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Cloning and Expression Analysis of *atp6* Genes between Cytoplasmic Male Sterile Line and Its Maintainer Line in Bunching Onion

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Abstract In order to further study the relationship between *atp6* gene and Cytoplasm male sterility (CMS) of Bunching onion, the sequence of *atp6* gene was obtained from male sterile line and maintainer line of Zhangqiu onion bunching using homologous cloning. The protein secondary structure and the expression of *atp6* gene in different growth stages were compared. The results showed that there was one polymorphic locus (A/T) at the 171st base of the open reading frame of *atp6* gene in CMS line and maintainer line, which changed the proportion of protein secondary structure slightly, but did not change the transmembrane structure of ATP6 protein. The expression of *atp6* gene in roots and leaves/flowers at seedling, bolting, flowering and late flowering stage was detected by real-time fluorescence quantitative polymerase chain reaction. The results showed that the expression of *atp6* gene in root showed a trend of increasing at first and then decreasing during the whole development of the plant, reaching the maximum at the flowering stage, and the expression of the maintainer line was 1.14 times higher than that of the CMS; The expression of *atp6* gene in leaves/flowers decreased gradually during the whole growth period, and reached the highest level at the seedling stage, and the maintainer line was 1.96 times higher than that of the CMS.

Keywords Bunching onion; *atp6* gene; Cloning; Expression analysis

Bunching onion (*Allium fistulosum* L.) is a very important spicy vegetable. It is an essential vegetable and condiment in the daily life of Chinese. It has pungent taste, warm nature, and is suitable for raw or cooked food. It has health functions such as appetizing and digestion, anti-cancer, anti-tumor, cardiovascular protection, lowering blood pressure, improving human immunity and preventing aging.

Cytoplasmic male sterility (CMS) is a maternal genetic trait, which is mainly manifested in the inability of plants to produce functional pollen. This phenomenon was first discovered by Jones in *Allium cepa* L. in 1936 (Jones and Emsweller, 1936; Jones and Clarke, 1943). Studies have shown that plant CMS is related to mitochondrial gene abnormalities. In 1976, Levings and Pring found that there are obvious differences in enzyme digestion patterns between mitochondrial DNA in male fertile cytoplasm and male sterile cytoplasm of maize (*Zea mays* L.), which directly proved that maize CMS is closely related to mitochondrial DNA gene differences at the molecular level. The mitochondrial genomes of higher plants are often rearranged or recombined in the process of evolution, resulting in more chimeric open reading frames (ORFs), in which genes that induce CMS may be produced (Hanson and Bentolila, 2004). *atp6* gene is an important component of F1F0-ATP synthase system (Ji et al., 2013). Sequence mutation will affect the synthesis of coding amino acids, affect the function of ATP synthase, interfere with pollen development, and lead to the occurrence of plant CMS (Dietrich et al., 2003).

In order to further study the relationship between *atp6* gene and cytoplasmic male sterility in bunching onion, the sequence of *atp6* gene in bunching onion was obtained by homologous cloning, and its protein secondary

structure and *atp6* gene expression at various stages of growth and development were analyzed and compared, so as to provide a theoretical basis for revealing the molecular mechanism of CMS in bunching onion.

1 Results and Analysis

1.1 Quality detection of total RNA in root, leaf and flower of bunching onion

The extracted total RNA was determined by UV spectrophotometer. The ratio of OD_{260} to OD_{280} was in the range of 1.9~2.0, and the ratio of A260 to A230 was in the range of 2.0~2.3, indicating that the purity of the extracted RNA was high. After 1% agarose gel electrophoresis (Figure 1), the results showed that the 28S and 18S bands were clear, indicating that the RNA integrity was good and could be used for subsequent tests.

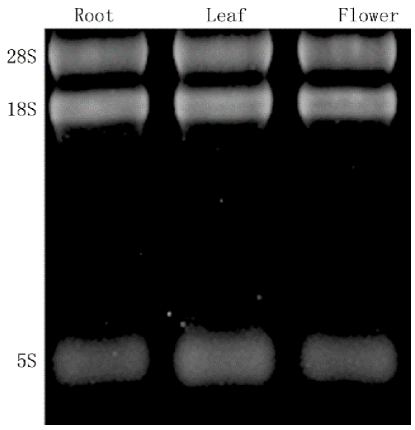

Figure 1 Extraction and detection of total RNA of root, leaf and flower from bunching onion

