Diurnal variation of melatonin content in sweet cherry leaves

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Abstract. In order to explore the accumulation characteristics of melatonin in sweet cherry leaves, the melatonin content was measured every 4h for a whole day in mature leaves of 'Hongdeng' and the expression patterns of related synthetic genes were investigated. The melatonin content displayed double peaks at 14:00 and 22:00, respectively, speculating that high temperature and strong light intensity promote the synthesis of melatonin in leaves. In addition, expression pattern of 5 melatonin synthesis genes *TDC*, *T5H1*, *T5H2*, *SNAT* and *ASMT* were investigated by qRT-PCR. Among them, expression level of *PacTDC*, *PacASMT*, *PacT5H1* and *PacSNAT* was positively related to the accumulation of melatonin. This study provides new information on melatonin synthesis in plants, especially in sweet cherry.

1 Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a newly discovered indoles hormone, widely found in animals, plants and microorganisms. It is an amphiphilic molecule that can move in and out of cells freely and react with hydroxyl radicals and peroxides [1,2]. It was found a powerful internal (ROS)/reactive nitrogen (RNS) scavenger [3]. Melatonin can regulate biological processes, a case in point is that studies [4] have found that melatonin not only participates in the adaptation response to improve fruit yield, but also plays a certain role in improving fruit quality [5].

Fruit of sweet cherry is rich in nutrients such as vitamins, calcium, iron and so on. Therefore, it has high nutritional value. At the same time, sweet cherry is one of the fruits containing highest melatonin content compared with other fruits, which is 6-12 times that of sour cherries, 10-20000 times that of strawberries and bananas, and higher than that of mammals [6].

In recent years, melatonin has been attracted increasing attention from scientists and consumers due to its benefit function to human health. Until now, researches on melatonin in fruit trees at home and abroad mainly focus on the effect of melatonin on plant stress resistance, which fails to provide new ideas and methods for improving fruit quality and product value. Therefore, as the tree species with the highest endogenous melatonin content in fruit trees, it is of great theoretical value to study the mechanism of melatonin biosynthesis in sweet cherries. In this study, we analyzed the accumulation of melatonin and the expression of related synthetic genes in sweet cherry leaves, providing theoretical basis for the quality improvement of sweet cherry and other fruits.

2 Materials and methods

2.1 Material preparation and treatment

The test material is the mature leaf of 'Hongdeng' sweet cherry and the experiment was based on Sichuan Agricultural University. From 10:00 a.m. on the first day to 6:00 a.m. on the second day, sixty pieces of leaves were collected every 4 hours (10:00 a.m., 14:00 p.m., 18:00 p.m., 22:00 p.m., 02:00 a.m and 06:00 a.m) and were put into the ice box. Then the leaves were brought to the laboratory, and quick-frozen with liquid nitrogen in time. Then the experimental materials were stored in the refrigerator at -80°C for the determination of melatonin content and the extraction of RNA.

2.2 Determination of melatonin content

High performance liquid chromatography-fluorescence assay was used to determine the melatonin content. The 0.5g leaves were ground into homogenate in 5ml methanol without light, then extracted by 200W ultrasonic oscillation for 30min and centrifuged at 10000r/min for 15min. 2ml of supernatant was extracted and 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.

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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 designed by using Primer3 based on our transcriptome 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). 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. The cDNA was used as a template. The test procedure was performed in accordance with the instructions of the SYBR® Premix Ex Taq™ II kit (reaction system for SYBR Premix Ex Taq™ 10.0 µL, primers respectively 0.4 µL, ddH₂O 8.2 µL). PCR amplification was performed by 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 handling

Microsoft Excel 2016 was used to calculate the experiment data. Statistical was performed using software SPSS 22.0.

3 Results and discussion

3.1. Changes of melatonin content in sweet cherry leaves

As is shown in figure 1A, the content of melatonin in leaf extract was determined by HPLC. The retention time of melatonin standard sample was at 5.1 min. The chromatographic results of sweet cherry leaf extract were shown in figure 1B. The retention time of target peak was consistent with that of standard sample.

Figure 1. HPLC chromatogram of melatonin for (A) standard sample, (B) sample of sweet cherry leaves. The retention time of melatonin was at 5.1 min.

Within 24h, the melatonin content in the leaves of ‘Hongdeng’ appeared two peaks with the passage of sampling time. The first peak occurs at 14:00 p.m (925.7 ng·g⁻¹ FW) and the second peak occurs at 22:00 p.m (822.7ng·g⁻¹ FW). The first peak of melatonin may be related to high temperature and high light intensity. The melatonin content decreased sharply to 218.3 ng·g⁻¹ FW from 22:00 to 02:00 of the next day, which is about 23.6% at 14:00. Then increased slowly (the melatonin content was 277.1 ng·g⁻¹ FW at 06:00), and rose rapidly to 855.3 ng·g⁻¹ FW from 06:00 to 10:00 (Figure 2).

