Cloning and Analysis of MoTPS and its Promoter in Morinda officinalis How

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Abstract To reveal the sequence characteristics of MoTPS gene of Morinda officinalis and its expression pattern under auxin (IAA) treatment. The coding sequence of three MoTPS genes were obtained by RT-PCR, they were named separately MoTPS1, MoTPS2 and MoTPS3. Gene sequence analysis showed that the full-length sequence of three MoTPS genes were separately 2 841 bp, 2 126 bp and 2 144 bp, and contained separately 2 481 bp, 1 668 bp and 1 943 bp open reading frame (ORF), encoding separately 826, 555 and 640 amino acids. The sequence analysis results showed that proteins encoded by three MoTPS genes were hydrophilic unstable protein and amino acid sequences of three genes contained terpenoid specific aspartic acid (DDxxD) enrichment motif, RxR conservative motif, and terpenoid class I superfamily domain. Homology analysis showed that amino acid sequences of three MoTPS genes were similar to TPS gene matched in Coffea Arabica, and these plants belonged to Rubiaceae. Phylogenetic tree analysis showed that MoTPS1 belongs to TPS-f subfamily, but MoTPS2 and MoTPS3 belong to TPS-d subfamily. Among the three MoTPS genes, MoTPS3 gene was screened for promoter isolation, the length of 5’end promoter sequence was 739 bp, including a variety of regulatory elements related to light response, stress response and hormone response, such as G-box, Sp1, P-box, TGA-element etc. Fluorescence quantitative PCR analysis showed that the expression of three MoTPS genes were stable and tissue-specific in roots, shoots and leaves. the relative expression levels of three MoTPS genes were significantly up-regulated after IAA treatment. Therefore, this study has speculated that three MoTPS genes are involved in the synthesis of terpenoid secondary metabolites in root of Morinda officinalis and auxin IAA was predicted to play important role in the regulation of MoTPS gene expression.

Keywords Morinda officinalis How; Terpene synthase; Promoter; Gene cloning; Bioinformatics analysis

Morinda officinalis how, belonging to the genus Morinda genus in the Rubiaceae family, is distributed in Fujian, Guangdong, Hainan and other provinces in China and other subtropical regions in the world (Zhang et al., 2015). Dried root of M. officinalis, an important Chinese herbal medicine, tastes sweet, pungent and mild. it contains a lot of chemical components with medicinal value, such as anthraquinones, terpenes and polysaccharides, these components has the effect of Tonifying kidney and strengthening muscles and bones (Shao et al., 2019). Hu et al. (2019) and Kong et al. (2019) reported that the root extract of M. officinalis can improve the osteoporosis of chicken and mouse. Terpenoids are important components in medicinal ingredients, Zhai et al. (2018) reported that seven new pentacyclic triterpenoids extracted from the root of M. officinalis have antibacterial effect on two human osteosarcoma cell lines, Gram-positive bacteria and Gram-negative bacteria.

Terpenoids are the most common secondary metabolites in plants, including monoterpenes, sesquiterpenes, diterpenoids or triterpenoid derivatives (Hegazy et al., 2020), and many terpenoids have medicinal value, such as α-pinene, limonene, etc. Ateba et al. (2018) reported that costunolide and triptolide can inhibit the proliferation, migration, anti-apoptosis, tumor angiogenesis or metastasis of different breast cancer cells or tumors. Li et al. (2006) reported that granzyme, granulin and perforin levels of cell were significantly increased by using α-pinene and other substances contained in the volatiles to cultivate Nk-92mi cells. 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 1-deoxy-D-xylulose-5-phosphatesynthase (DXS),
3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and terpene synthases (TPS) are the key enzymes in the synthetic pathway of mevalonate (MVA) and 2-methyl-d-erythritol-4-phosphate (MEP).

Terpene synthases are direct catalytic enzymes for the formation of target products, the study of TPS gene plays a significant role in the regulation mechanism of terpenoids synthesis. Therefore, more and more TPS genes have been cloned in different plants, such as *Arabidopsis* (Aubourg et al., 2002), tobacco (Lv et al., 2015), rice(Ge., 2015), maize(Li., 2015) etc. But TPS gene of *M. officinalis* has not been cloned. In this study, based on the transcriptome data of *M. officinalis*, MoTPS gene and its promoter were isolated and cloned by RT-PCR, and we reported that sequence information of TPS gene and promoter, and gene relative expression profiles in root, shoot and leaf of *M. officinalis* and IAA treatment. This study will provide a theoretical basis for the regulation of terpenoids synthesis in root of *M. officinalis* and a reference for developing and utilizing the medicinal value of *M. officinalis* more scientifically and TPS gene research in other plant in the future.