1.2 Sequence analysis of *atp6* gene in bunching onion

Primers atp6-F1 and atp6-R1 were designed by using the *atp6* pseudogene sequence of bunching onion in GenBank (GenBank Accession No.JQ283733.1) to amplify the total DNA of male sterile line 980128A and maintainer line 980128B in bunching onion. The PCR amplification products were detected by 1.0% agarose gel electrophoresis. About 800 bp fragments were obtained on both male sterile line and maintainer line (Figure 2), which were consistent with the expected fragment size and clear bands. The sequencing results showed that the amplified fragments were 806 bp in length. The flanking sequences of 5 'and 3' were obtained by TAIL PCR. The start codon and stop codon were found, and the full-length sequence of *atp6* gene was 819 bp (Gao et al., 2018). Through monoclonal sequencing, it was found that S-type (sterile) cytoplasm of bunching onion had *T-atp6* genotype (GenBank No. KR973431), N-type (fertile) cytoplasm had *T-atp6* and *A-atp6* genotype (GenBank No. KR973430), and some N-type cytoplasm had only *A-atp6* genotype (Figure 3).

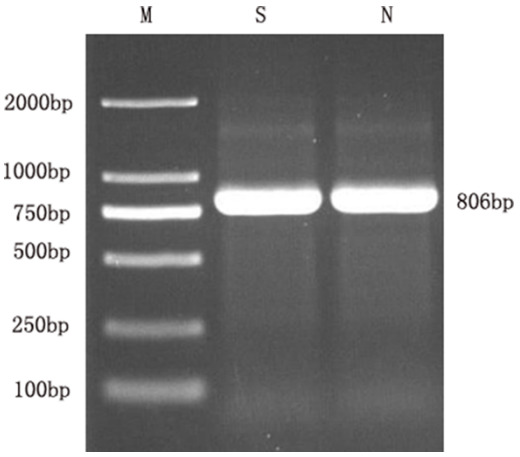

Figure 2 PCR electrophoresis results of *atp6* gene in bunching onion

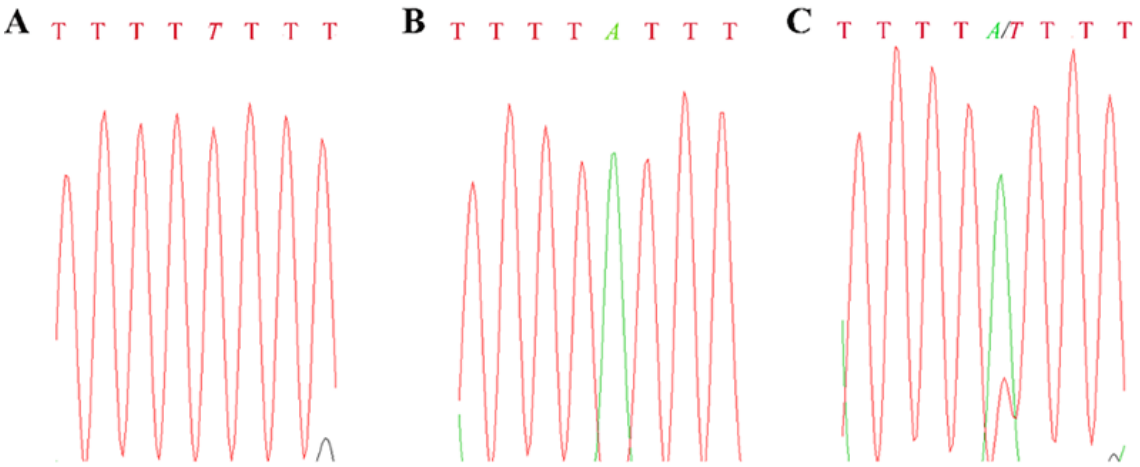

Figure 3 Polymorphic locus (A/T) at the 171st base of the *atp6* gene by monoclonal sequencing
Note: A: CMS line; B、C: maintainer line

1.3 Amino acid sequence analysis of *atp6* gene male sterile line and maintainer line in bunching onion

We compared the differential sequences of the two *atp6* genes and *atp6* pseudogene sequences (GenBank No. JQ283733.1) (Figure 4). It was found that *A-atp6* and *T-atp6* had one more T base at site 170 than JQ283733.1 sequence, thus the JQ283733.1 sequence that could not encode amino acids became a complete *atp6* gene. There is a G/T base difference at site 194. The sequence of JQ283733.1 is T, and both *A-atp6* and *T-atp6* are G. There is an A/T base difference between *A-atp6* and *T-atp6* at position 171. Both *A-atp6* and JQ283733.1 sequences are A, and *T-atp6* is T. The *atp6* gene of bunching onion encodes 272 amino acids. Leu (L; *A-atp6*) at position 57 is mutated to Phe (F; T-atp6) due to the existence of 171 A/T polymorphism.