Figure 2. The melatonin content in sweet cherry leaves.

3.2 Diurnal changes in the expression of melatonin synthesis genes in sweet cherry leaves

The expression levels of five melatonin synthesis genes in leaves of ‘Hongdeng’ were measured. The results showed that five genes displayed the different expression patterns. Among them the gene expression curves of *PacASMT*, *PacT5H1* and *PacT5H2* showed double peaks (Figure 3). *PacASMT* and *PacT5H2* reached their two peaks at 10:00 and 18:00 respectively, and both had highest peak at 18:00. *PacT5H1*’s peak at 18:00 was 5.14, which was 3.1 times of the first peak. And *PacASMT*’s peak at 18:00 was 1.6 times of the first peak. *PacT5H2* reaches its two peaks at 10:00 and 22:00, whose two peaks are close to each other around 1.4. In addition, both *PacTDC* and *PacSNAT* have single peak. The expression of *PacTDC* reaches the maximum value of 2.8 at 10:00. And the expression of *PacSNAT* reaches the maximum value of 2.9 at 18:00, which is about 6 times of its minimum value. On the whole, the expression levels of the genes for melatonin synthesis in sweet cherry leaves were significantly different. And the expression levels of *PacT5H1* were the highest, about 2-3 times that of other genes. The expressions of *PacTDC*, *PacASMT* and *PacSNAT* were similar, all of their maximum values were about 3. The expression of *PacT5H2* was the lowest and its maximum value was about 1.5. The expression of all genes was at a high level around 18:00, and at the minimum value from 02:00 to 06:00 at early morning.

Figure 3. The transcript level of five genes involved in melatonin biosynthesis in sweet cherry leaves.

4 Conclusions

Some studies have found that the melatonin content of cherry fruit reaches the highest in a day during the time of the highest temperature and the strongest light [7]. Light stimulates the synthesis of melatonin, thus making the cherry resist light damage. And the melatonin

content reaches the second maximum at the end of the photoperiod. In our study, similar results were obtained. Melatonin synthesis in 'Hongdeng' mature leaves presented a "double peaks" pattern within 24 hours (14:00 and 22:00). Melatonin peaks for the first time at the highest ambient temperature and maximum light intensity (14:00). The increase in natural light intensity leads to an increase in ultraviolet radiation. High temperature stress and ultraviolet radiation can induce plants to produce a large amount of reactive oxygen species (O_2), especially hydrogen peroxide (H_2O_2). In plants, melatonin can significantly reduce the content of O_2 and H_2O_2 , and increase the synthesis and accumulation of antioxidant enzymes and antioxidants. In this way, melatonin can reduce the harm of high temperature stress and ultraviolet radiation to seedlings [8]. In sweet cherry leaves, high temperature and high light intensity also induce the biosynthesis and accumulation of melatonin to resist oxidation and light damage. The second peak occurs at 22:00, probably caused by darkness. The melatonin levels peak again at the end of the photoperiod.

Up to now, the metabolic pathways of melatonin biosynthesis have been clearly concluded [9], but the expression rules of the genes in these pathways are still unclear, so it is of great significance to study the expression of these genes in melatonin synthesis. In our study, we found that over a period of 24 hours, the peak expression time of three genes *PacASMT*, *PacT5H1* and *PacANNAT* appeared some time earlier relative to melatonin accumulation. And the three genes expression curves were consistent with melatonin biosynthesis. It indicated that the genes *PacASMT*, *PacT5H1* and *PacANNAT* positively regulate the biosynthesis of MT in sweet cherry leaves through some regulatory mechanism. *PacTDC* and *PacSNAT* reached their maximum before the two peaks of melatonin synthesis. They were involved in the regulation of MT biosynthesis in sweet cherry leaves. This result has not been confirmed its universality and further validated through experiments.

In the current study, the expression of *PacTDC*, *PacT5H1*, *PacT5H2*, *PacSNAT* and *PacASMT* was prior to the synthesis of melatonin in both fruit development and 24-hour rhythm studies. Previous studies [9] have shown that *PacTDC* and *PacT5H* can catalyze the formation of serotonin (a key intermediate in melatonin synthesis) from 5-methoxytryptophan, by *PacSNAT* and *PacASMT*. Then melatonin was produced by serotonin catalyzed with *PacSNAT* and *PacASMT*. Therefore, up-regulation of these five genes leads plants to produce melatonin. Moreover, our results showed that the five genes expression was positively correlated with the secretion of melatonin. And the expression of the genes was earlier than the synthesis of melatonin, indicating that these genes were involved in the synthesis of melatonin. This study provides new information on the rate-limiting enzymes in melatonin synthesis in plants, especially sweet cherries.

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