Astaxanthin accumulation difference between non-motile cells and akinetes of *Haematococcus pluvialis* was affected by pyruvate metabolism

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**Abstract**

Background: *Haematococcus pluvialis* is the best source of natural astaxanthin, known as the king of antioxidants. *H. pluvialis* have four cell forms: spore, motile cell, non-motile cell and akinete. Spores and motile cells are susceptible to photoinhibition and would die under photoinduction conditions. Photoinduction using non-motile cells as seeds could result in a higher astaxanthin production than that using akinetes. However, the mechanism of this phenomenon has not been clarified.

Results: Transcriptome was sequenced and annotated to illustrate the mechanism of this phenomenon. All differentially expressed genes involved in astaxanthin biosynthesis were up-regulated. Particularly, chyb gene was up-regulated by 16-fold, improving the conversion of \( \beta \)-carotene into astaxanthin. Pyruvate was the precursor of carotenoids biosynthesis. Pyruvate kinase gene expression level was increased by 2.0-fold at the early stage of akinetes formation. More changes of gene transcription occurred at the early stage of akinetes formation, 52.7% and 51.9% of total DEGs in control group and treatment group, respectively.

Conclusions: Genes transcription network was constructed and the synthesis mechanism of astaxanthin was clarified. The results are expected to further guide the in-depth optimization of the astaxanthin production process in *H. pluvialis* by improving pyruvate metabolism.

**Keywords:** Astaxanthin, *Haematococcus pluvialis*, Pyruvate, Transcriptome

**Background**

Astaxanthin is regarded as the best biological antioxidant. Its antioxidant activity is 10, 65, 100 and 550 times of \( \beta \)-carotene, vitamin C, \( \alpha \)-tocopherol, and vitamin E, respectively (García-Malea et al. 2006; Olaizola 2000; Ranjbar et al. 2008; Zhang et al. 2009). As the best source of natural astaxanthin, *Haematococcus pluvialis* could accumulate 4–5% astaxanthin of dry weight under certain conditions (Wan et al. 2015). Industrial production of *H. pluvialis* was successfully achieved by a two-stage model. First stage is cell proliferation phase (also called the growth phase), in which the algae cells grow rapidly until to a high cell density. The second stage is photoinduction stage, aiming to promote the accumulation of astaxanthin in *H. pluvialis* (He et al. 2007; Lorenz and Cysewski 2000).

Previous studies mainly focused on the optimization of the second stage to improve astaxanthin accumulation ability. Temperature (Wan et al. 2014b), strong light (Lv et al. 2016), high salinity (Sarada et al. 2002), plant hormone (Gao et al. 2012a), nitrogen deprivation (Wang et al. 2013), oxygen stress (Gu et al. 2013), metal ion stress (Yu et al. 2015) and ethanol (Wen et al. 2015) were reported in the second stage to promote *H. pluvialis*
accumulate astaxanthin. Li et al. (2019a) and Choi et al. (2011) have shown that the appropriate cell type for photoinduction was the non-motile cell, however, there was no article has interpreted this mechanism.

Transcriptome sequencing was regarded as an efficient approach for exploring the mechanism of astaxanthin accumulation. The gene transcription changes of H. pluvialis have been discussed in many studies, e.g., high light intensity could increase the activity of β-carotene keto-lase (BKT) and IPP isomerase (Linden 1999); astaxanthin synthesis-related genes were significantly up-regulated in H. pluvialis mutant under 15% CO2 (Li et al. 2017); the astaxanthin accumulation ability of H. pluvialis could be improved by gibberellin (GA3), accompanied by increasing the transcription of ipi (isopentenyl pyrophosphate isomerase), psy (phytoene synthase), pds (phytoene desaturase) and bkt genes (Gao et al. 2013). However, these researches were mainly focused on the regulation mechanism of astaxanthin synthesis responding to various stresses, e.g., light, salinity, temperature, hormone, iron and inorganic carbon (Lee et al. 2016; Wen et al. 2015).

