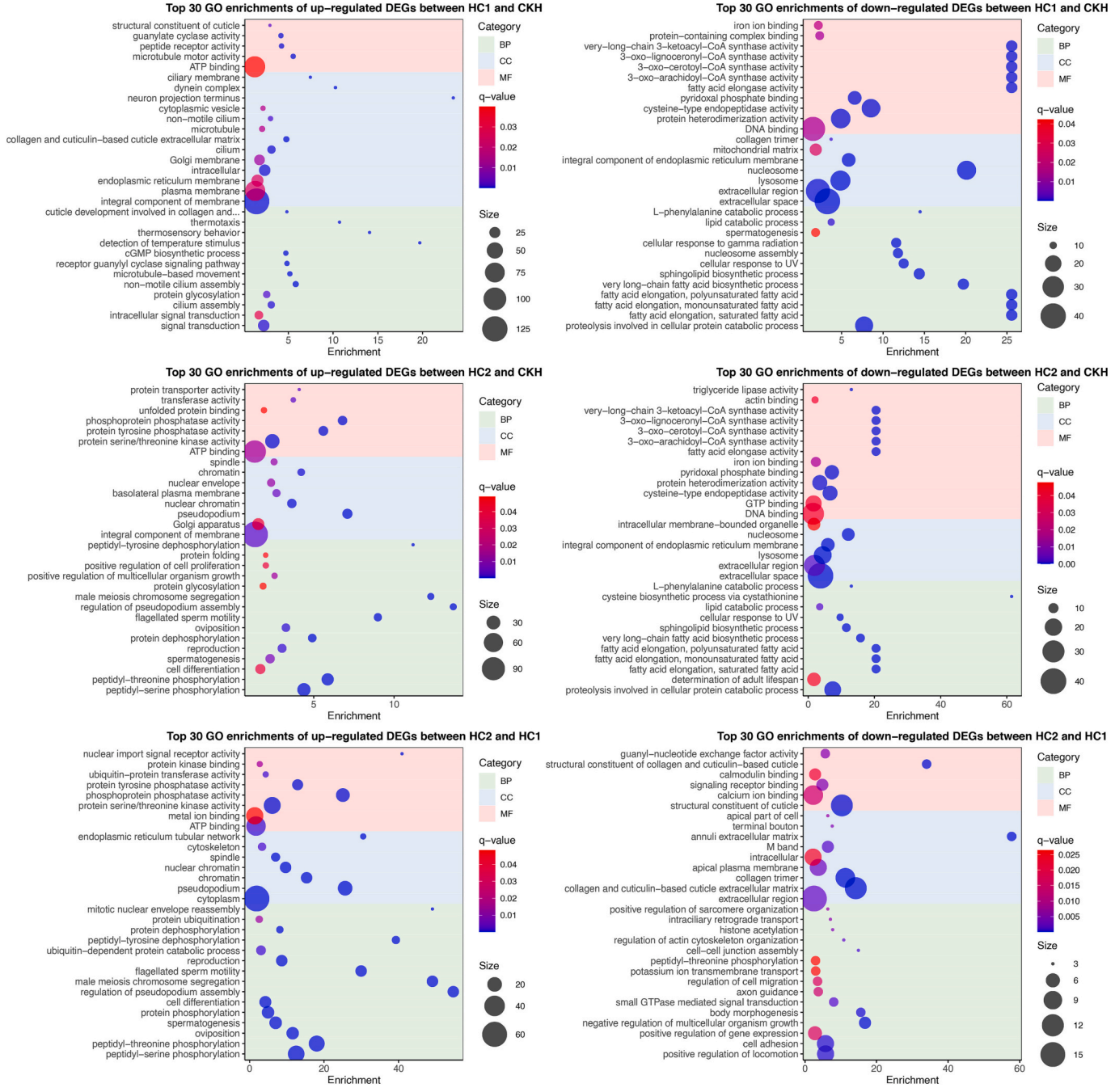

Fig. 4. Top 30 gene ontology (GO) enrichment of differentially expressed genes treated with 0.08 μL/mL octanoic acid. (a) up-regulated genes in HC1 compared with CKH; (b) down-regulated genes in HC1 compared with CKH; (c) up-regulated genes in HC2 compared with CKH; (d) down-regulated genes in HC2 compared with CKH; (e) up-regulated genes in HC2 compared with HC1; (f) down-regulated genes in HC2 compared with HC1.

treatment time on the number of DEGs. We also compared the HC2 and HC1 groups, and the LC2 and LC1 groups to elucidate the dynamic effects of different treatment times of octanoic acid on *M. incognita*. With increasing treatment time, 732 (476 up-regulated and 256 down-regulated) and 1065 (710 up-regulated and 355 down-regulated) DEGs were found under high-dose octanoic acid and low-dose octanoic acid treatments, respectively.

