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Preharvest Application of Melatonin Affects the Color, Strength, and Antioxidant Capacity of Pear Peels by Regulating Phenylpropane Metabolism

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Abstract: Melatonin is an important regulator of fruit growth and development. To explore the physiological mechanism whereby preharvest melatonin application regulates the polyphenol content of 'Yuluxiang' pear peel, we sprayed 0.1 mM melatonin during the first fruit expansion and early color change periods, and the control group were sprinkled with fresh water. Then, we measured the contents of anthocyanin, lignin, and major monomeric phenolics and the activities of key enzymes associated with phenolic metabolism. The results showed that melatonin application significantly increased the content of total phenolics, total flavonoids, total anthocyanins, and lignin in the peel from the color change to mature development stages. Near maturity, the activities of all key enzymes, except dihydroflavonol-4-reductase, were higher than those in the control samples, but significant differences in enzyme activity occurred at different time points. Compared with the control group, the fruit peels of the melatonin-treated plants exhibited a higher antioxidant activity and accumulated more flavonols. Thus, preharvest spraying of melatonin can alter the activity of key enzymes associated with phenolic metabolism, increasing the total phenol, flavonoid, anthocyanin, and lignin contents, which in turn, affects the color, strength, and antioxidant capacity of pear peels.

Keywords: preharvest; melatonin; anthocyanins; lignin; antioxidant capacity

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1. Introduction

Pear is one of the world's major temperate fruits, loved for its unique flavor and high nutritional value. In 2021, global pear production was approximately 25,659,000 t, of which China, as a major pear-producing country, produced 18,978,000 t, accounting for 73.9% of the world's total pear production [1]. The 'Yuluxiang' pear is of high quality, with a reddish skin, rich juice and tender flesh. It is reputed as the highest quality pear planted in northern China [2]. However, the peel of the 'Yuluxiang' pear is extremely thin, which is a good indicator of quality, but it offers little protection during storage and transportation, resulting in injury to the fruit. Further, natural coloring is poor during production. These issues have seriously affected the commodity value of 'Yuluxiang' pears.

Polyphenols are important secondary metabolites found in pear peels that can affect fruit quality; they act as natural antioxidants, reducing oxidative damage in injured plants [3]. Polyphenol anabolism mainly involves the phenylalanine metabolic pathway, of which anthocyanin and lignin metabolism are important branches. Phenylalaninammonialyase (PAL) is the first enzyme in the phenylalanine metabolic pathway, catalyzing the generation of trans-cinnamic acid from l-phenylalanine. PAL then enters the phenylpropane metabolic pathway to generate secondary metabolites, such as flavonoids and

lignin. Studies have shown that it has an important effect on the metabolism of phenolic substances [4]. Chalcone isomerase (CHI), dihydroflavonol-4-reductase (DFR), and flavonoid 3-0-glucosyltransferase (UFGT), key enzymes involved in flavonoid metabolism [5], are associated with anthocyanoside synthesis [6–8]. Further, the enzymes cinnamate-4-hydroxylase (C4H), 4-coumarate:coenzyme A ligase (4CL), cinnamyl-alcohol dehydrogenase (CAD), and peroxidase (POD) are closely related to lignin synthesis; the overexpression or silencing of the regulatory genes of these enzymes can notably affect the content or type of lignin produced [9–12].

Melatonin (MT), N-acetyl-5-methoxytryptophan amide, is abundant in plants. MT has recently emerged as an applied plant growth-regulating compound. Studies have shown that MT plays a wide range of physiological roles in plant growth and development [13–15], such as increasing resistance to adversity [16,17] and improvement in the storage quality of fruits [18–21]. In terms of promoting fruit coloration, researchers found that spraying MT at an early stage of color change could accelerate coloration in southern fruit pears, though it had little effect on the intrinsic quality of the fruit [22]. In addition, they found that MT could affect the accumulation of anthocyanosides through RBOH (respiratory burst oxidase homologs)-mediated ROS production [23]. Other studies have shown that MT can also promote the accumulation of anthocyanidins in fruits through the ethylene or ABA metabolic pathways [24,25]. Lignin plays a crucial role in enhancing cell strength as a key component of the cell wall. Previous studies have shown that exogenous melatonin treatment can significantly increase lignin content in the flower stalks of Chinese rose, thus improving the resistance of stalks to felling [26]. Another study found that spraying melatonin at an appropriate concentration increased the total phenolics, flavonoids, and lignin to enhanced the resistance of cherry tomatoes to disease [27]. Other studies have shown that postharvest melatonin application not only maintains fruit quality during storage in loquat [28] and kiwifruit [29], but also reduces pulp lignification during cold storage in both.

The antioxidant system is a key factor in the resistance of plants to adverse effects. Melatonin has been found to significantly increase the antioxidant capacity of crops to cope with stress and damage in several studies [30–33]. A previous experiment showed that spraying 0.1 mM melatonin promoted fruit coloring and improved the peel strength of 'Yuluxiang' pear [34]. Studies have shown that anthocyanin content is closely related to fruit coloring [35–37], while lignin, an important constituent of the cell wall, influences the mechanical strength of the cell wall [38]. However, the mechanism underlying the regulatory effects of preharvest melatonin on anthocyanins, lignin, and other phenolic substances in pear peels remains unclear. Therefore, the present study aimed to investigate the effects of preharvest melatonin spraying on the metabolism of primary phenolic metabolism in 'Yuluxiang' pear peel to elucidate the mechanism whereby melatonin physiologically regulates the metabolism of phenolic substances such as anthocyanin and lignin. Our findings help elucidate the mechanism whereby preharvest melatonin application regulates the content of phenolics and consequently affects pear fruit quality.