The direct precursor of fatty acid biosynthesis is acetyl-CoA (Shtaida et al. 2015). Acetyl-CoA carboxylase (ACACA) can enhance the carboxylation of acetyl-CoA to malonyl-CoA (Shtaida et al. 2015). This step was considered a critical step in the lipid biosynthetic pathway (Huerlimann and Heimann 2013). Lipid can be used for the esterification of astaxanthin (Karsten et al. 2009; Schoefs et al. 2001). Pyruvate metabolism plays an important role in glycolysis to form acetyl-CoA. Cheng et al. (2017) reported that most significant differences were found in unigenes related to photosynthesis, carotenoid biosynthesis and fatty acid biosynthesis pathways when photoinduction with high light under 15% CO2. Above studies indicated astaxanthin synthesis mechanism is complicated and has not been systematically clarified.

To understand the molecular mechanism of astaxanthin synthesis and to dissect the mechanism that photoinduction using non-motile cells as seeds could result in a higher astaxanthin production than that of using akinetes, transcriptome sequencing of microalgal culture process was conducted. The metabolic network between astaxanthin synthesis and pyruvate metabolism was proposed. The mechanism by which high astaxanthin production was obtained by non-motile cells as seeds was dissected. The results provide a new entry point to improve the astaxanthin productivity.

Methods
Algal strains
Haematococcus pluvialis ZY-18 was obtained from State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology (Shanghai, China).

Seed culture conditions
The basic seed medium and culture methods were the same with Hata et al. (2001). H. pluvialis cells were obtained from broths at different incubation times. H. pluvialis cells were used for the next step in photoinduction experiments.

Photoinduction culture conditions
Haematococcus pluvialis cells from cell proliferation phase were inoculated to the NIES-N medium (Kang et al. 2005) and then were turned into the photoinduction stage. The initial cell concentration was approximately 0.3 g/L in all the experiments. 1-L column bioreactors (height: 45 cm, and diameter: 7 cm) for photoinduction was a cylindrical glass tube with a conical bottom (height: 6 cm). 5% CO2 mixing was conducted by sparging air supplemented with a flow rate of 0.2 L/min. A gas sparger was centrally placed at the bottom. Throughout the experiment, the light intensity was about 540 μmol/(m2 s) (Wan et al. 2014b) and the culture temperature was controlled at 28 °C. All experiments have two biological replicates.

Measurement of dry weight
V ml broth containing algal cells was obtained by centrifuging the culture at 2683×g for 10 min, and collected into an empty tumbler (W1) after being washed twice with distilled water and then dried at 85 °C for 24 h (W2). The dry weight was calculated in terms of the equation:

\[
C_x = \frac{(W_2 - W_1)}{V} \times 1000, \tag{1}
\]

where \(C_x\) (g/L) is dry weight of broth, \(W_1\) is the weight of the empty tumbler, and \(W_2\) (g) is the weight after being dried, and \(V\) (ml) is the volume of the initial sample, respectively.

Determination of astaxanthin content
The astaxanthin content was measured by a modified Boussiba method (Borowitzka et al. 1991; Wan et al. 2014a). V ml culture sample was centrifuged for 10 min at 2683×g. 4–6 pieces of glass beads and 1 ml of dimethyl sulfoxide were added to each centrifuge tube, and subsequently were vortex oscillated for 30 s, and then heated with 45 °C water bathing for 15 min. Later, 1 ml acetone was added into the mixture solution and centrifuged for 10 min at 2683×g. Then the supernatant was collected and transferred into a volumetric flask. Above-mentioned acetone extraction and supernatant collection were conducted repeatedly until the supernatant becomes transparent and the precipitate becomes white. The absorbance values of the extracts were determined at 474 nm using acetone as reference.
The astaxanthin content was calculated in terms of the equation:

\[ C_{\text{Car}} = \frac{\text{OD}_{474} \times V_1 / V_2 \times \text{dilution ratio}}{210}, \]

\[ C_{\text{Asta content}} = \frac{C_{\text{Car}}}{C_x} \times 85, \]

where \( C_{\text{Car}} \) is the concentration of carotenoid (mg/L), \( C_x \) is the dry weight of the algal (g/L) and \( C_{\text{Asta content}} \) is the content (%) of astaxanthin, \( V_1 \) is the volume of the volumetric flask (ml), and \( V_2 \) is the volume of the initial sample (ml).