Thirty-six DEGs were presented in both the high and low dose treatments at different processing times. The expression heat map of these 36 DEGs was clustered by treatment time and showed four types of expression modules (Supporting information 4, Fig. S3). Of these, 12 DEGs were annotated with possible biological functions involved in signal transduction, protein synthesis, lipid metabolism and lysosomal function. TNF signaling pathway-related gene, *pgam-5* was up-regulated in the treatment group, while the HC group was up-regulated twofold compared to the LC group, with an average change of 5-fold. Notably, two *elo-4* genes, predicted to be involved in the biosynthetic process of fatty acids and sphingolipid, were significantly up-regulated in the low-dose treatment, with an approximately 2-fold increase in the HC group and a 14 to 31-fold increase in the LC group. Except for the lysosomal proteases *cpr-1* and nuclear hormone receptors *nhr-100* were both down-regulated in the treatment group.

3.3.3. GO enrichment

Regarding the up-regulated genes, in the comparison between the HC group and CK groups, as shown in Fig. 4(a, c), DEGs related to the overall

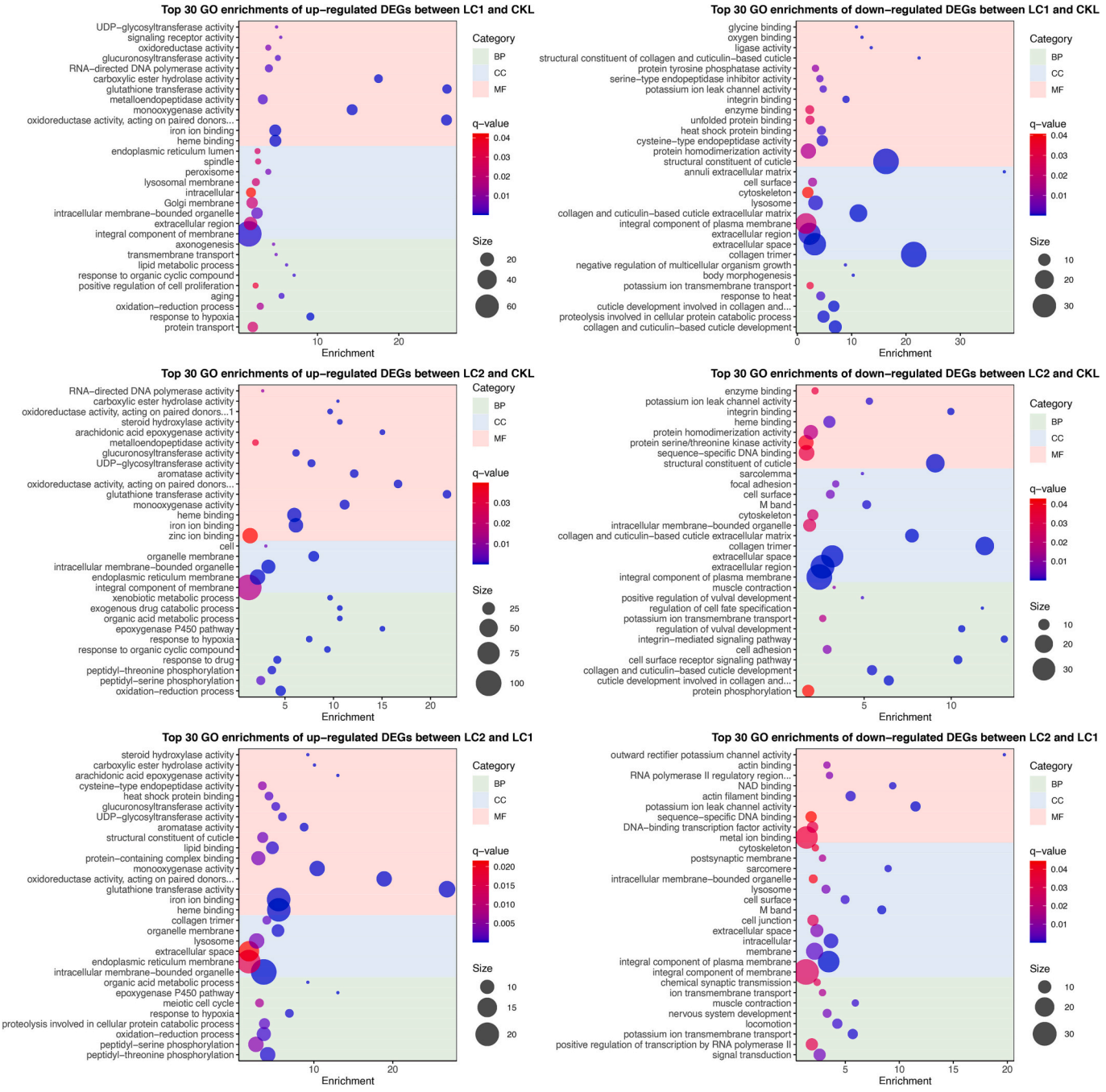

Fig. 5. Top 30 gene ontology (GO) enrichment of differentially expressed genes treated with 0.03 μL/mL octanoic acid. (a) up-regulated genes in LC1 compared with CKL; (b) down-regulated genes in LC1 compared with CKL; (c) up-regulated genes in LC2 compared with CKL; (d) down-regulated genes in LC2 compared with CKL; (e) up-regulated genes in LC2 compared with LC1; (f) down-regulated genes in LC2 compared with LC1.

composition of the membrane and protein synthesis and modification were all enriched in both HC1 and HC2. Furthermore, DEGs related to signal transduction were up-regulated when nematodes were treated with 0.08 μL/mL octanoic acid for 5 min, while, the expression of cell proliferation and differentiation could be up-regulated by treating nematodes with 0.08 μL/mL for 10 min. In addition, DEGs related to cell development and signaling, such as cytoplasm, ATP binding, metal ion binding, protein serine/threonine kinase activity, cell differentiation, and oviposition, were significantly up-regulated in HC2 compared to HC1. Many DEGs involved in lysosomes, extracellular regions and spaces, and very-long chain fatty acid biosynthetic and elongation were down-regulated in both HC1 and HC2 groups compared to CK. High concentrations and prolonged octanoic acid treatment may also lead to down-regulation of collagen and cuticle structural components over time compared to HC1.

Under low concentrations of octanoic acid treatment, as shown in Fig. 5, many DEGs related to cuticle structure and development, extracellular space and region, motibility, ions transmembrane transport and lysosome were down-regulated, whereas DEGs related to peroxidase, monooxygenase, oxidoreductase, metalloendopeptidase, glutathione transferase, carboxylate hydrolase, glucuronyltransferase were up-regulated, regardless of the length of treatment. As the treatment time was extended to 10 h, the nematode responses to organic cyclic compounds, exogenous drug catabolism, heterologous metabolism and organic acid metabolism were also significantly up-regulated in addition to multiple enzymes being up-regulated. Compared to LC1, the GO