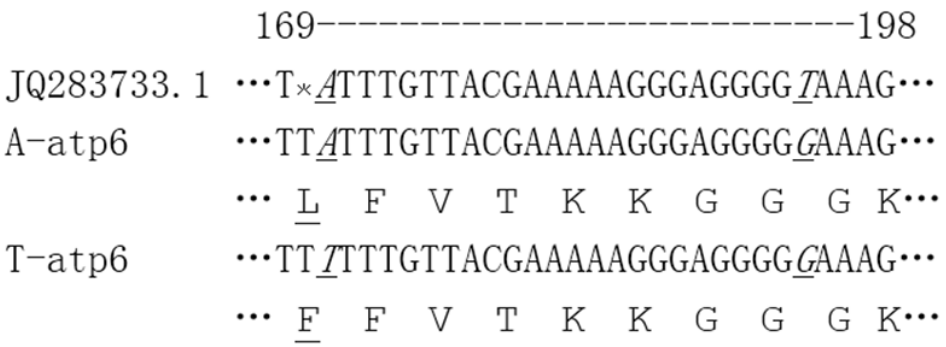

Figure 4 Sequence analysis of difference between *atp6* genes and JQ283733.1 in bunching onion

1.4 Characteristics of *atp6* gene coding protein in bunching onion

The secondary structure of *atp6* gene protein in bunching onion was predicted by SOPMA online software. The results showed that the secondary structure of ATP6 protein of A/T haplotype was composed of α-helix, extend strand, β-turn and random coil (Table 1). Because the 171st base of *atp6* gene sequence was mutated from a maintainer line to T of sterile line, leucine (Leu) at 57th position of amino acid sequence maintainer line was mutated to phenylalanine (Phe) of sterile line, so that the secondary structure proportion of ATP6 protein in bunching onion changed slightly.

Table 1 Prediction of amino acid composition of *atp6* gene encoded protein in bunching onion

Type	Alpha helix(%)	Extend strand(%)	Beta turn(%)	Random coil(%)
A-ATP6	47.79	19.12	4.78	28.31
T-ATP6	47.43	18.01	5.88	28.68

The transmembrane structure of the two haplotypes of A-ATP6 and T-ATP6 in bunching onion was predicted by the online analysis software TMHMM. The results showed that the number of transmembrane domains of the two haplotypes of *atp6* gene coding proteins did not change due to A/T mutation, and the 5 transmembrane domains still existed (Figure 5).