1 Results and Analysis

1.1 Molecular cloning of *MoTPS1*, *MoTPS2* and *MoTPS3* gene

According to core sequence of different TPS genes of *M. officinalis*, the full-length cDNA sequences of *MoTPS1*, *MoTPS2* and *MoTPS3* gene are obtained by 5' and 3'-RACE amplification and sequencing, they were separately 2 841 bp, 2 126 bp and 2 144 bp,. The NCBI database was used to compare and analyze three genes, the results showed that three genes were different TPS genes, and open reading frame length of *MoTPS1* gene was 2 481 bp, encoding 826 amino acids; *MoTPS2* gene was 1 668 bp, encoding 555 amino acids; *MoTPS3* gene was 1 923 bp, encoding 640 amino acids. Primers were designed using open reading frames of three genes, cDNA as template, to exert RT-PCR amplification. After 1.5% gel electrophoresis, clear and untailed bands were obtained, and the size of the bands was consistent with the prediction (Figure 1). The amplified fragment was purified by Omega Gel Extraction Kit method, and then it was transferred to pET-28a vector for monoclonal culture. Positive monoclonal solution was sent to Fuzhou Boshang sequencing company for sequencing. Sequencing results showed that the gene sequence matched the transcriptome sequence.

![Figure 1](http://hortherbpublisher.com/index.php/mpr)

Figure 1 The cloning of *MoTPS1*, *MoTPS2* and *MoTPS3* gene

Note: M: 1 kb plus DNA Maker; 1: *MoTPS1* gene; 2: *MoTPS2* gene; 3: *MoTPS3* gene

1.2 Bioinformatics analysis of *MoTPS1*, *MoTPS2* and *MoTPS3* gene

1.2.1 Homology analysis and phylogenetic tree construction

The amino acid sequences of three genes were compared with other plants by Blastp tool in NCBI website. The results showed that the homology of the three genes with TPS gene of *Coffea arabica* was over 75%. The amino acid sequences of three *MoTPS* genes and TPS genes of other plants were used to construct phylogenetic trees by neighbor joining method of MEGA 7.0 software. The results show that amino *MoTPS1*, *MoTPS2* and *MoTPS3* are grouped with TPS genes of *Coffea arabica* (Figure 2), and they are closest to *Coffea arabica* and belong to Rubiaceae. *MoTPS1* gene and S-linalool synthase-like gene belong to subfamily TPS-f, *MoTPS2* and *MoTPS3* belong to the same subfamily of limonene synthase gene and belong to subfamily TPS-d. Three genes are far from subfamily TPS-b represented by alpha terpene gene.
1.2.2 Analysis of amino acid composition and physicochemical properties

The amino acid composition and physicochemical properties of MoTPS1, MoTPS2 and MoTPS3 gene were analyzed by the online tool of ExPASY protparam, the results showed that the relative molecular mass of protein encoded by MoTPS1 gene was 74.56 kD, theoretical isoelectric point was 6.61, amino acids with high proportion were separately (11.9%), serine (8.0%), glutamic acid (7.5%); The relative molecular mass of protein encoded by MoTPS2 gene was 64.63 kD, and its theoretical isoelectric point was 5.23, its amino acids with high proportion were separately leucine (10.1%), glutamic acid (7.9%) and aspartic acid (7.2%); The relative molecular mass of protein encoded by MoTPS3 gene was 94.74 kD, and its theoretical isoelectric point was 5.57. Its amino acids with high proportion were respectively leucine (11.3%), serine (7.4%) and glutamic acid (7.3%). Instability coefficients of amino acid of three genes were higher than 40, so they belonged to instable proteins. Total average hydrophobic coefficient (GRAVY) of MoTPS1, MoTPS2 and MoTPS3 were -0.276, -0.338 and -0.303 respectively, so proteins encoded by three genes were speculated to be hydrophilic proteins.