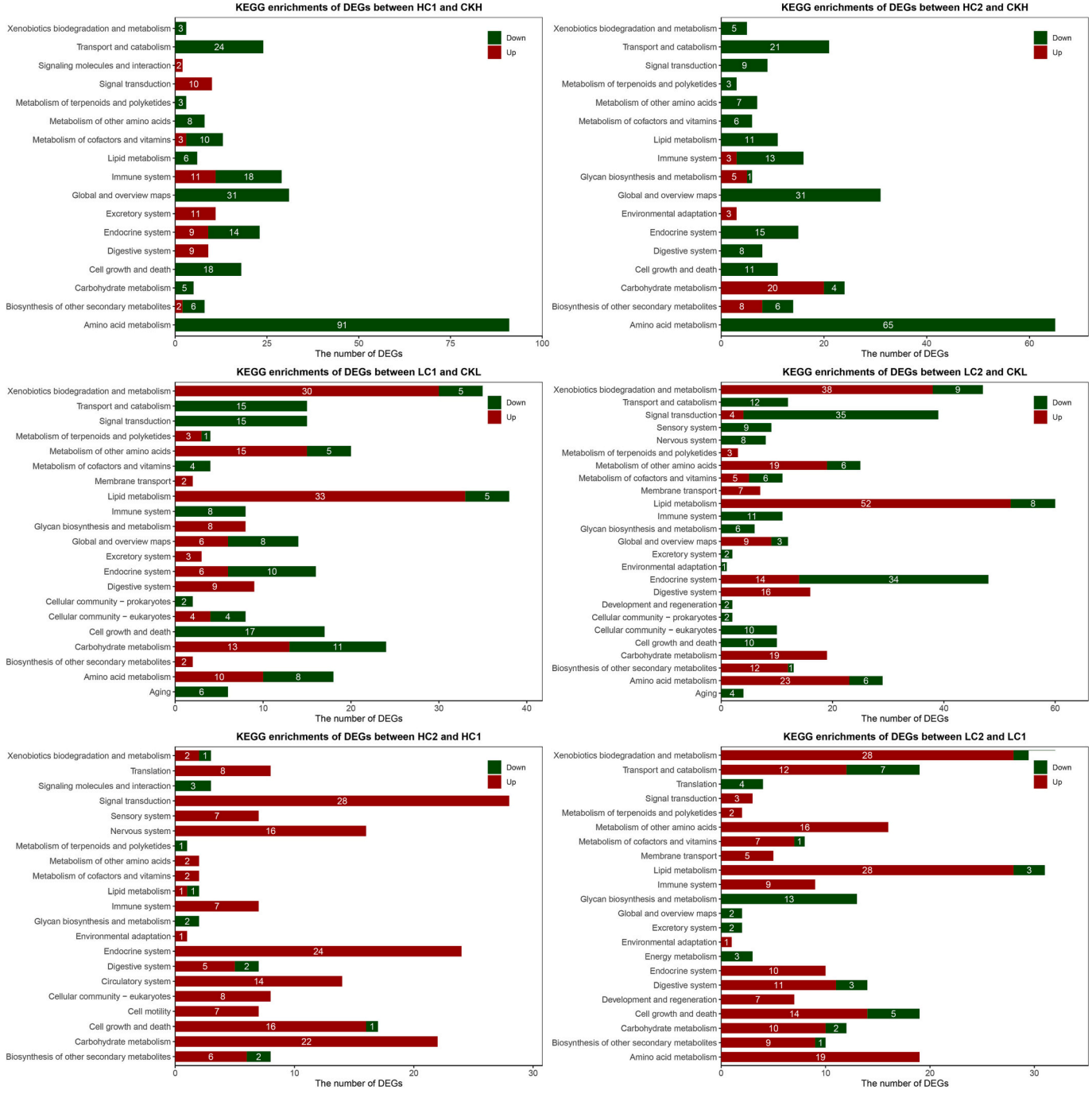

Fig. 6. Top 20 most enriched KEGG pathways under level 3 treated with octanoic acid. (a) HC1 vs CKH; (b) HC2 vs CKH; (c) LC1 vs CKH; (d) LC2 vs CKH; (e) HC2 vs HC1; (f) LC2 vs LC1.

functions down-regulated by LC2 were mainly enriched in neural, motibility, signal transduction and cell membrane composition, while glutathione transferase, monooxygenase and oxidoreductase activities were significantly up-regulated.

3.3.4. KEGG enrichment

Fig. 6 shows the results of KEGG pathways enrichment under level 2. As shown in Fig. 6(a) and Fig. 6(b), there is a clear trend of down-regulation in the HC group. Nine down-regulated pathways were present in both HC1 and HC2 treatments compared to CK. Most of the down-regulated genes were associated with biodegradation and metabolism of xenobiotics, transport and catabolism, metabolism of terpenoids and polyketides, metabolism of other amino acids, lipid metabolism, global and overview maps, endocrine system, cell growth and death, especially amino acid metabolism (91 and 65 unigenes in HC1 and HC2 respectively). In contrast, as shown in Fig. 6(e), the KEGG pathways between HC1 and HC2 showed an obvious up-regulated trend including translation (8 unigenes), signal transduction (28 unigenes), sensory system (7 unigenes), nervous system (16 unigenes), immune system (7 unigenes), endocrine system (24 unigenes), circulatory system (14 unigenes), cell motility (7 unigenes), carbohydrate metabolism (22 unigenes), as well as cell growth and death (16 unigenes up-regulated, 1 unigenes down-regulated). As shown in Fig. 6(c) and Fig. 