RNA isolation, cDNA library preparation, qRT-PCR and sequencing

Totally, the transcription profiling of samples at six time points were evaluated, with two biological replicates at each time point. In the proliferation stage, three samples in different time points were selected: (1) Sample 1, cells at 100 h (green motile cells accounted for the vast majority); (2) Sample 2, cells at 250 h (non-motile cells accounted for the vast majority); (3) Sample 3, cells at 550 h (brown akinetes accounted for the vast majority). In the photoinduction phase, three samples were also selected as follows: (1) Sample 4, Sample 2 cells were transferred to weak light for 0.5 day under photoinduction condition (green non-motile cells were converted to brown akinetes); (2) Sample 5, Sample 3 cells were transferred and exposed to weak light for 6 days under photoinduction condition (red akinetes accounted for the majority, astaxanthin content was high, and astaxanthin content was no longer increased); (3) Sample 6 was the Sample 4 cells continued photoinduction until the 6th day (red akinetes account for the vast majority. The astaxanthin content was high, and the astaxanthin content was no longer increased). Therefore, there are two cultivation routes named treatment group (green non-motile cells were used for photoinduction in the order: Sample 1, 2, 3 and 5) and control group (brown akinetes were used for

Transcriptome mapping, annotation, and differential transcription analysis

The software of cut-adapt was used to remove adapters, poly-N strands, and low-quality reads. Then all filtered reads were examined by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to confirm data quality. Transcript assembly used Trinity with a K-mer 25 bp. Every transcript was compared with the NCBI non-redundant protein database and eggNOG (evolutionary genealogy of genes: non-supervised Orthologous Groups) using the Blast algorithm (version 2.2.30+). Transcripts with same gi number were classified as a uni-gene and only the longest transcript was kept. After that, filtered reads were mapped to unigenes with Bowtie2 (v2.2.4) and the reads per kilobase of exon model per million (RPKM) mapped fragments was used to represent gene transcription.

Differential transcription analysis between samples was conducted using the R package, DESeq (version 1.18.0). Genes with |fold changes| > 2 and \( P \) value < 0.05 were considered as statistically significant. Functional analysis of differential transcription genes was performed by Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. GO
Fig. 2 Induction effect and images of *H. pluvialis* using different cell type as seeds. **A** Induction effect of *H. pluvialis* using different cell type as seeds. Astaxanthin content during induction process; **B** astaxanthin concentration during induction process; **C** dry weight during induction process. Hollow circle represents green non-motile cells as seeds, and solid square represents brown akinetes as seeds; **b, c** represent SEM and TEM images of control group (B, 1235 route) and treatment group (C, 1246 route), respectively. Sample 1, Sample 2 and Sample 3 were the heterotrophic cells at 100 h, 250 h, and 550 h, respectively; Sample 4 was Sample 2 cells induction for 0.5 day; Sample 5 was Sample 3 cells induction for 6 days; Sample 6 was the Sample 2 cells induction for 6 days. SEM: scanning electron microscope (scale bar = 10 μm); TEM: transmission electron microscope (scale bar = 5 μm). Data are shown as mean ± SD, and number of replications is two (*n* = 2).
analysis of each unigene was carried out using Blast2go software and KEGG enrichment analysis was performed using KASS and KEGG automatic annotation server, respectively.

**Statistical analysis**

All exposure experiments were repeated two times independently, and data were recorded as the mean with standard deviation (SD). For mRNA-sequencing were analyzed with the two biological replicates. Statistical analyses were performed using the Spearman correlation analysis (SPSS19.0). For all of the data analysis, a \( P \)-value < 0.05 was considered as statistically significant.

**Results and discussion**

The comparison of photoinduction differences

*Haematococcus pluvialis* have four cell forms—spore, motile cell, non-motile cell and akinete (Fig. 1). To compare the photoinduction result of non-motile cells group and brown akinetes group, the astaxanthin content, astaxanthin concentration and dry weight were detected. Comparing with control group, a better photoinduction result was acquired using non-motile cells as seeds. The astaxanthin content and astaxanthin concentration of non-motile cells group were 3.40% and 25.7 mg/L, respectively (Fig. 2a). The results were much higher than those in brown akinetes group. Our previous studies showed that spores and motile cells were susceptible to photoinhibition and would die under photoinduction...
conditions. Therefore, non-motile cells of heterotrophic culture are the best cell type for photoinduction. Li et al. (2019a) and Choi et al. (2011) also demonstrated that the appropriate cell type for photoinduction was the non-motile cells.