The amino acid phosphorylation sites of three genes analysis showed that three genes contained more phosphorylation sites with high reliability, such as 21 (15 serine, 5 tyrosine and 1 threonine) in MoTPS1, 15 (8 serine, 6 tyrosine and 1 threonine) in MoTPS2, and 18 (13 serine, 4 tyrosine and 1 threonine) in MoTPS3. These phosphorylation sites were not modified by specific modified kinase, which indicated that phosphorylation sites in amino acid sequence of three genes contained multiple modification types.

The signal peptide of amino acid sequences of three genes analysis showed that the shear site value, S value and Y-max value of signal peptide sites of three genes were lower than 0.2, which meant that they have no signal peptide site. The transmembrane domain analysis showed that the peptide chains encoded by three genes were out of the membrane without transmembrane domain. The subcellular localization predicted that proteins encoded by three genes played a role in the chloroplast membrane. However, the chloroplast transporter peptide was predicted that there was no chloroplast transporter peptide, but there were amino acid residues of 10–30 bp leading peptide. Protein conserved domain and secondary and tertiary structure analysis
Protein conserved domains analysis showed that the class I terpene synthetase superfamily domain and aspartic acid sequence (conserved domain DDXXD) were contained in amino acid sequences of three MoTPS genes. Protein secondary structure analysis showed that protein secondary structures of MoTPS1, MoTPS2 and MoTPS3 gene were mainly composed of alpha helix and random coil, the proportion of alpha-helix is more than 50%, and the proportion of irregular coil is more than 15% (Figure 3). Meanwhile, the proportion of irregular curl of MoTPS1 was 26.88%, and the proportion of alpha-helix of MoTPS2 was 61.44%. The tertiary structure analysis showed that MoTPS1 protein was predicted to be abietadiene synthase with sequence consistency of 28.70%; MoTPS2 protein was predicted to be sesquiterpene synthase with sequence consistency of 44.38%; MoTPS3 protein was predicted to be 4S-limonene Synthase), the sequence consistency was 48.52% (Figure 4).

Figure 3 The encoding protein tertiary structure prediction of MoTPS1, MoTPS2 and MoTPS3 gene

![MoTPS1 tertiary structure](image1.png)

![MoTPS2 tertiary structure](image2.png)

![MoTPS3 tertiary structure](image3.png)

Figure 4 The encoding protein tertiary structure prediction of MoTPS1, MoTPS2 and MoTPS3 gene

Note: A: MoTPS1 encoded protein tertiary structure; B: MoTPS2 encoded protein tertiary structure; C: MoTPS3 encoded protein tertiary structure
1.3 Promoter analysis of MoTPS3 gene

Combined with the results of bioinformatics analysis to select a representative gene (MoTPS3) in three MoTPS genes for promoter cloning. Three specific primers were designed by Primer primer 5.0 software according to the coding sequence of MoTPS3 gene, and the upstream promoter region was cloned by Genome Walking Kit method, its length was 739 bp (Figure 5). The bioinformatics analysis showed that the promoter contained typical conservative cis-elements (TATA box and CAAT box), which indicated that the sequence has promoter activity. It also contained many light response elements, such as box 4. G-box and GT1 motif etc, and contained many hormone response elements, such as CGTCA motif and TGA element. In addition, it contained some cis-elements related to the binding sites of MYB and Myc, such as flavonoid synthesis gene regulatory transcription factor MBS and drought induced gene regulatory transcription factor MBS, which indicated that promoter of MoTPS3 gene can respond to environmental changes (such as hormone action, stress) and regulate gene expression.

Figure 5 The sequence of upstream promoter element of MoTPS3

1.4 Fluorescence quantitative expression analysis of MoTPS1, MoTPS2 and MoTPS3 gene

The relative expression analysis of MoTPS1, MoTPS2 and MoTPS13 gene in the root, shoot and leaf of M. officinalis showed that the relative expression trend of three MoTPS genes in each tissue part was consistent, and the trend was consistent with the trend of FPKM (reads per kilobase per million) value in the transcriptome data. the relative expression of TPS gene was higher in root than that in shoot and leaf, which indicated that TPS gene had tissue-specific and stable expression in M. officinalis. In addition, the relative expression of three genes was significantly increased after IAA treatment. Compared with the control group, the expression levels of MoTPS1 and MoTPS2 gene were significantly increased on the 5th day after treatment. The expression levels of MoTPS1 and MoTPS2 genes were still up-regulated on the 7th and 10th day after treatment, but MoTPS3 gene tended to be stable (Figure 6).

