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Telomerase Activity of Apple Plantlets *in Vitro* Kept Relative Stable after Conserved for Years

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Abstract In order to understand the telomerase characteristics in relation to the proliferation and aging of the tissue culture plantlets of apple, the leaves, stem segments and callus sampled from *Malus domestica* 'Golden Delicious', *Malus domestica* 'Fuji' and *Malus domestica* 'Gala' plantlets that subcultured in vitro for years were analyzed for the telomerase activity and the amount of telomerase reverse transcriptase gene expression by using modified TRAP and Quantitative Real-time PCR. The results showed that the telomerase activity in the leaves and stem segments had no significant difference among the plantlets that subcultured for different times. During a given subculture time, the telomerase activity in leaves showed a tendency of increasing first and then declining along with the leaf aging in a ninety-day period. The telomerase activity varied in different materials, among which *Malus domestica* 'Golden Delicious' callus and *Malus domestica* 'Gala' leaves appeared the highest amount, respectively. The expression of telomerase reverse transcriptase gene was consistent with the change of telomerase activity.
Keywords Apple; Plantlet *in vitro*; Subcultured times; Plantlet aging; Telomerase activity; Telomerase reverse transcriptase

Plant tissue culture is one of the most important methods to preserve fruit germplasm resources, but frequent and long-term subculture of germplasm may cause problems such as growth, proliferation capacity change and genetic variation. Telomere is a special nucleic acid protein complex at the end of linear chromosome in eukaryotes, which plays an important role in maintaining chromosome stability and preventing degradation or fusion of chromosome ends (Blackburn, 1991; Riha et al., 1998). In the process of cell division, due to the semiconservative DNA replication, a telomere sequence will be lost after each replication and division, resulting in the shortening of telomere length, weakening of cell differentiation and gradual aging and death. Telomerase is an important biological enzyme that maintains normal cell division and affects cell life. It is mainly composed of telomerase reverse transcriptase, telomerase RNA and related proteins (Blackburn et al., 2006), and among them, telomerase reverse transcriptase has the ability to synthesize telomere end structure, which can prevent the loss of telomere structure at the end of chromosome, slow down cell aging and maintain the stability and integrity of genome (Wang et al., 2019).

The study of telomerase in plant cells started late, and different plant species, different tissue parts and different environments will cause changes in telomerase. Studies on *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Hordeum vulgare* and other plants have found that telomerase is mainly distributed in meristems and organs with vigorous cell division (Riha et al., 1998). The callus of *Hordeum vulgare* and *Arabidopsis thaliana* with strong differentiation ability has higher telomerase activity (Mcknight et al., 2002). Highly differentiated *Ginkgo biloba* leaves and dormant embryos have lower telomerase activity (Mu et al., 2014). The telomerase activity of *Ginkgo biloba* trees also has seasonal specificity and different response characteristics with tree age (Song et al., 2010). *Arabidopsis thaliana* with loss of telomerase activity can survive for 10 generations, and its telomere DNA is reduced by 500 bp in each generation. Somatic cells from the sixth generation begin to divide abnormally, and agronomic traits have also been mutated (Fitzgerald et al., 1999). Some scholars believe that telomerase activity can be used as an effective evaluation index for the division and proliferation ability of plant tissues and cells (Mu et al., 2014).

Our team has been engaged in the rapid propagation of apple tissue culture and the preservation of germplasm *in vitro* for more than 30 years. The stem tip explants of apple were collected from the same mother plant in different years for inoculation and culture. The plantlets *in vitro* of *Malus domestica* 'Fuji', *Malus domestica* 'Gold Delicious' and *Malus domestica* 'Gala' with different subcultured times and different preservation years were accumulated, the growth and proliferation characteristics of which had no significant difference (Shi et al., 2007; Liu et al., 2011). In this study, the leaves, stem segments and callus of apple plantlets *in vitro* with different subcultured times and different plantlet ages were analyzed for the telomerase activity and the amount of telomerase reverse transcriptase gene expression, and the relationship between telomerase activity and cell aging and plant growth was also analyzed, which provided a theoretical basis for apple germplasm resources to conserve *in vitro* for a long term.

