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Promoting Effect of Choline-Phosphate Cytidylyltransferase Gene (*pcyt-1*) on Departure of Pinewood Nematode from *Monochamus alternatus*

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Abstract: In order to study the key gene in internal causes of pinewood nematode (PWN), *Bursaphelenchus xylophilus*, a departure from its vector beetle, *Monochamus alternatus*, we collected PWNs extracted from newly emerged *M. alternatus* and beetles 7 days after emergence. The total RNAs of the two groups of PWNs were extracted, transcriptomes sequencing was performed, and gene expression differences between the two groups of PWN were analyzed. It was found that the expression of the choline-phosphate cytidylyltransferase gene (*pcyt-1*) was markedly up-regulated. After inhibition of *pcyt-1* expression by RNA interference, the rate of lipid degradation in PWN decreased significantly, and the motility of PWN also decreased significantly. The analysis identified that phosphatidylcholine could promote the emulsification and degradation of neutral lipid granules in PWN, which provides sufficient energy for PWN departure from *M. alternatus*. The up-regulation of the gene *pcyt-1* is an important internal factor for PWN departure from its vector.

Keywords: departure; emulsification; lipid; motility; pinewood nematode

1. Introduction

Pine wilt disease (PWD) constitutes one of the most serious conifer diseases worldwide, affecting *Pinus* spp. from the Far East forestlands (Japan, China, Korea) [1,2], and to Europe (Portugal and Spain) and North America [3–7]. PWD is caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, with PWN transmission being dependent on a vector insect, such as *Monochamus alternatus* Hope, the main vector in East Asia [8,9]. The feeding period of adult *M. alternatus* is an important stage in the life cycle of the beetles as well as a key step in PWN transmission [10,11]. After emergence, adult *M. alternatus* feed on healthy pine trees, a process accompanied by the transmission of PWN.

PWN has two developmental forms in its life cycle, namely the propagative and dispersive forms. Under favorable conditions, PWN molt into their propagative form and then reproduce rapidly [12,13]. However, under unfavorable conditions—e.g., high temperature, starvation, or high population density—PWN (as propagative second-stage juveniles) will molt to produce the dispersive third-stage juveniles, which will aggregate around the pupal chamber of the beetles [14,15]. The dispersal third-stage juveniles molt to produce fourth-stage juveniles, which enter the tracheal system of the vector as the vector beetles emerge [14–17]. The fourth-stage juveniles then depart from the beetle and to be transmitted to healthy pine trees through feeding or ovipositing wounds caused by the vector [18–21].

At present, there is no unified view on the mechanism of PWN departure from the vector. However, it has been found that some volatile chemicals play an important role in

the process of PWN departure from the vector. It has been reported that the monoterpenes released from healthy pine trees, such as *β*-myrcene and *α*-pinene, have the strongest attraction to PWN, these chemicals playing an important role in the process of PWN departure from vector and invasion of healthy host trees as well as in the movement of PWN within pine trees [22,23]. Ishkawa et al. found that *β*-myrcene on agar plate held an obvious attraction for dispersive third-stage juveniles [24]. Enda et al. dried *Pinus* twigs at 70 °C to remove volatile substances, then treated twigs with *β*-myrcene and fed them to the beetles, these twigs greatly promoted the departure of PWN from *M. alternatus* [25]. Stamps et al. showed that various volatile pine chemicals did not significantly affect PWN departure from its vector and the departure of PWN is a spontaneous act [26]. Aikawa et al. fed beetles with fresh pine twigs and twigs treated with high temperature (121 °C, 40 min, to remove volatiles from the pine twigs), respectively, and found that the pine twigs without volatile chemicals were more likely to cause PWN departure from vector, whereas twigs with volatiles had inhibitory effects [27].

The departure of PWN may depend on the nematode's own factors, environmental factors, or a combination of the two. Stamps et al. suggested that the level of neutral lipids in PWN may determine whether they depart from the vector [21]. In PWN with low concentrations of neutral storage lipids, departure was induced by the pine volatile *β*-myrcene, whereas PWN with high concentrations of neutral lipids continued to be retained within the vector [21]. Previous studies showed that intrinsic factors play an important role in regulating PWN departure, whereas *β*-myrcene is simply a directional signal that promotes PWN escape from vector insects [26]. Wang et al. suggested there was no difference in the time of PWN departure from *M. alternatus* which between fed after starvation (Starvation treatment for 4 d, no volatile irritation.) and fed directly after emergence, and the motility of PWN carried by beetles 1 week after emergence was greater than that of PWN carried by newly emerged beetles (almost resting) [28]. It was suggested that PWN departure is related to the motility but not associated volatiles and that increased motility required energy, which mainly comes from the degradation of neutral lipids [21,28]. Because dispersive PWN accumulates a large amount of neutral lipids in their body and depends on the energy stored in lipids for existence [29].

