Daily accumulation of melatonin and gene expression in chinese cherry

Xin Wang¹, Tian Shen¹, Peng Hu¹, Fangren Liu¹, Xuefeng Zhang¹, Gui-hong Zhou², Dong Liang^{1,3} and Hui Xia^{1,3,a}

¹College of Horticulture, Sichuan Agricultural University, Chengdu, Sichuan, China

²Science Technology & Agriculture Livestock Bureau of Wenchuan county, Aba Tibetan and Qiang Autonomous Prefecture, Sichuan 623000, China

³Institute of pomology and Olericulture, Sichuan Agricultural University, Chengdu, Sichuan, China

Abstract. To explore the accumulation characteristics of melatonin and underline mechanism in leaves of chinese cherry, the daily changes of melatonin content and the expression of synthetic genes were determined. The results showed that melatonin content increased gradually during the day and midnight, reaching the peak at 2:00 a.m, and then began to decrease, indicating that light regulate the synthesis of melatonin in plants. The expression profile of 5 melatonin synthesis genes was investigated by qRT-PCR, among them the mRNA transcript of *PacSNAT*, *PacASMT* and *PacT5H2* increased and then decreased with time prolonging, in accordance with changes of melatonin content, indicating their key role in melatonin synthesis.

1 Introduction

Melatonin, a new found phytohormone, has been confirmed in a wide range of biological functions in plants. It can effectively eliminate hydroxyl free radicals, peroxide free radicals, superoxide anion free radicals and singlet oxygen, and its antioxidant capacity is higher than that of carotenoids, glutathione, VC and VE [1]. Studies have shown that the melatonin content of cherry fruit reaches the highest in a day at 14:00 when the temperature is highest and the light is strongest [2], indicating that light stimulates the synthesis of melatonin in plants, or that melatonin in plants accumulates continuously during the day and finally reaches the peak [3]. Studies in grapes have also found a circadian rhythm in melatonin metabolism, which is conducive to the accumulation of melatonin in the dark environment and promotes the synthesis of melatonin in the daytime with high light intensity [4]. The synthesis of melatonin in hyacinth also has a rhythm, showing that the melatonin content reaches the highest before sunset, while the melatonin content decreases at night [5]. Previous studies have concluded that the accumulation of melatonin in plants is closely related to light exposure, but there may be significant differences among different plants [6].

In this study, the diurnal variation of melatonin content in cherry leaves and the expression of genes were analyzed, aiming to explore the relationship between the accumulation of melatonin in cherry leaves and the day and night, as well as the expression rule of genes involved in melatonin synthesis.

2 Materials and methods

2.1 Material preparation and handling

In this experiment, cherry leaves were used as test materials. When the fruit is ripe, the ripe leaves were collected every four hours, from 10:00 on the day to 6:00 on the next day (10:00, 14:00, 18:00, 22:00, 2:00, 6:00), and put into an ice box. A total of 6 sets of leaves were collected, 60 pieces for each set (10 tablets for one repeat, a total of 6 repeats), and it is promptly sent to the laboratory for packaging, quick-frozen with liquid nitrogen, stored in the refrigerator at -80°C, the content of melatonin is determined, and RNA is extracted.

2.2 Melatonin content determination

High performance liquid chromatography-fluorescence assay was used. The 0.5g leaves were ground into homogenate in 5ml methanol without light. Then it was extracted by 200W ultrasonic oscillation for 30min and centrifuged at 10000r/min for 15min. The supernatant was taken 2ml, filtered with 0.22um organic filter membrane for chromatographic analysis. The samples were separated on a nertsil ODS-3 C18 column (4.6×250mm) with mobile phase (Water: methanol: acetic acid =44.9:55:0.1) at a flow rate of 0.8ml·min⁻¹. Melatonin was detected at 280nm excitation and 384nm emission wavelengths.

^a susanxia_2001@163.com

2.3 Gene expression

The expression patterns of five genes involved in melatonin synthesis, including *TDC*, *T5H1*, *T5H2*, *SNAT* and *ASMT* were investigated by qRT-PCR. The primers were design using Primer3 based on our transcription data. Total RNA was extracted from samples according to the instructions of RNA prep pure Plant Kit. The concentration of total RNA samples was detected by nucleic acid protein analyzer, and the integrity was detected by 1.2% gel electrophoresis.

According to the instructions of the Prime Script™ Reverse Transcriptase Kit, total RNA was used as a template for Reverse transcription into cDNA. After the mixing centrifugation, reverse transcription was performed on a PCR instrument (37 °C for 15 minutes, 85 °C for 5 seconds), then centrifugation was performed. The samples were diluted 10 times and the concentration of RT products was determined by Nnd-2000C nucleic acid protein detector. According to the results, all RT products were diluted to the same concentration for PCR amplification.

Table 1. Primers used in this study.

Primer	Sequence
<i>PacTDC</i> -real	AACCGGGAAGATTTTGCTT CACATGCCTCTCCTCAGTCA
<i>PacT5H1</i> -real	CAAAGCAATTCTGCAGGACA TTGGTTGAGGTCGCTCTCTT
<i>PacT5H2</i> -real	CAAATGCCACTCGACAGAGA ACCCTGGCACTACCAATCTG
<i>PacSNAT</i> -real	GATCAGAGGGGAATGACCAA GCCCTGATAACCAGGATCAA
<i>PacASMT</i> -real	GATGAGCCTAGCTCCAATGC GGCAAAGTCCCAAATCTCAA
<i>Actin</i>	CTTGCATCCCTCAGCACCTT TCCTGTGGACAATGGATGGA

Primers for the expression analysis of four genes were designed based on the sequences of the genes cloned by the research group. By reverse transcription of cDNA template, reference SYBR® Premix Ex Taq™ II kit instructions operate (reaction system for SYBR Premix Ex Taq™ 10.0 µL, primers respectively 0.4 µL, ddH₂O 8.2 µL). PCR amplification was performed using BIO-RAD iQ5™ real-time quantitative PCR instrument. The reaction procedure was: pre-denaturation at 95°C for 10min; Denatured at 95°C for 10s; Annealing at 60°C for 31s and 72°C extension 20s, a total of 40 cycles. IQ5™ real-time PCR Detection System was used to analyze gene expression data.