Figure 6 Relative expression of MoTPS1, MoTPS2 and MoTPS3 gene in different tissues and under IAA treatments

Note: "*" indicates significant difference relative to the control; "**" indicates extremely significant difference relative to the control.
2 Discussion

There are many iridoid glycosides in the root of *M. officinalis*, which can prevent osteoporosis, anti-fatigue and depression (Yang et al., 2018; Zang, 2019; He, 2019). The study of terpene synthase gene can provide theoretical support for the maximum utilization of *M. officinalis*. In this study, based on the transcriptome data of *M. officinalis*, we have cloned full-length cDNA sequences of three *MoTPS* genes. The amino acid sequence analysis showed that amino acid sequences of *MoTPS1*, *MoTPS2* and *MoTPS3* gene contained aspartic acid conservative motif (DDXXD), which conformed to the sequence characteristics of common terpenoids, TPS gene will be inactivated when the aspartic acid conserved motif in TPS gene mutates (Mau and West, 1994). Further analysis showed that *MoTPS2* and *MoTPS3* had RXR motifs at 35 amino acids upstream of DDXXD motif. RXR motif, a conserved amino acid sequence of TPS gene, is involved in the catalysis of terpenoids (Huang et al., 2019). In addition, the cyclization of *MoTPS1* gene may be affected due to it has no N-terminal double arginine motif (RRX8W). Three *MoTPS* genes are similar in protein secondary structure, they mainly consisted of α helix and irregular curl. But in the third structure prediction, *MoTPS1* gene was predicted to be abietadiene synthase belong to diterpenoids, which also verifies that it is not a monoterpane.

Homology comparison and phylogenetic tree analysis showed that three genes were highly homologous with TPS genes of *Coffea arabica*. Combined with amino acid sequence of TPS gene in different plants through NCBI matching to construct phylogenetic tree, they have formed three branches (TPS-a, TPS-f and TPS-g), and three *MoTPS* genes have been classified into these branches. Bioinformatics analysis showed that the similarity between *MoTPS1* and S-linalool of *Coffea arabica* was not high, so *MoTPS1* was not monoterpenoids due to linalool belonged to monoterpensoids (Aubourg et al., 2002). And monoterpenoids usually encodes 560–640 amino acids (Chen et al., 2011), which also was indicated *MoTPS1* encoded 826 amino acids not to be monoterpenoids. This special situation also occurs in other plants, for example, some TPS genes in *Arabidopsis thaliana*, a model plant, were not located in the subfamily containing other TPS genes with the same function, but close to each other (Huang et al., 2019). These abnormal situation might be caused by the establishment of species-specific homologous TPS gene clusters or these TPS genes are still evolving. Studies have shown that some sequence mutations of TPS gene lead to slight structural differences in their target products, these mutations have been accumulated in plant evolution and resulted in thousands of different TPS genes (Chu et al., 2009).

Fluorescence quantitative expression analysis showed that *MoTPS1*, *MoTPS2* and *MoTPS3* gene were stably expressed in root, shoot and leaf, and the expression in root was higher than that in leaf and shoot, which is consistent with the relative expression of TPS gene in *Fallopia multiflora* (Sheng et al., 2010), *Panax ginseng* (Yoon et al., 2016) and *Salvia miltiorrhiza* (Ma et al., 2012). Root is the main nutrient storage part of these plants, and root is also the important medicinal part of *M. officinalis*, which confirmed that there are many terpenoids in the root of *M. officinalis*. Hormones can play an important role in the regulation of plant metabolism, and there are many hormone response elements in the promoter of TPS gene. *ZmTPS6* gene of maize can be significantly up regulated in GA, MeJA and ABA treatments (Li et al., 2006). And we have found that *MoTPS3* gene promoter also has many hormone response elements (Table 1), such as auxin, GA, MeJA response elements. the gene expression level of *M. officinalis* was significantly up-regulated in IAA treatment. It is speculated that hormone plays an important role in the regulation of TPS gene in *M. officinalis* and provided a reference for improving the content of terpenoids in *M. officinalis*. In this study, the TPS gene and its expression pattern in the terpenoid synthesis pathway of root in *M. officinalis* have been preliminarily explored, but TPS genes are diverse and a single gene may regulate the production of one or more terpenoids. Therefore, the functional verification of TPS gene of *M. officinalis* and expression patterns under treatment of other hormones need to further explored.
Table 1 Predicting cis-acting elements of MoTPS3 promoter