PWN departure from vector beetle requires sufficient energy to provide sufficient power to overcome the resistance of the beetle trachea [30]. Production of sufficient energy requires a large amount of lipid granules (the lipid form in PWN) to be degraded. The emulsification of lipid granules can increase the contact area between lipases and lipids, which greatly promotes lipid degradation [31]. Therefore, the emulsification of lipid granules is very important for PWN to escape from vector insects. In the present study, transcriptome sequencing was performed on PWN carried by newly emerged *M. alternatus* and beetles 1 week after their emergence. Important genes related to lipid emulsification metabolism were screened, RNA interference (RNAi) was carried out, and the effect on lipid degradation and PWN motility was determined.

2. Materials and Methods

2.1. PWN Collection and RNA Extraction

In April 2019, dead specimens of *Pinus massoniana* trees infested by *M. alternatus* larvae and PWN were collected at Bocun Forest Farm, Huangshan City, Anhui Province, China. The trees were cut into logs and maintained in outdoor insect cages (Figure 1A). *M. alternatus* were collected daily (every 8 h) during the period of adult beetles emergence. The collected *M. alternatus* were divided into two groups, from one group, PWN was extracted immediately using the Baermann funnel method [32], whereas another was fed (with fresh pine twigs) for 7 days (Figure 1B) before the PWN was extracted. Extraction of total RNAs from the two groups of PWN (each group was represented by three independent biological replicates) was performed using the Trizol method [33].
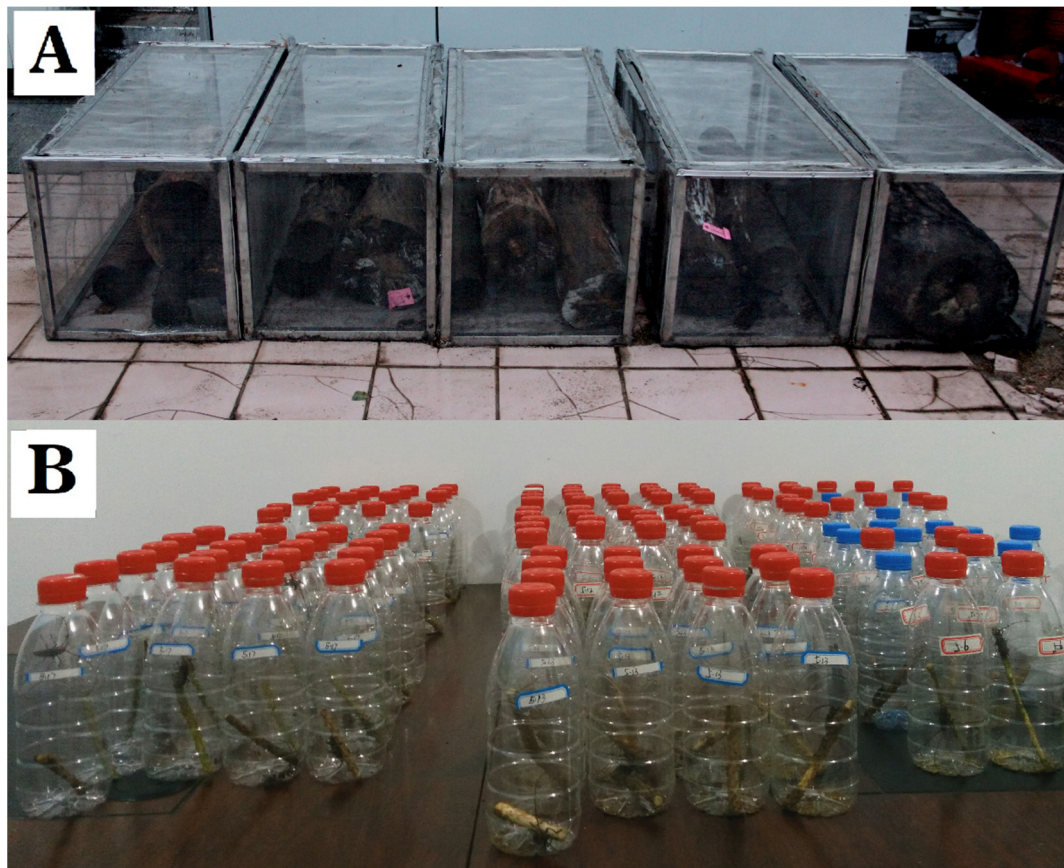
Figure 1. Collection and feeding of *Monochamus alternatus.* (**A**): Pine trees were cut into logs and maintained in outdoor insect cages, *Monochamus alternatus* were collected daily (every 8 h) during the period of adult beetle emergence. (**B**): The collected *Monochamus alternatus* were put into small bottles and fed with fresh twigs of pine.