6(d), the changes in the KEGG pathway were more complex in the LC treatment group compared to the HC treatment group. Among these pathways, the biodegradation and metabolism of xenobiotics and lipid metabolism were most affected in both LC1 and LC2. Pathways related to transport and catabolism, immune system, cell growth and death, aging were all down-regulated both in LC1 and LC2 compared to CK. In addition, the sensory system and nervous system were also down-regulated in LC2. Between LC2 and LC1, important and representative pathways including biodegradation and metabolism of xenobiotics, immune system, metabolism of other amino acids, lipid metabolism, environmental adaptation, endocrine system, digestive system, development and regeneration, carbohydrate metabolism, biosynthesis of other secondary metabolites, and metabolism of amino acids were significantly up-regulated with increasing treatment time.

The 20 most enriched (those with <20 are all shown) KEGG pathways under level 3 are listed in supporting information 4, Fig. S4 and Fig. S5. It is important to highlight that the down-regulated DEGs in both HC and LC groups were enriched in apoptosis and lysosome. More interestingly, the pathways in the HC group and LC group showed unique characteristics. The down-regulated DEGs in the LC2 group was concentrated in signaling pathways such as MAPK, Neurotrophin, PPAR, Wnt, Insulin, FoxO, which were rarely found in HC group. Furthermore, a clear trend showed that most pathways relevant to metabolism and degradation were down-regulated in the HC group, while most were up-regulated in the LC group.