1 Results and Analysis

1.1 Telomerase activity in the apple plantlets *in vitro*

1.1.1 Telomerase activity in the apple plantlets *in vitro* subcultured for different times

The determination of telomerase activity was performed on the 30-day leaves sampled from *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Fuji' plantlets that subcultured *in vitro* for 143, 71, 34 times and from *Malus domestica* 'Gala' plantlet that subcultured *in vitro* for 165, 71, 34 times. The results showed that the number and brightness of leaf strips in *Malus domestica* 'Golden Delicious', *Malus domestica* 'Fuji' and *Malus domestica* 'Gala' plantlets that subcultured *in vitro* for different times were basically the same, indicating that the telomerase activity of which had no significant difference (Figure 1).

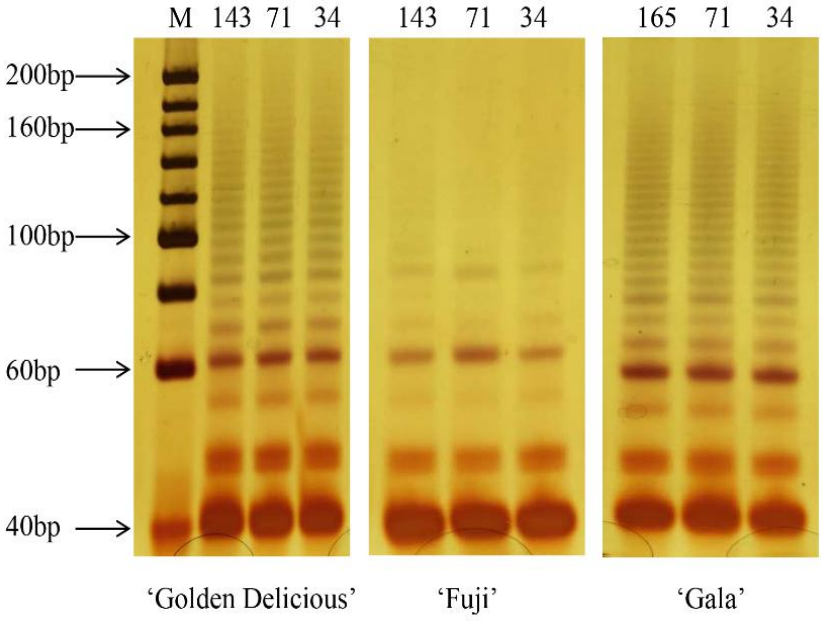

Figure 1 Telomerase activity in the apple plantlets *in vitro* subcultured for different times

Note: M: DL500 DNA Marker

1.1.2 Telomerase activity in the different age apple plantlets *in vitro*

The determination of telomerase activity was performed on the leaves sampled from the apple plantlets *in vitro* with the same subculture times and different plantlet ages of 10 d, 30 d, 50 d and 90 d (Figure 2). During the ninety-day period, the telomerase activity in leaves showed a tendency of increasing first and then declining along with the leaf aging. The telomerase activity of the leaves sampled from *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Gala' plantlets *in vitro* was the highest at the plantlet age of 30 d, and the telomerase activity of the leaves sampled from *Malus domestica* 'Fuji' plantlets *in vitro* was the highest at the plantlet age of 50 d.

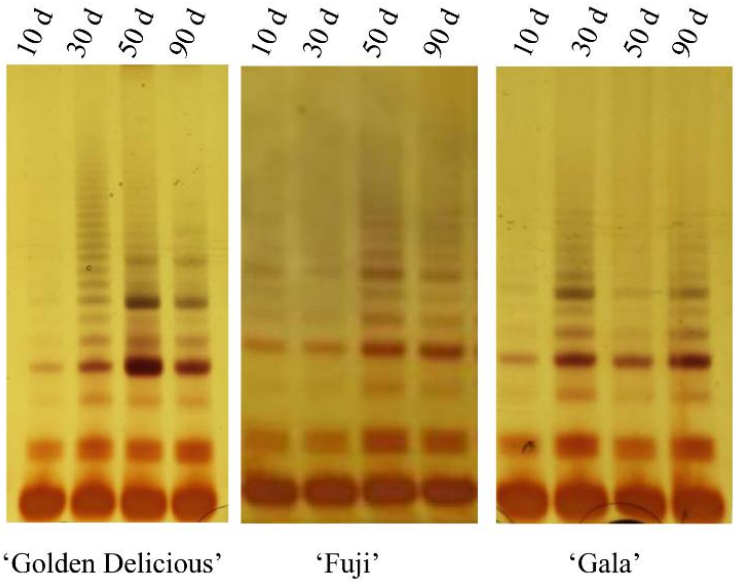

Figure 2 Telomerase activity in the different age apple plantlets *in vitro*

1.1.3 Telomerase activity in the different materials of the apple plantlets *in vitro*

The determination of telomerase activity was performed on 30-day leaves and stem segments and 20-day callus sampled from *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Gala' plantlets *in vitro* (Figure 3). The results showed that the telomerase activity varied in different materials of these two varieties, among which *Malus domestica* 'Golden Delicious' callus and *Malus domestica* 'Gala' leaves appeared the highest amount, respectively.