2.2. Transcriptome Sequencing (Biomarker Technologies, Beijing, China)

2.2.1. RNA Quantification and Qualification

RNA degradation and contamination were monitored on 1% agarose gels. RNA purity and concentration were measured using NanoPhotometer® spectrophotometer (IMPLEN, Rohnert Park, CA, USA) and Qubit® 2.0 Flurometer (Life Technologies, Rohnert Park, CA, USA) respectively. RNA Nano 6000 assay kit was used to evaluate the integrity of RNA.

2.2.2. Clustering and Sequencing

A total of 1 μg RNA was used as input material per sample. The NEBNext®UltraTM RNA Library Prep Kit was used to generate Sequencing libraries and added index codes to the attribute sequence.

The TruSeq PE Cluster Kit v4-cBot-HS (Illumia) was performed to cluster the index-coded samples. After that, the Illumina Hiseq x-ten platform was used to sequence library preparations and generate paired-end reads.

2.2.3. Quality Control

The raw reads of fastq format were processed through an internal script. In this step, Delete reads containing ploy-N and adapter, and low-quality reads from raw reads to obtain clean data. High-quality clean reads were the basis of all the downstream analyses.

2.2.4. Comparative Analysis and Gene Functional Annotation

Clean data were mapped to the reference genome of PWN (Bursaphelenchus xylophilus (ID 11822)-Genome-NCBI (nih.gov)). The data with a perfect match or only one

mismatch was analyzed and annotated. The tool software HISAT2 was used to map with a reference genome. The functions of genes were annotated based on these databases: Nr, Nt, KOG/COG, Swiss-Prot, KO, GO.

2.2.5. Differential Expression Analysis

The DESeq R package (1.10.1) was used to analyze differential expressions of the two PWN groups. DESeq R package provides statistical routines for determining the differential expressions in digital gene expression data using a model based on the negative binomial distribution. Resulting p-value was modified by Benjamini and Hochberg's control of the false discovery rate (FDR). Gene with a modified p-value < 0.05 founded by DESeq was identified as differentially expressed.

2.2.6. Pathway Enrichment Analysis

The KEGG database was used to identify biological pathways which were enriched in gene lists more than would be expected by accident. The software KOBAS [34] was used to test the degree of statistical enrichment of differentially expressed genes (DEGs) in the KEGG pathway.

Based on the sequencing results, highly expressed differentially genes related to lipids emulsification were screened.

### 2.3. RNA Interference (RNAi) and Quantitative Real-Time PCR (qPCR)

To interfere with the choline-phosphate cytidylyltransferase gene (*pcyt-1*) (DNA sequence: Supplementary Material S1), the interfering gene fragment is 5′-GGTGGACTTTAT CGCTCAT-3′ and negative control sequence is 5′-GGTGGGCTTTAACGCTCAT-3′. To prepare the double-stranded RNA (dsRNA), which was digested by ribozyme to obtain the small interfering RNA (siRNA) (dissolved in 20 μL of RNAase-free water), the in vitro Transcription T7 Kit (for siRNA Synthesis) (Takara Bio Inc., Shiga, Japan) was used [35].

PWN was cultured using the *Botrytis cinerea* (aging *B. cinerea* is not conducive to PWN growth) [36] as shown in Figure 2D. After 48 h of culture, PWN was extracted for no more than 2 h using the Baermann funnel method [32]. Extracted PWN was soaked in 0.05% (w/v) streptomycin sulfate for 10 min and washed three times with diethyl pyrocarbonate (DEPC)-treated water. PWN (10,000 individuals) were soaked in the siRNA solution for 48 h (Figure 2E), then washed three times with RNAase-free water. The total RNAs (one preparation per replicate per group) were extracted by the Trizol method [33]. Synthesis of cDNA was carried out using HiScript® II Q RT SuperMIX for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). qPCR was carried out with SYBR® Green I chimeric fluorescence, using ChamQ™ SYBR® qPCR Master Mix (Low ROX Primixed) (Vazyme, Nanjing, China). Then q-PCR validation was performed using the 7500 Real-Time PCR System (SeqGen, Inc., Torrance, CA, USA) and analyzed differences in gene expression. The reference gene primers were F: 5′-GCAACACGGAGTTCGTTGTA-3′, R: 5′-GTATCGTCACCAACTGGGAT-3′. Target primer: F: 5′-AGGCCTACAACAACTCAGCC-3′, R: 5′-TCAACTGATTCGCGTGTCCA-3′.