3.4. RNA-Seq data validation by qPCR

The relative expression levels of selected DEGs in *M. incognita* J2s treated with different concentrations of octanoic acid and different incubation times were assessed by qPCR. The expression levels of all selected DEGs were significantly different compared to CK (Fig. 7). Genes involved in lysosome and cuticle were significantly down-regulated in both low and high concentration treatments. Also, genes involved in the biosynthesis process of very long-chain fatty acids were also significantly down-regulated in the HC treatment. In addition, genes related to xenobiotic metabolic processes and innate immune response of nematodes were significantly up-regulated in the LC treatment group. These qPCR validation results are consistent with those shown in the corresponding transcriptome data.

3.5. The enzyme activity and related gene expression of J2s

It was found that exposure to octanoic acid resulted in significant changes in the activities of four enzymes associated with nematodes resistance. As shown in Fig. 8a, AchE activity in the high-dose treatment decreased rapidly at 5 min and continued to decrease until 10 min. In the LC treatment, AchE activity increased slightly at 5 h and then decreased sharply at 10 h. However, no significant correlation was found between gene expression and AchE activity assay. In the HC treatment, CarE activity did not change in the first 5 min and then increased significantly at 10 min. However, the trend of change in the LC treatments was reversed, with CarE activity first increasing and then decreasing with increasing exposure time. Consistent with the enzyme activity data, the gene expression levels of CarE showed similar changes, for example, Minc3s02429g29988, Minc3s00638g15461 and Minc3s02993g32346 were significant up-regulated in the HC2 treatment group, meanwhile, Minc3s03123g32876, Minc3s00168g06605, Minc3s00340g10527 were significant down-regulated in the LC2 treatment group (Fig. 8b). Compared with CK, CAT activity in HC treatments showed an increasing trend, and correspondingly, Minc3s00546g14073, Minc3s00005g00340, and Minc3s01021g19931 were significantly up-regulated in both HC1 and HC2 (Fig. 8c). For low-dose treatments, no differences of CAT activity were found at the first 5 h, but significantly lower compared with the CK after incubation until 10 h. As shown in Fig. 8d, GST activity remained low level in HC treatments; however, in LC treatment, GST activity increasedit significantly increased with prolonged exposure time in LC treatments as the continuous up-regulation of Minc3s00686g16074, Minc3s00048g02611.

4. Discussion

In the present study, we performed detailed toxicity, physiological and biochemical assays as well as transcriptome analysis to demonstrate that the adverse effects of octanoic acid on *M. incognita* are multifaceted,

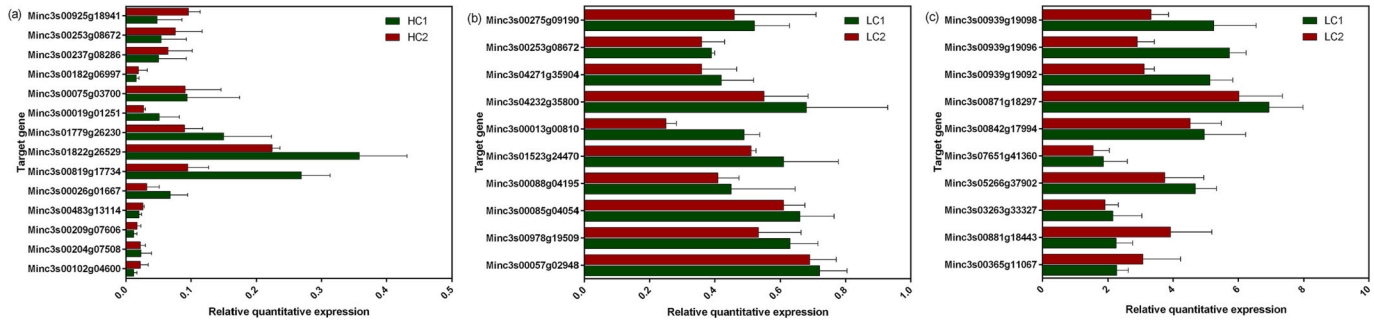

Fig. 7. RNA-Seq data validation by qPCR. (a) genes involved in very long-chain fatty acid biosynthetic process structural constituent of cuticle and lysosome in the HC treatment group; (b) genes related to lysosome and cuticle in the LC treatment group; (c) genes involved in xenobiotic metabolic process and innate immune response in the LC treatment group.