Scanning electron microscope (SEM, Fig. 2b, c) and transmission electron microscope (TEM, Fig. 2b, c) image of *H. pluvialis* were carried out during two culture stage to further explore the changes of non-motile cells and akinetes as photoinduction seeds. From the TEM images, more lipid drops were observed when using non-motile cells as seeds. Cheng reported that massive astaxanthin was esterified in the endoplasmic reticulum, and deposits in cytoplasmic lipid droplets to avoid the feedback inhibitor of carotenoids biosynthesis (Cheng et al. 2017). Thus, the result confirmed the better photoinduction result of non-motile cells.

The comparison of gene transcription differences

To have a comprehensive understanding of astaxanthin synthesis, transcriptome sequencing on microalgae sequence culture process was conducted. De novo assembly of transcriptomes revealed the genomic and transcriptional features of two culture routes (1235 route and 1246 route). A total of 0.3 billion clean reads, and 43,583 unigenes were generated, respectively. According to the standard of *p* < 0.05, 2455 upregulation and 2576 downregulation unigenes were identified in treatment group (1246 route, Fig. 3a), while 3209 upregulation and 3745 downregulation unigenes were identified in control group (1235 route, Fig. 3b). Among the differentially expressed genes, the number of upregulation genes in glycolysis declined during culture, while the number of upregulation genes in astaxanthin synthesis increased (Fig. 3c, d). The products of glycolysis can be used for astaxanthin synthesis. Therefore, the early stage of culture may be important for astaxanthin synthesis.

Transcriptome and pathway analysis involved in astaxanthin biosynthesis

The genes participated in carotenoid biosynthesis and astaxanthin synthesis have been fully studied (Lee et al. 2016; Wen et al. 2015). The conversion of geranylgeranyl-pp (GGPP) into astaxanthin synthesis is successively catalyzed by BKT, CHYB (β-carotene-3-hydroxylase), LCYB (lycopene beta cyclase), PSY and ZDS (ζ-carotene desaturase). These enzymes have been reported as essential enzymes in astaxanthin synthesis (Huang et al. 2006; Zhong et al. 2011).

The condensation of two GGPP molecules into phytoene is catalyzed by PSY. Compared with control, *psy* gene was up-regulated by 2.4-fold at the end of photoinduction (Table 1). The conversion of phytoene into β-carotene is successively catalyzed by ZDS and LCYB. The two genes were both up-regulated during photoinduction stage (Figs. 4, 5a, b). β-Carotene is the precursor of astaxanthin synthesis. The reactions catalyzed by BKT and CHYB are regarded as the rate-limiting steps. The *bkt* and *chyb* genes were both up-regulated during

| Table 1 Annotation and transcription changes of significantly different unigenes related to astaxanthin, fatty acid, pyruvate metabolic pathway and photosynthesis | KEGG | 3 vs. 4 FC | P-value | 5 vs. 6 FC | P-value |
|---|---|---|---|---|---|
| Astaxanthin biosynthesis pathway | | | | | |
| bkt | β-Carotene ketolase (EC 1.14.99.63) | K09836 | 3.8 ** | 1.9 *** |
| chyb | β-Carotene 3-hydroxylase (EC 1.14.15.24) | K15746 | 16 *** | 2.6 *** |
| lcyb | Lycopene beta cyclase (EC 5.5.1.19) | K06443 | 2.9 *** | 1.9 *** |
| psy | 15-cis-Phytene/all-trans-phytoene synthase (EC 2.5.1.32) | K02291 | 2.4 *** | 2.4 *** |
| zds | ζ-Carotene desaturase (EC 1.3.5.6) | K00514 | 2.7 *** | 2.0 *** |
| Pyruvate metabolic pathway | | | | | |
| ppc | Phosphoenolpyruvate carboxylase (EC 4.1.1.31) | K01595 | 1.0 * | 2.1 *** |
| pdhD | Dihydrolipoamide dehydrogenase (EC 1.8.1.4) | K00382 | 1.6 *** | 1.7 *** |
| PK | Pyruvate kinase (EC 2.7.1.4) | K00873 | 2.0 *** | 1.3 *** |
| fumA, fumB | Fumarate hydratase, class I (EC 4.2.1.2) | K01676 | 0.50 *** | 1.6 *** |
| MDH2 | Malate dehydrogenase (NADP-) (EC 1.1.1.22) | K00051 | 0.90 * | 1.5 *** |
| pdhC | Pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) (EC 2.3.1.12) | K00627 | 2.0 *** | 1.7 *** |
| pdhA | Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1) | K00161 | 0.50 *** | 1.1 * |
| NADP-ME | Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) (EC 1.1.1.40) | K00029 | 1.1 * | 1.8 *** |