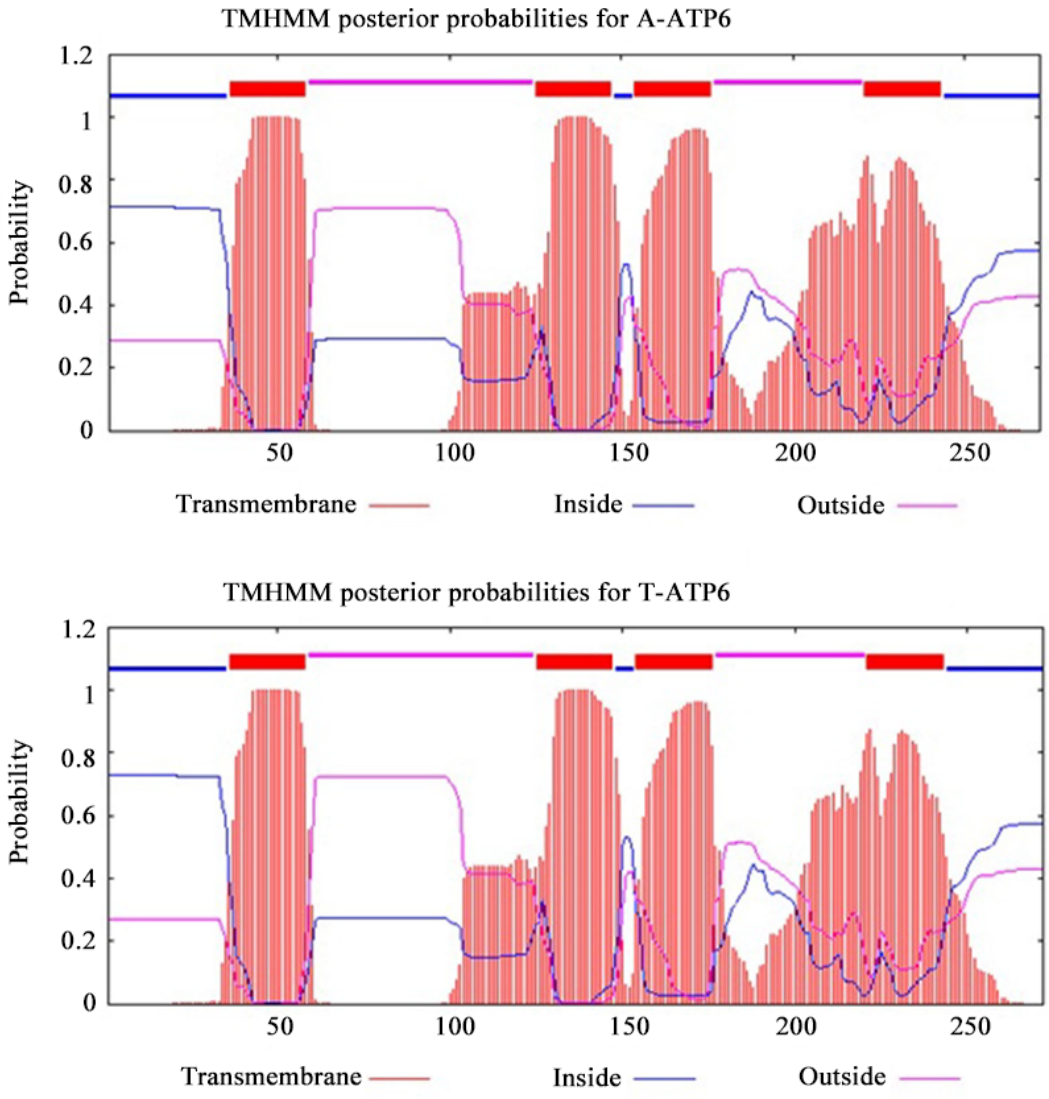

Figure 5 Prediction and analysis of transmembrane structure of protein encoded by *atp6* gene in A and T haplotype bunching onion

1.5 RT-PCR analysis of *atp6* gene male sterile line and maintainer line of bunching onion at different developmental stages

Quantitative expression analysis of *atp6* gene in male sterile line and maintainer line of bunching onion (Figure 6) showed that during the whole plant development process, the expression of *atp6* gene in root system showed a trend of first increasing and then decreasing, reaching the maximum value at flowering stage, and the maintainer line was 1.14 times than that of male sterile line; The expression of *atp6* gene in leaf/flower decreased gradually during the whole growth period, and reached the highest at seedling stage. The maintainer line was 1.96 times than that of male sterile line.