2.4 Data processing and analysis

Test data were processed and analyzed by Excel2016.

3 Results and analysis

3.1. Diurnal variation of melatonin content

The content of melatonin in leaves of cherries with different diurnal cycles was determined by HPLC. With the time prolonging, the melatonin content increased gradually, reaching the maximum 214.2 ng·g⁻¹ FW between at 02:00, and then decreased rapidly (135.8 ng·g⁻¹ FW at 06:00). The increase of melatonin content is mainly divided into two stages, the first stage is from 10:00 to 18:00, the second stage is from 22:00 to 2:00 the next day, and the content gradually decreases after 2:00 (figure 1).

Figure 1. The change of melatonin content in cherry.

3.2 Gene expression profiles

The expression levels of five genes in melatonin synthesis in Chinese cherry leaves were measured with a 4-hour interval (figure 2). The expression levels of the five genes all peaked at 22:00 and then decreased rapidly. Except for *PacTDC*, which reached a peak first at 14:00 p.m, exhibiting a double peak pattern. Except *PacTDC*, the daily expression trend of the other four genes was more similar to the daily variation trend of melatonin content. The difference was that the time of the peak was different, with the gene expression peak at 22:00 and the peak of melatonin content at 2:00. The expression levels of the five genes showed a tendency of first rising and then falling, and the expression levels fluctuated greatly before and after reaching the peak. Besides *PacTDC*, the expression levels of the other four genes were relatively consistent, averaging 1.77. The expression of *PacTDC* has the largest change range, with the maximum value of 3.65, which is about 12 times of the minimum value of 0.3.

Figure 2. The expression pattern of melatonin synthesis genes.

4 Conclusion

The diurnal variation of melatonin content in cherry leaves was measured, and the regularity showed that the melatonin content first rose and then fell. During the day, the melatonin content reached the peak at 2:00 a.m. And then gradually decreased. Studies have found that melatonin accumulation in plants in the dark phase [4], which is consistent with the conclusion of this experiment. In general, the expression level of several genes generally peaked at 22:00, while the peak value of melatonin content appeared at 2:00. The main reason may be that it took some time between the gene expression and the synthesis of melatonin, so the peak value of content change occurred later. The expression level of *PacTDC* in the five genes was significantly different from that of the other four genes, the difference mainly occurred between 10:00 and 14:00, and the expression level of *PacTDC* was much higher than that of the other four genes and the 24-hour study showed a double peak. Therefore, it was speculated that the increase of melatonin content in the first stage was mainly related to the expression of *PacTDC*, while the increase of melatonin in the second stage was caused by the co-expression of the five genes. A double peak of melatonin synthesis was found in studies on sweet cherries, suggesting that darkness and oxidative stress could induce melatonin synthesis [2]. In this experiment, the expression of *PacTDC* also showed a double peak, which was speculated to be the synthesis of dark induced melatonin. Studies have found that in the process of cherry fruit development of *PacTDC* gene expression and the accumulation of melatonin is consistent. In the short term, the diurnal variation of the expression of *PacTDC* quantity related to the accumulation of melatonin is also [2]. And the experimental research object for the cherry leaves, the results show that *PacTDC* with other four kinds of differences in gene expression. But with the change trend of melatonin levels is consistent, and closely related to the synthesis of melatonin, show the positive correlation. The accumulation rule of melatonin content in cherry leaves is that it rises first and then falls, and the increase of melatonin content is mainly divided into two stages. The increase of melatonin content in the first stage is mainly related to the expression of *PacTDC*, and the increase of melatonin content in the second stage is the joint influence of *PacTDC*, *PacSNAT*, *PacASMT*, *PacT5H2*, *PacT5H1* and other genes. Changes in melatonin content are regulated by the expression of genes for melatonin synthesis.

Acknowledgement

This work was financially supported by the Sichuan Science and Technology Project under Grant 2018JY0461 and Sichuan Innovation Training Program for College Students under Grant S201910626108.

References

1. T. DunXian, R. Hardeland, L.C. Manchester, jr. Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science. *J. Journal of Experimental Botany*, **63**, 2: 577-597. (2012)
2. Z. Yu, T. DunXian, L. Qiong, jr. Melatonin and its potential biological functions in the fruits of sweet cherry. *J. Journal of Pineal Research*, **55**, 1: 79-88, (2012)
3. W. Meng, F. XiaoYuan, D. Ying, jr. Research progress on melatonin and its nutritional function in cherry fruit. *J. Food science*, **35**, 19: 307-311. (2014)
4. T. YaJun, G. Yulu, Z. Heng, jr. Effects of circadian rhythm changes and fruit bagging on melatonin in grapes. *J. Chinese and foreign grapes and wine*, **4**: 38-41. (2018)
5. T. DunXian, L.C. Manchester, M.D. Paolo, jr. Novel rhythms of N1-acetyl-N2-formyl-5-methoxykynuramine and its precursor melatonin in water hyacinth: importance for phytoremediation. *J. FASEB Journal*, **21**, 8: 1724-1729. (2007)
6. L. Ding. A preliminary study on melatonin accumulation and TDC function of synthesis gene in paeonia lactiflora. D. Yangzhou university, (2017).