*** indicates statistical significance at *P*-value ≤ 0.001; ** indicates statistical significance at *P*-value ≤ 0.005; * indicates statistical significance at *P*-value ≤ 0.05
photoinduction stage (Figs. 4, 5a, b). At the initial stage of akinetes formation, the gene transcription difference was much more obvious than that at the end of photoinduction compared with control (Fig. 3). The chyb gene transcription level was 16-fold up-regulated of the control at the early stage of akinete formation (Table 1). Li also showed that psy, CrtO (β-carotene oxidase) and LcyB have relatively large changes during the early stage of akinetes formation (Li et al. 2019b). The result indicated that the early stage of akinete formation may be important for astaxanthin synthesis.

From Figs. 4, 5a, b, it can be known that all genes related to astaxanthin synthesis were lowly expressed in the cell proliferation phase and highly expressed in the photoinduction stage in both routes. Nevertheless, the transcription level of genes related to astaxanthin synthesis in treatment group (1246 route) was much higher than that in control group (1235 route) (Fig. 5 and Table 1). The results explained that the appropriate cell type for photoinduction was non-motile cells. The high astaxanthin synthesis ability of non-motile cells was attributed to the high transcription level of astaxanthin synthesis-related genes.

Lycopene is the common intermediate substrate for astaxanthin and lutein synthesis. During photoinduction stage, the upregulation of lcyB gene expression was accompanied by the downregulation of lcyE gene expression (Fig. 4). This phenomenon would decrease lutein metabolism competition for the lycopene, suggesting more carbon flow to astaxanthin rather than lutein.
Fig. 5 RPKM and unigene transcription changes involved in astaxanthin biosynthesis pathways of *H. pluvialis*. a, b represent RPKM changes (a) and relative transcript level changes (b) involved in astaxanthin biosynthesis pathways of *H. pluvialis* in control group (1235 route) and treatment group (1246 route), respectively. To calculate genes transcript level, Sample 1 was regarded as control; comparing with control group (1235 route), the gene transcription changes of the treatment group (1246 route) were presented in KEGG pathway (c). Sample 1, Sample 2 and Sample 3 were the heterotrophic cells at 100 h, 250 h, and 550 h, respectively; Sample 4 was Sample 2 cells induction for 0.5 day; Sample 5 was Sample 3 cells induction for 6 days; Sample 6 was the Sample 2 cells induction for 6 days. Data shown as mean ± SD, and number of replications is two (n = 2).
Fig. 6 RPKM and unigene transcription changes involved in pyruvate metabolism pathways of *H. pluvialis*. a, b represent RPKM changes (a) and relative transcript level changes (b) involved in pyruvate biosynthesis pathways of *H. pluvialis* in control group (1235 route) and treatment group (1246 route), respectively. To calculate genes transcript level, Sample 1 was regarded as control; comparing with control group, the gene transcription changes of the treatment group were presented in KEGG pathway (c). Sample 1, Sample 2 and Sample 3 were the heterotrophic cells at 100 h, 250 h, and 550 h, respectively; Sample 4 was Sample 2 cells induction for 0.5 day; Sample 5 was Sample 3 cells induction for 6 days; Sample 6 was the Sample 2 cells induction for 6 days. Yellow represents both down- and up-regulation gene, and red represents upregulation gene. Data shown as mean ± SD, and number of replications is two (n = 2).
et al. (2016) and He et al. (2018) also reported that the upregulation of lcyB was accompanied by the downregulation of lcyE.