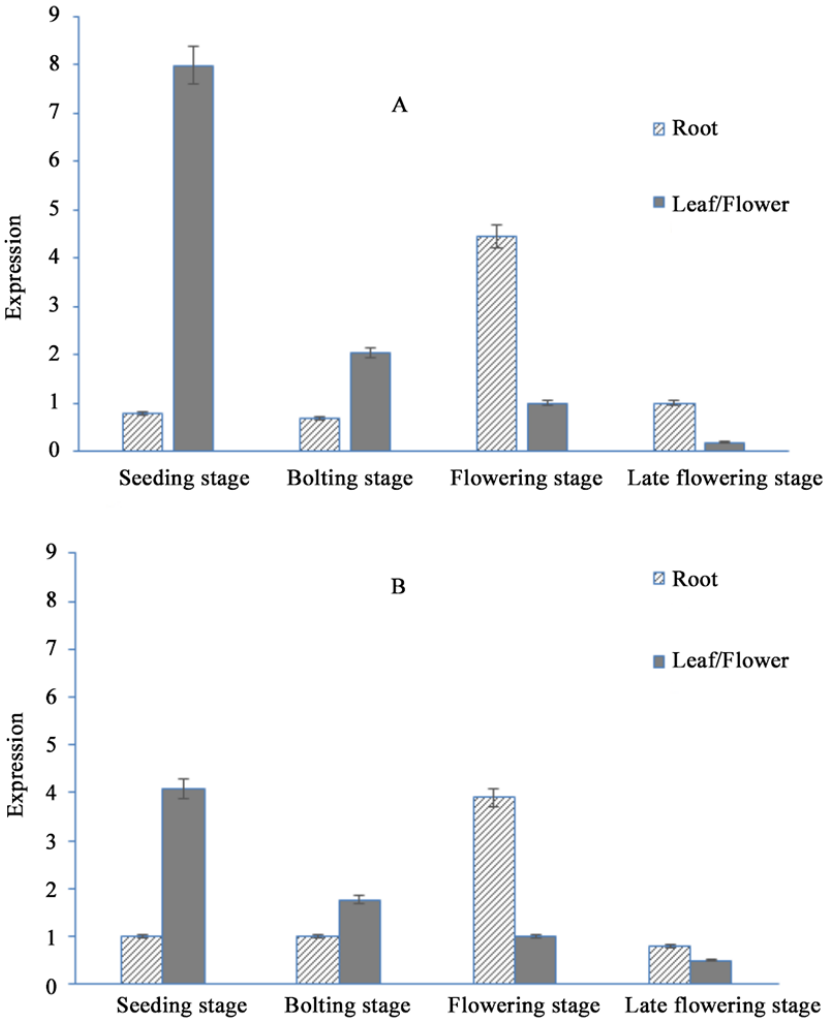

Figure 6 Relative expression of *atp6* gene in roots, leaves and buds of bunching onion at different growth stages

Note: A: maintainer line; B: CMS line

2 Discussion

Many studies have shown that *atp6* gene is an important candidate gene affecting plant CMS. Wang et al. (2015) compared and analyzed 33 mitochondrial genes and 22 open reading frames in K-type and V-type male sterile lines and maintainer lines of wheat (*Triticum aestivum* L.) and found that *atp6* gene has great differences between maintainer lines and male sterile lines. Tan et al. (2018) and Fabio et al. (2020) sequenced and assembled the mitochondrial genome of fennel, and found two types of *atp6* gene sequences, of which *atp6*- only exists in the male sterile mitochondrial genome, and *atp6*+ only exists in the male fertile mitochondrial genome. However, *atp6*+ was also detected in the male sterile material, which was speculated to exist in the nucleus. This study showed that *A-atp6* existed only in male fertile materials of bunching onion, and *T-atp6* existed not only in male sterile materials of bunching onion, but also in some male fertile materials. Because this study is based on the total DNA of bunching onion, it is uncertain whether *T-atp6* in some male fertile materials exists in the cytoplasm or in the nucleus, and whether there are differences in pollen fertility and fertility between plants with *A-atp6* gene and plants with partial *T-atp6* gene. These contents need to be further studied and observed.

Some single base mutations in some plant genes may lead to male sterility. Zhou et al. (2012) studied the *atp6* gene of bunching onion sterile line and maintainer line, and found that there was a single base mutation in the upstream and inside of bunching onion *atp6* gene respectively. The first A/G mutation was located at the 467th base upstream of the start codon, and the second A/C mutation was located at the 92nd base inside the *atp6* gene.

This mutation resulted in the mutation of the coding amino acid from serine (maintainer line, Ser) to tyrosine (sterile line, Tyr). Wang et al. (2014) also found the C/A mutation at the same site within the gene through the study on the *atp6* gene of bunching onion sterile line and maintainer line. The study believed that the mutation site appeared randomly between the sterile line and maintainer line, which may be related to the test material. This study found that there was A/T polymorphism in the *atp6* gene between the male sterile line and the maintainer line of bunching onion. Leu (L; *A-atp6*) was mutated to Phe (F; *T-atp6*), which led to a slight change in the proportion of protein secondary structure. Although it did not lead to the change of transmembrane domain, it could not be ruled out that this single base mutation might lead to male sterility, which needs to be verified by further genetic transformation.

Zhou et al. (2012) compared the expression of bunching onion *atp6* gene in male sterile lines and maintainer lines, and showed that in bunching onion flowering period, the expression of *atp6* gene in male sterile lines tended to increase first and then decrease, while that in maintainer lines tended to decrease first and then increase; There was little difference between male sterile line and maintainer line at small bud stage and large bud stage, but there was a great difference at middle bud stage. Tan et al. (2018) studied the expression of carrot *atp6* gene in male sterile lines and fertile lines at flowering stage. The expression of fertile lines at flowering stage tended to increase first and then decrease, while the expression of male sterile lines at the whole flowering stage changed little. The expression of *atp6* gene in root was the highest at flowering stage, and that in leaf was the highest at seedling stage. The maintainer lines were higher than the male sterile lines. However, there was no significant difference in the amount of expression in flower buds between male sterile lines and maintainer lines. It is possible that this sample was taken in the early stage of flowering, and the period of differential expression has not yet reached.