| Site name  | Position (chain) | Function                                                      |
|------------|------------------|---------------------------------------------------------------|
| Box 4      | +304, +363       | Conserved DNA module involved in light responsiveness          |
| CAAT-box   | +264, -581, +453, +733, +361, -698, -543, +734, +265, +609, -438, +712 | Common cis-acting element in promoter and enhancer regions     |
| CGTCA-motif| +51              | MeJA-responsiveness                                           |
| G-box      | +318             | Light responsive element                                       |
| MBS        | -553, +589       | MYB binding site involved in drought-inducibility             |
| GT1-motif  | -501, -502       | Light responsive element                                       |
| MBS I      | -96              | MYB binding site involved in flavonoid biosynthetic genes regulation |
| P-box      | +112, +123       | Gibberellin-responsive element                                 |
| Sp1        | +59              | Light responsive element                                       |
| TATA-box   | -676, -682, -680 | Core promoter element                                          |
| TGA-element| +218             | Auxin-responsive element                                       |
| TGACG-motif| -51              | MeJA-responsiveness                                           |
| Myb        | -553, +589       | MYB binding site                                               |
| MYC        | -132, +697       | MYC binding site                                               |

3 Materials and Methods

3.1 Materials and treatment

The experimental materials were planted in the backyard of the Institute of industrial materials, Fujian agricultural and Forestry University. The young leaves, shoots and roots of M. officinalis were picked in November 2019. Each of three tissue materials was set with three repetitions for gene cloning and total RNA extraction of tissue-specific fluorescence quantitative expression. At the same time, 10 growth-well and 3-year-old plants in the same block were irrigated with 100 μ mol / L IAA. Roots of each plant were picked at 0, 3, 5, 7 and 10 days and mixed samples were used to extract total RNA for fluorescence quantitative expression. The above samples shall be stored in the refrigerator immediately after picking.

3.2 Total RNA extraction and cDNA synthesis

Total RNA of leaves, shoots and roots of M. officinalis were extracted respectively by Total RNA Extraction Kit method. The quality, concentration and purity of total RNA were detected by 1% gel electrophoresis and spectrophotometer. The first strand of cDNA was synthesized by Reverse Transcription Kit method and stored in refrigerator at -20°C.

3.3 Gene cloning

Core fragments with open reading frame of three genes were screened by analyzing the transcriptome data of M. officinalis and comparison of NCBI blast online tool, three genes named MoTPS1, MoTPS2 and MoTPS3 respectively. 5‘ and 3‘-race specific amplification primers were designed by Primer premier 5.0 software (Table 2). The primers were synthesized by Fuzhou Boshang sequencing company. Amplification was exerted by RT-PCR, and the amplification system and procedure were in accordance with Xie et al. (2020). The amplified products were purified by Omega Gel Extraction Kit method after gel cutting. After purification, these products connected with the PEASY vector. And then products were cultured in monoclonal culture. The positive monoclonal bacteria were sent to Fuzhou Boshang company for sequencing. The full-length cDNA sequence of three MoTPS genes were obtained by splicing after sequencing.

3.4 Bioinformatics analysis of genes

The obtained full-length cDNA sequences of MoTPS1, MoTPS2 and MoTPS3 gene were analyzed by bioinformatics, the analysis method is as follows that the open reading frame of MoTPS genes were predicted by ORFFinder online tool of NCBI; the homology and conserved domain of protein sequences were predicted by NCBI blast P, NCBI CD and interpro online tools, and homology comparison of protein sequences were
compared by DNAMAN software; The phylogenetic tree construction of TPS gene sequences of different plants by neighbor joining algorithm of MEGA 7.0 software; the composition, physical and chemical properties and phosphorylation sites of amino acid sequences of *MoTPS* genes were analyzed and predicted by the online tools of ExPASy-Protparam, ExPASy-Protscale and Netpho3.1 server; the protein transmembrane domain and signal peptide were analyzed and predicted by TMHMM Server 2.0 and signalp online tools; The subcellular localization of *MoTPS* genes were analyzed by Plant mPLoc online tool; The existence of chloroplast transport peptide of *MoTPS* genes verified by ChloroP online tool; The protein secondary and tertiary structures of *MoTPS* genes were predicted by Predict Protein and SWISS-MODEL online tool.