Transcriptome and pathway analysis involved in pyruvate metabolism

In most microalgae, the direct product of photosynthesis is glucose. Then, glucose will be converted into lipid (Melis 2012). The dehydration condensation between lipid and free astaxanthin formed esterified form astaxanthin (Karsten et al. 2009; Schoefs et al. 2001). Astaxanthin esterification drove the formation and accumulation of astaxanthin (Chen et al. 2015). Pyruvate metabolism plays a major role in shifting carbon from glucose toward lipid synthesis (Chen et al. 2009; Orly et al. 2015). Pyruvate can interact with 3-phosphoglycerol aldehyde, yielding the substrate for producing isopentenyl pyrophosphate (IPP) through non-mevalonate pathway or mevalonate pathway (Saakov 2005; Ye et al. 2008). IPP is the precursor for astaxanthin synthesis (Lichtenthaler 1999). As such, pyruvate metabolism plays an important role in astaxanthin synthesis.

Pyruvate kinase (PK), a final enzyme of glycolysis, irreversibly converts phosphoenolpyruvate (PEP) into pyruvate with the concurrent generation of ATP (Shhtaia et al. 2015). Compared with control, the transcription level of pk gene was 1.3-fold up-regulated at the end of photoinduction (Table 1). Chen also reported that the increase of esterified form astaxanthin synthesis was accompanied by a 3.5-fold increase in pk (Cheng et al. 2017). Acetyl-CoA is the precursor for lipid synthesis. Pyruvate would be converted into acetyl-CoA, catalyzed by pyruvate dehydrogenase complex (PDH) (Li et al. 2014). Intriguingly, the upregulation of pyruvate dehydrogenase E1 component alpha subunit (pdhA), pyruvate dehydrogenase E2 component (pdhD), dihydrolipoamide dehydrogenase (pdhC) were observed when akinetes formation was used non-motile as seeds; whereas, in control only pdhA gene was up-regulated (Fig. 6a, b). Therefore, compared with control, non-motile cells have stronger pyruvate metabolism ability.

Additionally, malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) (NADP-ME) supplies carbon and NADPH for de novo fatty acid production. Compared with control, NADP-ME was up-regulated by 1.8-fold at the end of photoinduction (Table 1). Phosphoenolpyruvate carboxylase (PPC), which catalyzes PEP to oxaloacetate, was 2.1-fold up-regulated at the end of photoinduction (Table 1). The increased transcription level of malate dehydrogenase (NADP+) (MDH) accelerates the conversion of malate from oxaloacetate, thus providing additional carbon and NADPH for de novo fatty acid production (Shtaida et al. 2015). At the end of photoinduction, MDH was up-regulated by 1.5-fold when used non-motile cells as seeds (Table 1).

Compared with control, all genes related to pyruvate metabolism have a higher gene transcription level at the end of induction (Fig. 6c and Table 1). Above all, transcriptome analysis results further indicated that non-motile cells have stronger pyruvate metabolism ability. Whereas, pyruvate metabolism plays an important role in astaxanthin synthesis. The results further explained that non-motile cells have a strong ability to accumulate astaxanthin. The high astaxanthin synthesis ability of non-motile cells was ascribed to the high transcription level of genes related with pyruvate metabolism.

Conclusions

Above all, astaxanthin synthesis is closely related to pyruvate metabolism. The strong ability of non-motile cells to accumulate astaxanthin can be attributed to the improvement of pyruvate metabolism. The results are expected to further guide the in-depth optimization of the astaxanthin production process in H. pluvialis.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40643-019-0293-1.

Additional file 1: Table S1. Gene specific primers of qRT-PCR.

Abbreviations

DEGs: differentially expressed genes; PE: paired-end; NODE: National Omics Data Encyclopedia; GGPP: geranylgeranyl-pp; GA3: gibberellin; SEM: scanning electron microscope; TEM: transmission electron microscope.

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Authors’ contributions

MW and LF designed the study; LF analyzed the data and drafted the manuscript; JZ and ZF prepared the transcriptome sequencing samples and electron microscopy samples; MW reviewed and edited the article. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included in the main manuscript file and additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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