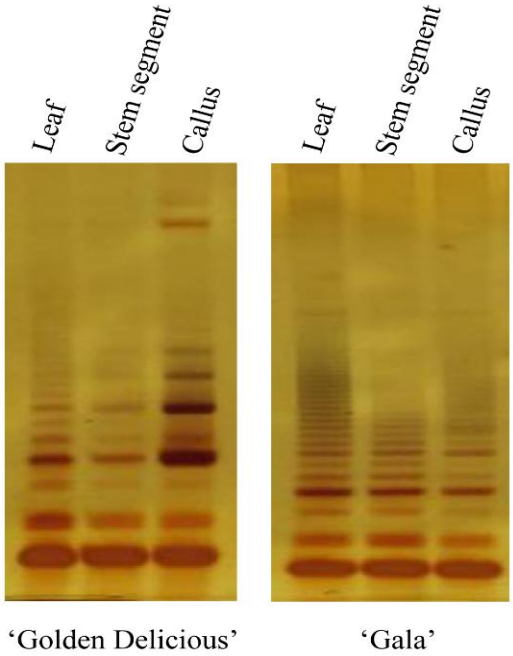

Figure 3 Telomerase activity in the different materials of the apple plantlets *in vitro*

1.2 Telomerase reverse transcriptase gene expression in the apple plantlets *in vitro*

1.2.1 Telomerase reverse transcriptase gene expression in the apple plantlets *in vitro* subcultured for different times

The telomerase reverse transcriptase gene expression was analyzed on the leaves sampled from *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Fuji' plantlets that subcultured *in vitro* for 143, 71, 34 times and from

Malus domestica 'Gala' plantlet (at the plantlet age of 30 d) that subcultured *in vitro* for 165, 71, 34 times (Figure 4). The results showed that the telomerase reverse transcriptase gene expression had no significant difference among these three apple varieties that subcultured for different times each, which was consistent with the change of telomerase activity.

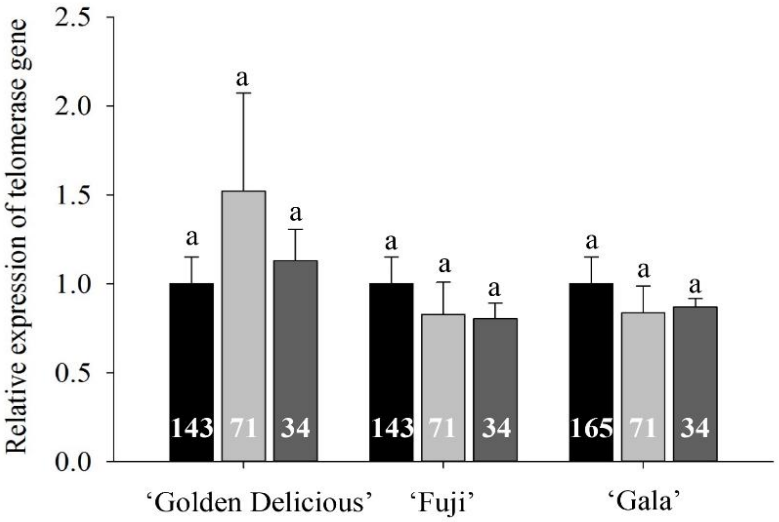

Figure 4 Telomerase reverse transcriptase gene expression in the apple plantlets *in vitro* subcultured for different times

Note: The number on the column in the column graph is the times of subculture; Different letters within a cultivar indicate significant differences (p<0.05)

1.2.2 Telomerase reverse transcriptase gene expression in the different age apple plantlets *in vitro*

The telomerase reverse transcriptase gene expression was analyzed on the leaves sampled from *Malus domestica* 'Golden Delicious', *Malus domestica* 'Fuji' and *Malus domestica* 'Gala' plantlets *in vitro* at different plantlet ages. During the ninety-day period, the telomerase reverse transcriptase gene expression in leaves showed a tendency of increasing first and then declining among these three apple varieties. The expression of the leaves sampled from *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Gala' plantlets *in vitro* was the highest at the plantlet age of 30 d, and the expression of the leaves sampled from *Malus domestica* 'Fuji' plantlets *in vitro* was the highest at the plantlet age of 50 d, which was consistent with the change of telomerase activity (Figure 5).