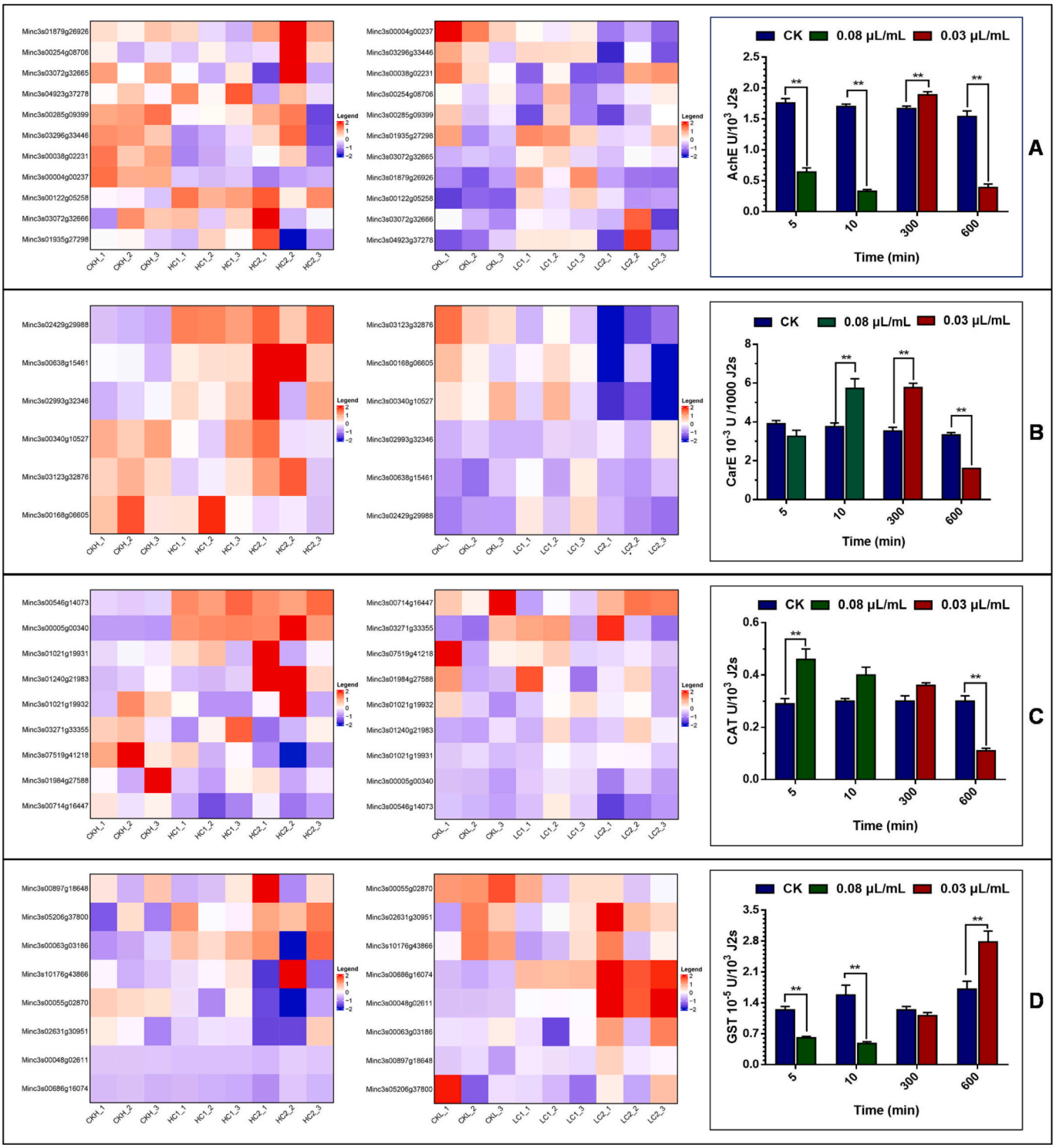

Fig. 8. Association analysis between enzyme activity determination and related gene expression. (a) AchE; (b) CarE; (c) CAT; (d) GST.

while nematodes can respond to octanoic acid treatment to some extent by initiating self-defense mechanisms.

In a preliminary experiment, different concentrations of octanoic acid were tested against J2s of *M. incognita*. The threshold effect of nematicidal activity of octanoic acid was observed at 0.03 μL/mL, and when at 0.08 μL/mL, nematodes can tolerate for 15 min, once greater than this concentration, death occurred within 1 min. Similar results were also reported by Rajasekharan et al., they founded that when 5-iodoindole was supplemented at 20 μg/mL, approximately 80% killing of J2 s was observed after treatment for 24 h, while at 50 μg/mL, death occurred instantaneously (Rajasekharan et al., 2020). More intriguingly, in the time required assays, we found that the death of nematodes under octanoic acid treatment occurs in an abruptly and completely way rather than in an increasing manner which is a unique feature which not observed in previous studies (Kwok et al., 1992; Yeon et al., 2019).