2.4. Comparison of Difference in Motility and Lipid Content of PWN after pcyt-1 RNAi

After 48 h of RNAi, differences of PWN motility were observed, with videos being taken under a microscope (Zeiss Axio lmager.M2, Carl Zeiss, Gottingen, Germany). After 48 h of RNAi, the lipids of PWN were stained with oil red O [37]. PWN was washed with M9 buffer in a 1.5-mL Eppendorf tube and allowed to stand for 10 min at 4 °C, then the supernatant was discarded and the washing was repeated once. The lipids of PWN were fixed with 4% paraformaldehyde at 4 °C for 30 min, frozen at −80 °C refrigerator for 15 min, then rapidly thawed in a 43 °C water bath. The sample was centrifuged for 1 min with 2000× g, and the supernatant was discarded. The sample was washed three times with 1× phosphate-buffered saline (PBS) (pH 7.2–7.4), after which the PBS was discarded, and soaked in 1% Triton X-100 and isopropanol oil red O saturated solution (ratio 2:3) for 30 min. The sample was washed three more times with PBS (1×), clearly differentiated

under the microscope using 60% isopropanol, and photographed (Zeiss Axio lmager.M2, Carl Zeiss, Gottingen, Germany).
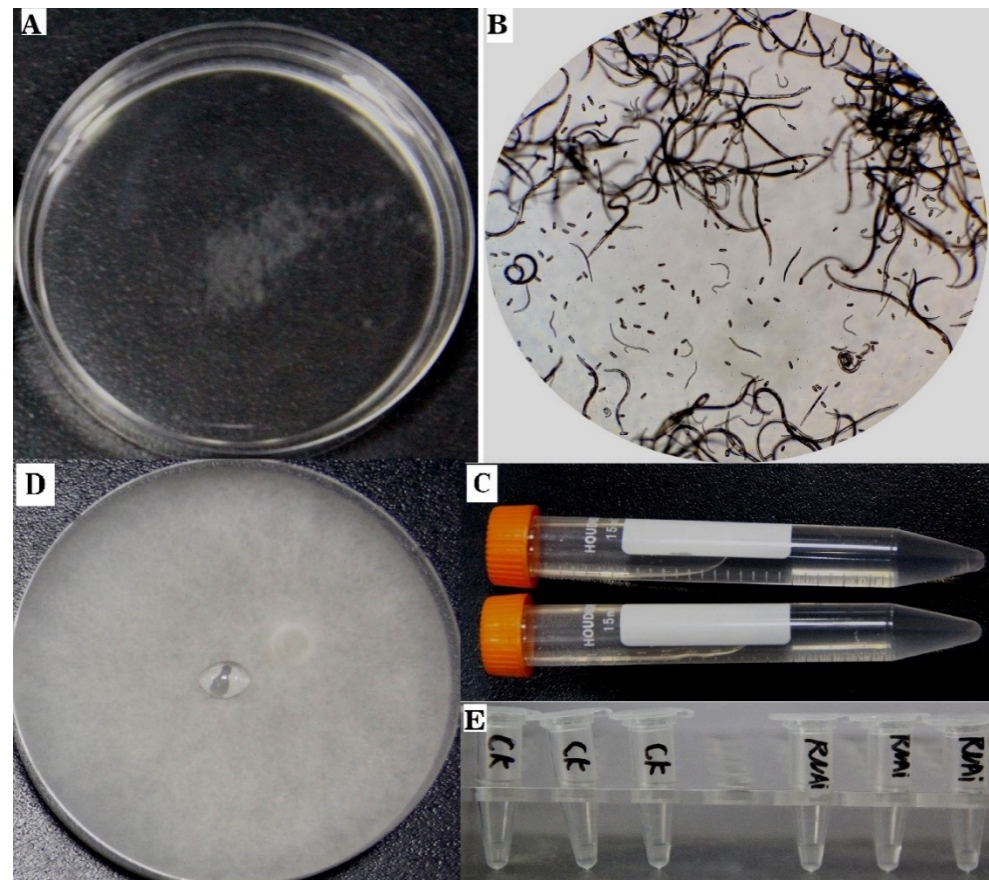
Figure 2. PWN culture and RNAi. (**A**): The PWN suspension was placed in a dish, and the eggs adhering to the bottom of the dish were collected every 6 h. (**B**): Eggs adhering to the bottom of the dish. (**C**): The collected PWN eggs were placed in an Eppendorf tube and incubated at 25 °C for 36 h. All the eggs hatched into second-stage PWN. (**D**): The second-stage PWN juvenile suspension was inoculated into the freshly grown *Botrytis cinerea* medium and cultured for 48 h (when the majority of nematodes were 4th stage juveniles). (**E**): RNAi was performed on PWN at 25 °C (immersion method).