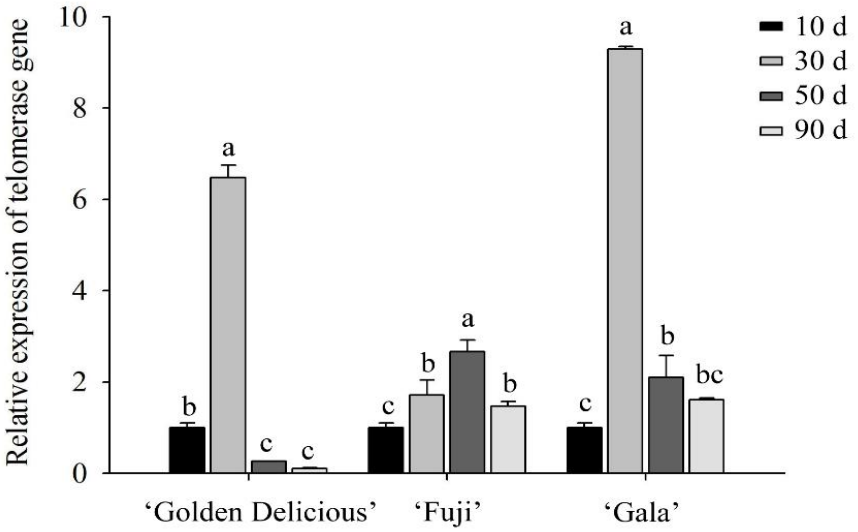

Figure 5 Telomerase reverse transcriptase gene expression in the different age apple plantlets *in vitro*

Note: Different letters within a cultivar indicate significant differences (p<0.05)

1.2.3 Telomerase reverse transcriptase gene expression in the different materials of the apple *in vitro*

The telomerase reverse transcriptase gene expression was analyzed on 30-day leaves and stem segments and 20-day callus sampled from *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Gala' plantlets *in vitro* (Figure 6). The results showed that the telomerase reverse transcriptase gene expression varied in different materials of these two varieties, among which the telomerase reverse transcriptase gene expression of *Malus domestica* 'Golden Delicious' was as follows: callus > leaf > stem segment, and the telomerase reverse transcriptase gene expression of *Malus domestica* 'Gala' was as follows: leaf > stem segment > callus, which was consistent with the change of telomerase activity.

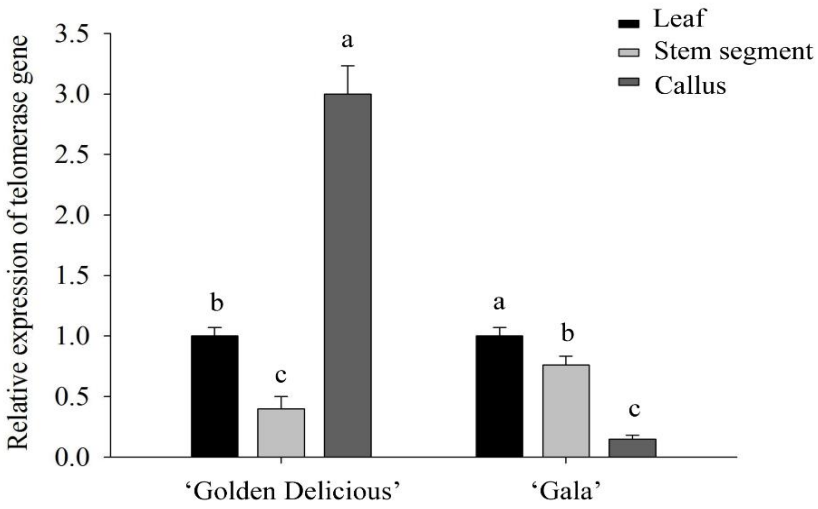

Figure 6 Telomerase reverse transcriptase gene expression in the different materials of the apple *in vitro*

Note: Different letters within a cultivar indicate significant differences ($p<0.05$)

2 Discussion

The test materials used in this study were *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Fuji' plantlets *in vitro* preserved for 8, 13 and 22 years, and *Malus domestica* 'Gala' plantlets *in vitro* preserved for 8, 13 and 25 years, which have maintained a good proliferation capacity for these years. The proliferation characteristics and phenotypes of these plantlets *in vitro* inoculated in different years and subcultured for different times had no significant differences, which provided a theoretical basis for apple germplasm resources to conserve *in vitro* for a long term.