Table 2 Primers used for cloning, promoter isolation, reference gene and quantitative of *MoTPS* gene

| Primer name | Primer sequence | Primer name | Primer sequence |
|-------------|-----------------|-------------|-----------------|
| MoT 5'R     | R1: CCAGCGAACAATATCGTTGGTGTC   | MoT 5'R     | R2: GTGACCATCTCGTGGTCCAGGTA |
| PS1 ACE 3'R | F1: GAGAAAGGCAATAGTGAAGGAC   | PS2 ACE 3'R | F2: GTGGAGGCTGGCCGAAATAGA |
| qPCR F     | TCACTGAAAGGTCTGTTGGCTGTG   | qPCR F     | TCTTCTGGAGATGCTTGGCTGTG |
| R1: TGAGGCAACATGTTGG  | R2: TTGCAAGCTCAAGCAGAACG |
| MoT Specific | SP1: TCTTCTGGAGATGCTTGGCTGTG | PS3 primer | SP2: TACATTTGCCCTGACGAGA |
| PS3 ACE 3'R | F1: CAGTCTGGAAATCACCACCTCACA | PS3 3-phosphate | R: AATCAACACGCCGAAACTG |
| qPCR F     | TCAAAGGCGCTTCCTTGATGGAGGA | qPCR F     | GCTGGCATTGCTTGGTGTG |
| R1: TCTTCTGGAGATGCTTGGCTGTG | R: AATCAACACGCCGAAACTG |

3.4 Bioinformatics analysis of genes

The obtained full-length cDNA sequences of *MoTPS1*, *MoTPS2* and *MoTPS3* gene were analyzed by bioinformatics, the analysis method is as follows that the open reading frame of *MoTPS* genes were predicted by ORFfinder online tool of NCBI; the homology and conserved domain of protein sequences were predicted by NCBI blast P, NCBI CD and interpro online tools, and homology comparison of protein sequences were compared by DNAMAN software; The phylogenetic tree construction of TPS gene sequences of different plants by neighbor joining algorithm of MEGA 7.0 software; the composition, physical and chemical properties and phosphorylation sites of amino acid sequences of *MoTPS* genes were analyzed and predicted by the online tools of ExPASy-Protparam, ExPASy-Protscale and Netpho3.1 server; the protein transmembrane domain and signal peptide were analyzed and predicted by TMHMM Server 2.0 and signalp online tools; The subcellular localization of *MoTPS* genes were analyzed by Plant mPLoc online tool; The existence of chloroplast transport peptide of *MoTPS* genes verified by ChloroP online tool; The protein secondary and tertiary structures of *MoTPS* genes were predicted by Predict Protein and SWISS-MODEL online tool.

3.5 Cloning and analysis of promoter

Three specific primers (SP1, SP2 and SP3) were designed by primer primer 5.0 software from 500 bp downstream of the starting codon (ATG) of the protein coding sequence for nested PCR (Table 2). Three times of thermo asymmetric PCR were performed with four special annexation primers provided by Genome Walking Kit. Reaction fluid (5 μL) at every times was detected by gel electrophoresis. The test results should be consistent with the primer primer when the primers were amplified to the inner primers. (SP3 was the innermost primer near the start codon). Finally, the third PCR amplification product was purified by Omega Gel Extraction Kit method, and then purified products were connected with the vector for monoclonal culture. Positive monoclonal solution was sent to Fuzhou Boshang sequencing company for sequencing. The cis-elements of promoter sequence were analyzed by plantCARE online analysis tool.
3.6 Quantitative expression analysis

According to the transcriptome data of *M. officinalis*, the qRT-PCR primers of *MoTPS* genes were designed by Primer primer 5.0 software. The stable GAPDH gene as reference gene. Using real-time fluorescent quantitative PCR instrument (Applied Biosystems™ 7500 of ThermoFisher) to carry out fluorescent quantitative PCR amplification, the fluorescent dye was SYBR green of ThermoFisher, and the test kit was SYBR Kit of Takara. Three parallel experiments and repetitions were applied for each sample. The relative expression of three genes were calculated by CT (2−ΔΔCT) method.

Authors’ contributions

Chengcheng Zhou is the executor of the experimental design and research of this study; Chengcheng Zhou, Dejin Xie and Youjie Ye had completed the data analysis and the writing of the first draft of the thesis; Chengcheng Zhou and Tianyou He had participated in the experimental design and the analysis of the experimental results; Yushan Zheng is the designer and person in charge of the project, guiding the experimental design, data analysis, thesis writing and modification. All authors read and approved the final manuscript.

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