3. Results

3.1. Comparison of Transcriptome Results

In the present study, 2950 DEGs were identified, of which 1531 were up-regulated.

The most enriched KEGG pathway is oxidative phosphorylation (Figure 3). There were 36 DEGs in this pathway, of which 35 were up-regulated. This indicates that there was a high level of oxidative phosphorylation process in PWN carried by *M. alternatus* 7 d after emergence. This requires a large amount of lipid degradation, as lipids are the energy storage material of PWN. In PWN, lipids exist in the form of lipid granules, which require emulsification to accelerate degradation.

According to the sequencing results, the choline-phosphate cytidylyltransferase gene (*pcyt-1*) was closely related to lipid catabolism. The expression level of *pcyt-1* in PWN carried by *M. alternatus* 7 d after emergence was significantly up-regulated (log2 FC = 9.5) relative to PWN from newly emerged beetles, as shown in Supplementary Material S2 (Ko00564, EC:2.7.7.15). This gene controls the production of phosphatidylcholine (PC), which acts as lipid emulsification in mammals [31]. PC is a powerful detergent and promotes lipid degradation [31]. Whether it has similar effects in PWN was further verified in this study.
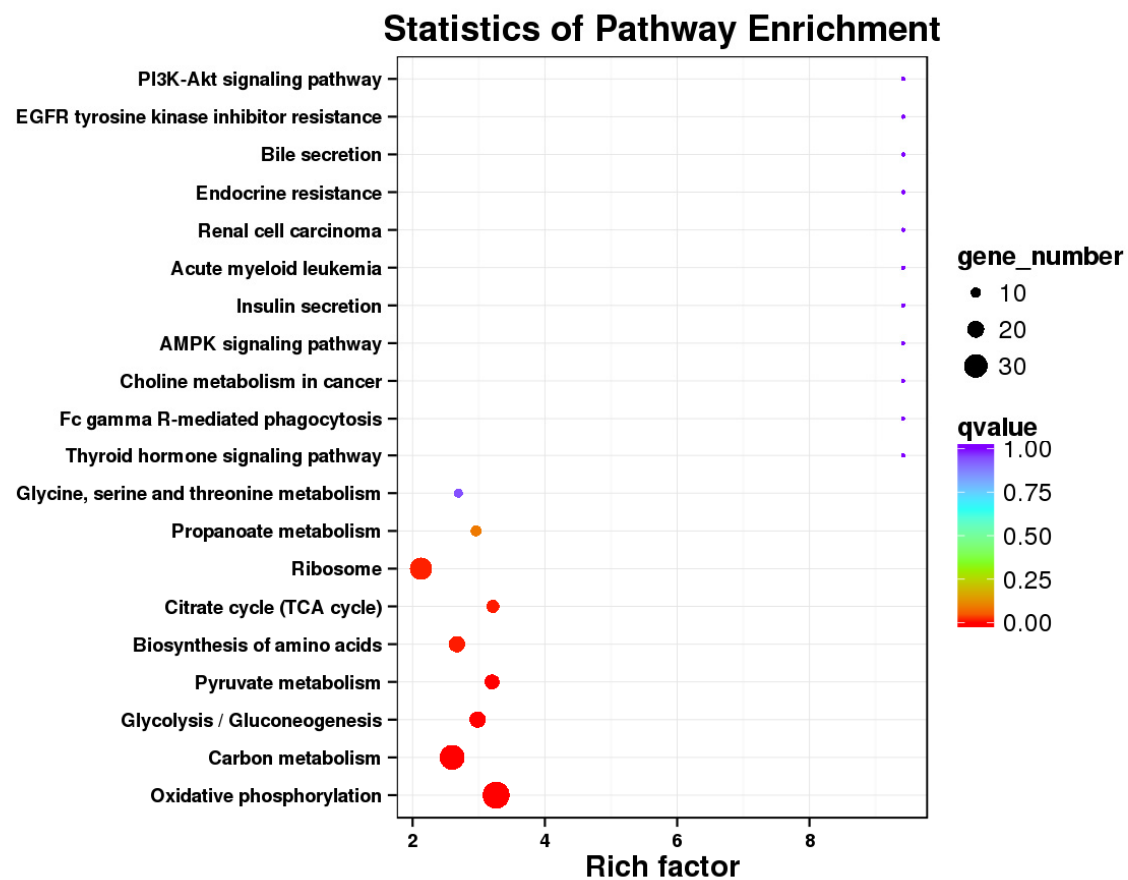
Statistics of Pathway Enrichment