Telomerase activity in tree core, needles and roots of *Pinus longaeva* aged from 20 to 3500 years showed a tendency of increasing or declining with the tree aging, but telomerase is present and remains active in all tree ages (Flanary and Kletetschka, 2005). In this study, telomerase was detected in the leaves of apple plantlets *in vitro* subcultured for different times, and had no significant difference, indicating that the increase of subculture times had no significant effect on telomerase activity, which was consistent with the proliferation characteristics and phenotypes of apple plantlets preserved for many years (Liu et al., 2011). And there were no signs of aging.

Telomerase is closely related to plant growth, cell proliferation, DNA repair and apoptosis inhibition (Fojtová et al., 2002). It is generally believed that telomerase activity is higher in tissues and organs with vigorous cell division. In the study of Song et al. (2010), telomerase was detected in embryo callus, microspore and leaves of *Ginkgo biloba*, and the telomerase activity in embryo callus was the highest. Studying on *Hordeum vulgare* and *Zea mays* seeds and the process of seed development. Killan et al. (1998) found that the young embryo had high telomerase activity and gradually declined during embryo development, and no telomerase was detected at the end of endosperm development, which also indicated that telomerase had high activity in tissues with strong differentiation ability. The results showed that the telomerase activity and the telomerase reverse transcriptase gene expression varied in different materials of *Malus domestica* 'Golden Delicious' and *Malus domestica* 'Gala' plantlets *in vitro*: among different materials, *Malus domestica* 'Golden Delicious' callus had the highest

telomerase activity, the telomerase reverse transcriptase gene expression of which was as follows: callus > leaf > stem segment, while *Malus domestica* 'Gala' leaves had the highest telomerase activity, the telomerase reverse transcriptase gene expression of which was as follows: leaf > stem segment > callus. Mu et al. (2014) showed that the telomerase activity of *Ginkgo biloba* callus subcultured for different times was variable. Only the callus subcultured for a certain number of times could maintain high and stable telomerase activity, and the proliferation ability would reach a relatively stable and high level. In this experiment, the callus was induced from the leaves of plantlets *in vitro* and subcultured for only seven times. The results of the two varieties were inconsistent. Was it related to the subculture times of callus? The reason remains to be further studied.

3 Materials and Methods

3.1 Plant materials

The stem tips of *Malus domestica* 'Gold Delicious' and *Malus domestica* 'Fuji' plantlets *in vitro* (subcultured for 143, 71, 34 times) inoculated in 1996, 2005 and 2010 and *Malus domestica* 'Gala' plantlets *in vitro* (subcultured for 165, 71, 34 times) inoculated in 1993, 2005 and 2010 were cultured routinely and subcultured eight times a year. From 2011 to 2017, the plantlets *in vitro* were cultured at low temperature and subcultured once a year. The subculture medium was MS + 1.0 mg/L 6-BA + 0.05 mg/L NAA + 35.0 g/L sucrose + 6.0 g/L agar, pH 5.8~6.0. In 2017, the callus was induced from the leaves of plantlets *in vitro* and subcultured every 20 d. The callus subculture medium was MS + 1.0 mg/L 6-BA + 0.1 mg/L NAA + 5 mg/L V_C + 35.0 g/L sucrose + 6.0 g/L agar, pH 6.0~6.5. The culture temperature was (25±3)℃, the light intensity was 2 000 lx, and the photoperiod was (day/night) 16 h / 8 h.

The test materials used in this study to determine telomerase activity and telomerase reverse transcriptase gene expression were as follows: the top leaves of 30-day apple plantlets *in vitro* subcultured for different times, the leaves of apple plantlets *in vitro* with the same subculture times and different plantlet ages of 10 d, 30 d, 50 d and 90 d, the leaves and stem segments of 30-day apple plantlets *in vitro*, and callus cultured for 20 d.