There have been extensive studies on the ability of nematicidal active substances to destroy the structure of nematodes in previous studies. For example, J2s became "granular" and accompanied by a progressive loss

of structure in the internal tissue when exposed to fluensulfone (Kearn et al., 2017). Nguyen et al. found that verrucarin A and roridin A isolated from *Myrothecium verrucaria* can destroy eggs and the larva died soon after hatching (Nguyen et al., 2018). Similarly, microscopic observations of J2s under octanoic acid treatment in the present study revealed severe alterations in shapes, cuticle and internal structures. The cuticle in nematodes is a thin and flexible outer covering composed of cuticlins, lipids, proteins and surface-associated carbohydrates, which is known to act as a physical barrier and protect nematode from xenobiotics (Fetterer and Rhoads, 1993; Niu et al., 2010; Davies and Curtis, 2011). In this study, we also found that both protein and carbohydrate were significantly reduced under octanoic acid treatment compared to CK. These results suggested that octanoic acid may act as a "dissolving agent" to attack nematodes. Furthermore, it is consistent with the microscopic studies revealed structural alterations in the cuticle of nematodes, genes related to the synthesis and development constituent of collagen and cuticle were also down-regulated in all treatments in comparison with CK (Supporting information 4, Fig. S6).

It is worthwhile to note that there is a lack of previous studies on the effect of nematicidal substances by transcriptome method against root knot nematodes although the whole genome sequencing of *M. incognita* was completed as far back as 2008 (Abad et al., 2008). Alternatively, researchers mostly use the model nematode *Caenorhabditis elegans* to study host-pathogen interactions as it has the advantages of simple growth requirements and short generation time (Brenner, 1974; Kaletta and Hengartner, 2006). However, the degree of correlation between saprophytic nematode and plant parasitic nematode are unknown which leads to the limitations of the research results. In this study, transcriptomic profiling was performed to observe the effects of octanoic acid on the root-knot nematodes *M. incognita*.

It is important to note that our results indicate that DEGs related to lysosomes were significantly down-regulated in all treatments when compared to CK (Supporting information 4, Fig. S7), and the inhibition of lysosomes demonstrated that it is one of the main targets of octanoic acid. For example, the expression level of lysosomal proteases encoding genes, including *cpr-1*, *cpr-4*, *cpr-5* and *cpr-6*, were significantly down-regulated in HC group. As an important dynamic organelle, lysosomes are responsible for macromolecule degradation and catabolite recycling, and it also act as centers of degradation, recycling, and signaling, lysosomes play a crucial role in various fundamental processes that maintain cell and tissue homeostasis (Lawrence and Zoncu, 2019; Carmona-Gutierrez et al., 2016). As the KEGG enrichment analysis shown, multiple signaling pathways was down-regulated in LC treatment group, meanwhile, therefore, it seems that octanoic acid can affect the signal transmission of *M. incognita* by inhibiting lysosomal activity. Previous studies have shown that lysosome function is essential for lifespan extension in *C. elegans*, and the maintenance of lysosome activity and dynamics may promote degradation of lipids, misfolded proteins and damaged organelles (Sun et al., 2020). In *C. elegans*, the aging process is subjected to regulation like other biological processes, one such pathway is the insulin/IGF-1 signaling pathway and extends the lifespan of nematodes when weakened (Kenyon et al., 1993; Anisimov and Bartke, 2013). In our research, we found the insulin signaling pathway as well as insulin secretion were up-regulated with the time in HC treatment group as shown in supporting information 4, Fig. S4 (a, e), and this could means that the inhibition of lysosomal activity leads to the enhancement of this signal pathway, which further affects the lifespan of nematodes longevity. Furthermore, it was shown that the survival of *Meloidogyne incognita* J2s outside a host plant is dependent on lysosome-mediated lipolysis (Lu et al., 2022). Consistently, we founded that DEGs related to lipid metabolism were significantly down-regulated in the HC group, and it appears that high concentration of octanoic acid could inhibit lipid metabolism in nematodes by suppressing lysosomal activity. In contrast, although lysosomal activity was also inhibited in the LC group, the lipid metabolism pathways were significantly up-regulated compared to CK. This may be because the stress of low concentrations of octanoic acid is relatively benign, so nematodes can respond by up-regulating lipid metabolism in a resistant manner.

Another characteristic effect of octanoic acid on nematodes in the present study was that genes associated with the extracellular region and space were significantly down-regulated in all HC and LC treatments. The extracellular region and space were involved in the secretion process of the myriad enzymes, which is consistent with the observation that the expression of multiple proteins was suppressed during the octanoic acid infection (Tani et al., 2007). On the other hand, in previous studies, researchers found that alarmin, a danger signal, was released into the extracellular space upon cell damage or death to recruit and activate resposive immune cells (Dziki et al., 2018). Combined with the above details, we infer that the extracellular region and space were associated with protein translation processes and the immune system in nematode, and that the down-regulation of these two terms implied the infection of it.