Figure content showing scatter diagram with the following y-axis pathway labels (top to bottom):
PI3K-Akt signaling pathway, EGFR tyrosine kinase inhibitor resistance, Bile secretion, Endocrine resistance, Renal cell carcinoma, Acute myeloid leukemia, Insulin secretion, AMPK signaling pathway, Choline metabolism in cancer, Fc gamma R-mediated phagocytosis, Thyroid hormone signaling pathway, Glycine, serine and threonine metabolism, Propanoate metabolism, Ribosome, Citrate cycle (TCA cycle), Biosynthesis of amino acids, Pyruvate metabolism, Glycolysis / Gluconeogenesis, Carbon metabolism, Oxidative phosphorylation

x-axis: Rich factor (2, 4, 6, 8)

Legend: gene_number (10, 20, 30); qvalue (1.00, 0.75, 0.50, 0.25, 0.00)

Figure 3. Scatter diagram of enrichment of DEGs KEGG pathways.

3.2. qPCR Gene Expression Studies

After *pcyt-1* RNAi was carried out in PWN, qPCR was used for verifying the effect of gene interference. The results shown in Figure 4 indicate that the expression of *pcyt-1* was significantly down-regulated after RNAi.
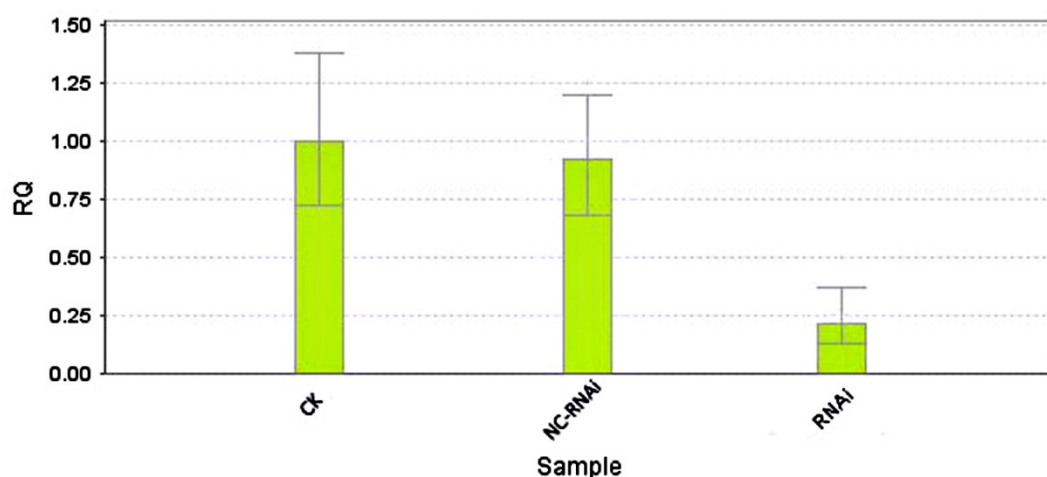
Bar chart with y-axis labeled RQ (0.00 to 1.50) and x-axis labeled Sample with categories CK, NC-RNAi, RNAi.

Figure 4. Relative expression of *pcyt-1* in control pinewood nematodes (PWN; CK), PWN exposed to RNA interference (RNAi), and negative control (NC-RNAi).