3.2 Determination of telomerase activity

The telomerase activity was determined according to the telomeric repeat amplification protocol (TRAP) method proposed by Kim et al. (1994), and in this study we made some improvements. Take 0.25 g samples from each plant material, add 700 μL pre-cooled CHAPS lysate after liquid nitrogen grinding, centrifuge at 14 000 r/min for 20 min at 4℃, take the supernatant and add 10% (m/V) PEG8000 (Fitzgerald et al., 1996), take an ice bath for 30 min after mixing, centrifuge at 14 000 r/min for 10 min at 4℃, discard the supernatant, add 175 μL pre-cooled CHAPS lysate, take an ice bath again for 30 min after mixing, centrifuge at 14 000 r/min for 10 min at 4℃, and then store the supernatant at -80℃ for later use.

Coomassie brilliant blue method was used to determine the content of telomerase protein. The design of forward primers and reverse primers referred to TS21 (5'-GACAATCCGTCGAGCAGAGTT-3') and RP (5'-CCCTAAACCCTAAACCCTAAA-3') researched by Fitzgerald et al. (1996).

First, telomere extension was performed and 5 μL TRAP buffer, 4 μL dNTP mixture, 1 μL TS21 and 100 ng telomerase protein was added, and then water was added to 48 μL. After mixing, it was put into PCR apparatus, and the extension condition was 26℃ for 45 min; pre-denaturation at 94℃ for 2 min. Then the telomerase was amplified, 1.6 μL RP and 0.4 μL *Taq* were added to the extension product, and the three-step method (Wang et al., 2012) was used for the PCR reaction: 94℃ 45 s, 48℃ 60 s, 72℃ 60 s, for 5 cycles; 94℃ 45 s, 50℃ 60 s, 70℃ 70 s, for 5 cycles; 94℃ 45 s, 56℃ 60 s, 72℃ 60 s, for 27 cycles; equilibrate at 72℃ for 10 min.

13.5% of the non-denaturizing polyacrylamide gel was used. After electrophoresis, the gel was dyed in the pre-prepared dyeing solution (0.5 g $AgNO_3$, ultrapure water was added to 500 mL) for 10 min. Rinse once with ultrapure water, and add developer (5 g NaOH, 2.5 mL formaldehyde, ultrapure water was added to 500 mL) and then develop for about 8 min. Finally, rinse with double distilled water. The telomerase activity was reflected by the number and depth of the ladder strip.

3.3 Real-time fluorescent quantitative PCR method to determine telomerase reverse transcriptase gene expression

Take 0.1 g samples from each plant material. Use OMEGA's Plant RNA Kit to extract the total RNA, and see the instructions for specific operations. Using the total RNA as a template, the One-Step gDNA Removal and cDNA Synthesis SuperMix kit from TransGen Biotech was used to perform the RNA reverse transcription reaction to prepare cDNA. The *ACT* gene was used as an internal control, and the fluorescent quantitative primer is TERT (Table 1), The Top Green qPCR SuperMix kit from TransGen Biotech was used to perform the real-time fluorescent quantitative PCR reaction. PCR system was 20 μL: 10 μL Top Green qPCR Mix, 1 μL forward primer, 1 μL reverse primer, 6 μL cDNA template, 2 μL H_2O. The reaction conditions were as follows: pre-denaturation at 95℃ for 5 min; denaturation at 95℃ for 10 s, annealing at 60℃ for 10 s, extension at 72℃ for 10 s, and for 40 cycles. ΔΔCT method was used for data processing, and three biological replicates and three technical replicates were set for all samples.

Table 1 The primer sequences used for gene expression analysis

Primers	Primer sequence (5'→3')
TERT(FP)	CGATTACCGCCACCTCCTCAA
TERT(RP)	ACACCAAGCCGACGAAGTTAGA
ACT(FP)	CACTATGCCGTGCTCAATG
ACT(RP)	GGTATGGGTCAGAAGGATGC

3.4 Data processing and statistical analysis

DPS data processing system version 12.01 and Excel 2010 were used for data statistical analysis.

Authors' contributions

LC participated in the execution of the research and data analysis, and wrote the manuscript. LYJ was the designer and executor of the research, and conducted data analysis. WL participated in the execution of the research and paper revision. SXX and DGQ were the creator and the person in charge of the research who directed the experiment, data analysis, manuscript writing and revision. All authors read and approved the final manuscript.

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