3 Materials and Methods

3.1 Test materials

The male sterile line and maintainer line of Zhangqiu bunching onion were planted in the experimental base of Vegetable and Flower Research Institute of Shandong Academy of Agricultural Sciences. Fresh leaves and roots of bunching onion were sampled at seedling stage and bolting stage; Flower buds and new roots were sampled at the flowering stage and late flowering stage.

3.2 Extraction of genomic DNA and RNA from bunching onion

The rapid plant genomic DNA extraction kit was used for the extraction of bunching onion genomic DNA; The plant total RNA extraction kit is used for extracting the RNA from bunching onion, and the FastQuant cDNA first strand synthesis kit is used to synthesize single strand cDNA. The total DNA, RNA extraction kit and cDNA first strand synthesis kit are purchased from Tiangen Biotech (Beijing) Co., Ltd. Methods refer to the operating instructions.

3.3 Primer design, PCR amplification and sequencing of *atp6* gene

According to the serial number of GenBank Accession No.JQ283733.1 in GenBank database, Primer Premier 5.0 was used to design primers atp6-F1 and atp6-R1 (Table 2). The primers were purified to PAGE and were synthesized by Boshang (Beijing) Biotech Co., Ltd. PCR reaction was carried out by TC-XP-D gene amplification instrument (produced by Bio-Rad Company), 20 μL in the reaction system, 2×Pfu Master Mix 10 μL. Upstream and downstream primers (10 μmol/L) were 1.0 μL, respectively. Sample DNA 50 ng; The reaction procedure, PCR product detection, recovery and purification of target fragments, cloning and other operations were carried out with reference to Gao et al. (2018), with slight changes. The TAIL-PCR amplification and the acquisition of two terminal sequences refer to Liu et al. (2007).

3.4 Sequence analysis of *atp6* gene in bunching onion

The nucleotide sequence of *atp6* gene was analyzed bioinformatics by DNAMAN and other software; The protein secondary structure and transmembrane structure were predicted by method of Liu et al. (2020).

3.5 RT-PCR analysis of *atp6* gene in bunching onion

According to the *atp6* gene sequence a pair of specific RT-PCR primers RT-atp6-F and RT-atp6-R were designed. Tublin was an internal reference, and the primer sequences were TUB-F and TUB-R (Table 2). The root, leaf and flower bud of CMS line and maintainer line in bunching onion were analyzed by Real-time quantitative PCR. RT-PCR reaction system was 25 μL system, including 12.5 μL SYBR Premix Ex TaqII (TAKARA, Japan), upstream and downstream primers (10 μmol/L) each 1.0 μL. CDNA template (after 10 times dilution) 1.0 μL，0.4 μL Rox Reference DyeII, 9.5 μL ddH_2O; The reaction procedure is as follows: pre denaturation at 95°C for 30 s; Then 95°C for 5 s, 60°C for 30 s, 40 cycles. Each sample was set to repeat for 3 times. The Real-time fluorescent quantitative PCR analysis was carried out using the Light Cycler system (Roche Light Cycler480). The relative expression of genes was calculated using the $2^{-\triangle\triangle Ct}$ method (Magnus et al., 2005).

Table 2 Primers

Name of Primer	Primer direction (5′-3′)
atp6-F1	ATGAGTGCTGAAAGGAGGAAAG
atp6-R1	TGACTCATTTTGATGGAGATTTG
RT-atp6-F	CATCCTTGTCTATGCTGCTC
RT-atp6-R	TCGTTTACCGGGTTCG
TUB-F	CAGGAGGTCCCTTGACATTGAA
TUB-R	TCGAAGGCTGAGTTGGTGATTT

Authors' Contributions

WQH is the executor of this experimental research, completing data analysis and writing the first draft of the paper; ZRT participated in the experimental design and the analysis of experimental results; JSJ gave suggestions on experimental design; GLM is the designer and person in charge of this project, guiding the experimental design, data analysis, thesis writing, and completing the revision and finalization of the article. All authors read and approved the final manuscript.

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