*3.3. Motility and Lipid Content of PWN*

After RNAi, the motility of PWN decreased significantly, as shown in Supplementary Material S3 (Video S1: RNAi group, Video S2: Control group). After RNAi, the lipid

particles were still arranged regularly and no obvious degradation, but obvious degradation had occurred in the control group (Figure 5). At the same time, it was found that the morphology of the control group changed and developed towards adulthood, whereas the experimental group was still at the 4th-stage juvenile phase. GO enrichment results also showed that growth/developmental process terms were highly enriched (Figure 6).
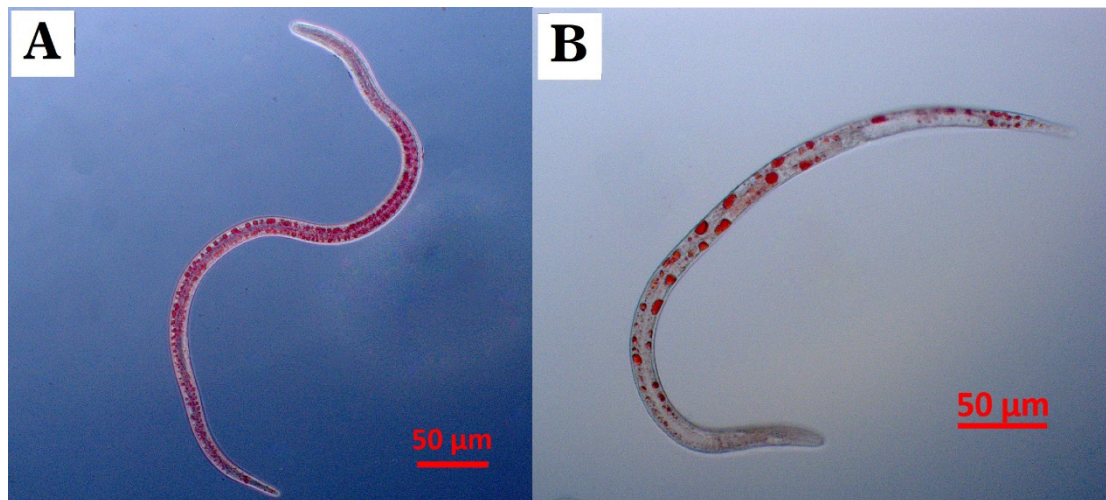
Figure 5. Pinewood nematode lipid content was shown by staining with oil red O in the presence and absence of *pcyt-1* RNA interference (RNAi). Red spots indicate lipid drops. (**A**): PWN after RNAi for 48 h. (**B**): Control nematodes after culture 48 h. many lipid droplets were still present in PWN in the experimental group, whereas the number of lipid droplets in the control group was markedly reduced.
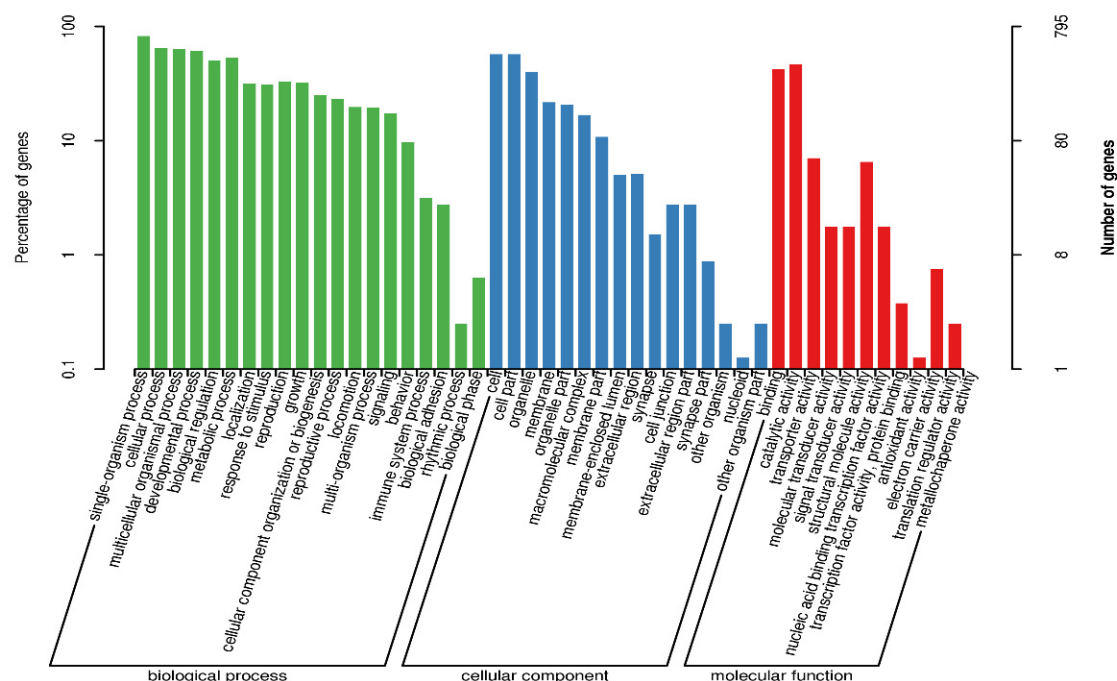
Figure 6. Statistical map of GO annotation classification of Up-regulate DEGs.