The lethal process caused by active substances is usually associated with changes in the activity of certain enzymes in the host (Wu et al., 2013). In the present study, we examined changes in the activity of four enzymes in nematodes under octanoic acid stress and further analyzed the correlation with the expression of related genes in the transcriptome. Acetylcholinesterase (AChE) is an important enzyme in the nervous system that catalyzed the hydrolysis of the neurotransmitter acetylcholine (ACh) to terminate nerve impulses, which once inhibited leads to paralysis and death due to the excessive accumulation of Ach (Li et al., 2016). AChE is the target of many widely used pesticides, such as organophosphorus and carbamate compounds (Fournier and Mutero, 1994). In this study, although lack of significant correlation between transcriptome data and enzyme activity measurements, the results of acetylcholinesterase inhibition in *Meloidogyne incognita* point to AchE as a potential target for octanoic acid. This is similar to reports indicating that C6, C9, C10, and C12 *2E*-alkenals can lead to 65% of AchE inhibition in pine wood nematodes (*Bursaphelenchus xylophilus*) (Kang et al., 2013). As previously reported, nematodes have three-stage defense, with metabolic enzymes expressed primarily in stages I and II to facilitate biotransformation of xenobiotics and excretion (Hartman et al., 2021). Three classes of genes (CYPs, UGTs, and GSTs) that may be involved in detoxification were particularly up-regulated in the LC group (Supporting information 4, Fig. S8). There are three major classes of reactions in the first phase: oxidation, reduction, and hydrolysis, with oxidation being the most common reaction and cytochrome P450 enzymes playing a central role. UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), sulfotransferases (SULTs), and *N*-acetyltransferases (NATs) are the four major families of enzymes in the secondphase. When confronted with the long-time threat of octanoic acid, GSTs and UGTs were activated and become part of the defense mechanism for detoxification. To gain more insight into the expression patterns of these defensive enzymes under different treatments, we compared their DEGs. All three kinds of defensive enzymes were significantly expressed in LC group, with processing time prolonged, more enzymes were responded and enriched. In LC2 treatment, up-regulated DEGs of CYP, UGT, and GST can high up to 29, 12 and 14, respectively. Menawhile, similiar as glutathione S-transferase (GST), carboxylesterase (CarE) are another important detoxification enzymes to degrade toxic substances (Hemingway, 2000; Zhang et al., 2011). In this study, the GST activity of nematodes in LC group was significantly increased, and this may be an adaptive response to low concentrations of octanoic acid stress, however, it decreases at high concentrations. Conversely, CarE activity was significantly increased with the prolongation of treatment time in HC group, but gradually inhibited in LC group. It seems that the nematodes initiate different defensive responses to the different concentrations of octanoic acid stress. Furthermore, transcriptome analysis indicates that octanoic acid exerted different effects on the nematode resistance and immune system, and most genes were up-regulated, especially in LC group. Meanwhile, it has been reported that the up-regulation of ABC transporter genes in animal- and

plant-parasitic nematodes is associated with drug resistance (Kooliyottil et al., 2020). In total, 32 multi-drug resistance (MDR) protein were identified and 20 of them were differently regulated. When compared to control, except one down-regulated MDR in HC2 treatment, all the other differently regulated MDRs were showed the same rising trend in LC group, with processing time prolonged, more MDRs were up-regulated and their expression level was higher (Supporting information 4, Fig. S8).

Based on the above results, we proposed a hypothetical model of the effect of the octanoic acid on *Meloidogyne incognita* J2s. Briefly, octanoic acid can directly affect the structural stability of nematodes, destroy the cuticle and internal organs of nematodes. Upon a molecular level, octanoic acid adversely affects lifespan, energy and signal transduction systems in *Meloidogyne incognita* J2s, which appear to be related to lysosomes. Meanwhile, nematodes can resist the damage by up regulating their own defense system and heterologous metabolic pathway when responding to low concentrations of octanoic acid. However, this hypothetical model is still incomplete, and more studies need to be implemented in the future, such as subcellular analyses of different organelles, or analysis of pathological changes at the tissue and cellular level by lectron microscopy or other microscopes with high resolution and magnification.

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Authorship contribution statement

All authors contributed to the study conception and design. Experiments designed, material preparation, data collection and analysis were performed by Jian-Yu Wang and Qiu-Yue Li. Jian-Ping Qu, Li Ren and Zheng Gao performed transcriptomic analysis. Cheng Guo helped perform the experiment. The first draft of the manuscript was written by Jian-Yu Wang and Hui-Fang Wang. Qian Zhang and Bo Zhou contributed to the conception of the study. All authors commented on previous version of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2023.105432.

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