4. Discussion

PWN does not immediately depart from vector beetle after emergence but starts to depart after a period of time. There have been reports that PWN starts to depart from *M. alternatus* at different times, such as 7–12 d after the emergence of beetles [38], 3–5 d after

adult beetle emergence [39], at 7 d after emergence [40], at 5 d after emergence [28,41], and at 10 d after emergence [42]. During the period between the emergence of *M. alternatus* and the start of the departure of PWN, the physiological characteristics of PWN may change, or they may be continuously stimulated by certain signaling chemicals. Different studies have reached different conclusions on the causes of PWN departure from *M. alternatus*.

Ishkawa et al. examined the attractant effect of volatile chemicals from *Pinus densiflora* on the dispersive PWN that leave *M. alternatus* and showed that *β*-myrcene played an important role in the transmigration of PWN from the vector to the pine tree [24]. To clarify the effect of *P. densiflora* volatiles on the departure of PWN from *M. alternatus*, Aikawa et al. fed *M. alternatus* with normal twigs and with twigs without volatiles and consequently suggested that PWN have a trait of spontaneous departure from *M. alternatus* [27]. Stamps et al. found that lipid content of PWN gradually decreased with the time after the emergence of *M. alternatus* and suggested that the concentrations of neutral storage lipids were correlated with PWN leaving their vector [19,21].

In studies on the effect of *M. alternatus* feeding behavior on PWN departure, Wang et al. found that there was no significant difference in the start time of PWN departure from *M. alternatus* following direct feeding or feeding after a starvation period following emergence [28]. However, it was found that PWN carried by newly emerged beetles was less motility than carried by beetles 7 d after emergence, and believed that increased motility is an important cause of PWN departure [28]. Increased motility requires a large amount of energy (in the form of ATP), and the degradation of neutral storage lipid droplets in PWN can release energy [21]. Therefore, the degradation of lipids may be an important endogenous cause of PWN departure. In the present study, we also found that the lipid droplets content of PWN was markedly higher after *pcyt-1* RNAi than the control, whereas the motility of PWN in the RNAi-treated group was markedly lower than that of those in the control group. This indicates that lipid degradation is a necessary pre-condition for PWN departure from *M. alternatus*.

Not all PWN carried by *M. alternatus* can leave their vector beetles, with about 70% of PWN failing to leave the vector [38]. Aikawa et al. showed that the percentage of PWN departure from the vector was higher in thick tracheae than in thin tracheae [30] suggesting that PWN in the thin trachea cannot get enough oxygen to release enough energy (ATP) to power their exit, eventually causing them to stay in the trachea for long periods [30]. After the emergence of *M. alternatus*, the energy resource (represented by limited lipid stores) of PWN carried by beetles is gradually exhausted [21]; at this time, even if sufficient oxygen was supplied, it would not generate enough energy to drive PWN motility and leave the vector. Wang et al. showed that greater motility was necessary for PWN to depart from the vector [28]. Sufficient oxygen supply over a limited period is vital for PWN to degrade lipids and produce enough energy (ATP) for the high motility levels required for departure.

Lipid granules (triacylglycerol) are the main energy store in PWN, with lipid being insoluble in water, whereas the enzymes that digest lipids are water-soluble; therefore, lipid digestion occurs only at the lipid–water interface [31]. The emulsification of lipid granules increases the lipid-water interface, which greatly accelerates digestion. Phosphatidylcholine (PC) has both hydrophilic and hydrophobic groups, making it a powerful "detergent" conducive to lipid emulsification and promoting lipid catabolism [31,43–45]. Choline-phosphate cytidylyltransferase (CCT) has high specificity and is the rate-limiting enzyme in phosphatidylcholine synthesis, controlling the synthesis of phosphatidylcholine [46–48]. In the present study, the results of transcriptome sequencing revealed that *pcyt-1* expression was significantly up-regulated in PWN in beetles 7 d after emergence relative to PWN extracted from beetles immediately after emergence (Supplementary Material S2: ko00564, EC: 2.7.7.15; Supplementary Material S3).

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