2. Materials and Methods

2.1. Plant Materials

The study was performed using 8-year-old 'Yuluxiang' pear (*Pyrus bretschneideri*) trees from an orchard at the Research Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning Province, China. All trees had experienced uniform growth, as well as daily management and fertilizer consistency; the only difference was the melatonin treatment. The treatments were set up in a randomized block design with one plot for one replicate of three trees, for a total of three plots and nine trees.

2.2. Experimental Treatments

Melatonin (0.1 mM) was applied via spray during the first fruit expansion period (approximately 25 days after blooming) and early color change period (approximately

30 days before harvest) to the treatment group (MT_{100}), while the control group (CK) was sprayed with fresh water. Fruit samples were collected at 0, 7, 14, 21, and 28 d after spraying was completed. The end of each spraying session was measured by dripping leaves and fruits. Four fruits were taken at a time from the periphery of the canopy center of each plot (two from the eastern and two from the western halves of each plot), yielding a total of 12 fruits. Subsequently, the fruits were stored in a cool box and taken back to the laboratory. Eight fruits were selected for peel sampling, while the remaining four were utilized for photography and paraffin sectioning. Before peeling, the fruits were washed with distilled water. The same person applied this protocol each time to ensure the homogeneity of sampling. One part of the peel samples was quickly wrapped with aluminum foil and frozen in liquid nitrogen, then it was pulverized and put into a −80 °C refrigerator for the measurement of phenolic substances, antioxidant capacity, enzyme activity and other indexes. The remaining parts of the samples were dried and ground for the determination of lignin content.

2.3. Measurement of Total Phenol, Flavonoid, and Lignin Contents

A total of 5.0 g of the frozen ground sample was extracted with 20 mL of ethanol in two batches, then adjusted to 25 mL. Then, 10 mL of the extract was aspirated on a rotary evaporator (BUCH AG, Switzerland, R-210) at 40 °C under reduced pressure evaporation to remove methanol. An HLB column (Waters Oasis®HLB, USA; 200 mg/6 CC) was activated sequentially with 5 mL of methanol and deionized water. The vial was washed with 2 mL of deionized water, which was subsequently poured into the HLB column for filtration, after which the resulting filtrate was discarded, and the process was repeated three times. Thereafter, the vial was washed with methanol in a similar manner and the filtrate was collected. Finally, 5 mL of the filtrate was blown with a stream of nitrogen and then passed through a 0.22 μm membrane filter prior to analysis.

Total polyphenol content was measured using the Folin–Ciocalteu method as previously described [39] with slight modifications. First, 0.2 mL of the sample cleanup solution was pipetted into a 15 mL centrifuge tube. Then, 3 mL of water, 1 mL of color developer, and 15% Na_2CO_3 were added. The reaction mixture was incubated at room temperature for 30 min and colorimetrically analyzed at 765 nm. Additionally, the standard curves of 0 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$, 200 $mg \cdot L^{-1}$, 300 $mg \cdot L^{-1}$, and 400 $mg \cdot L^{-1}$ gallic acid were established to calculate the total polyphenol content in the peel.

The total flavonoid content was calculated as previously described [40] with slight modifications, using catechin as the standard substance. A total of 0.5 mL of sample purification solution was mixed with 4 mL of water, 0.2 mL of 5% $NaNO_2$ solution, 0.2 mL if 10% $AlCl_3$, and 2 mL of NaOH (1 $mol \cdot L^{-1}$) solution. The solution was mixed thoroughly for colorimetric determination at 510 nm, wherein catechins of 0 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$, 200 $mg \cdot L^{-1}$, 300 $mg \cdot L^{-1}$, 400 $mg \cdot L^{-1}$, and 500 $mg \cdot L^{-1}$ of the standard curves were used to calculate total flavonoid content.

Determination of lignin was carried out as previously described in the literature [41]. A total of 5 mg of dried sample was weighed and added to 5 mL of 40% acetylbromide-glacial acetic acid solution (*w*/*w*) and 0.2 mL of perchloric acid; the mouth of the tube was sealed with a glass stopper, and the tube was placed in a 70 °C water bath for 30 min and placed in a rocker every 10 min. Subsequently, the reaction solution was completely transferred to a volumetric flask containing 20 mL of a mixture of 2 $mol \cdot L^{-1}$ NaOH/25 mL CH_3COOH. Glacial acetic acid was then added to adjust the volume to 100 mL, and the absorbance was assessed at 280 nm.

2.4. Analyses of Phenolics and Anthocyanosides

We used UPLC-PAD-MS (Waters, Milford, MA, USA) to measure the composition and content of phenolics and anthocyanins. The sample purification solution (2.0 mL) was aspirated into a brown injection bottle, and the conditions for the detection of various phenolics were set according to a previous study [42]. The chromatographic, spectroscopic,

and mass spectral characteristics of the anthocyanin component were compared with those of the standard substance to confirm its composition. Mass spectral ion-pair information was obtained from a previous studies [43]. To ensure accurate measurements, we performed comparisons with the contents and peak shape of the standard substance.

2.5. Antioxidant Capacity Measurement

For the DPPH method [44] with slight modification, 0.2 mL of a periplasmic sample was taken after 5-fold dilution and added to 3.8 mL of DPPH reaction solution (20 mg of DPPH fixed to 500 mL). Trolox was used as the standard, while methanol was used as the control for the configuration of standard substance markers with different concentrations, and the absorbance values were measured at 515 nm after the reaction in dark conditions; results are expressed as μmol TE·g^{-1} FW.

FRAP determination was performed as outlined in a previous study [45]. Briefly, the FRAP reaction solution was prepared by configuring sodium acetate buffer (30 mM, pH 3.6), 10 mM 2,4,6-tripyridin-2-yl-1,3,5-triazine (TPTZ) solution, and 20 mM Fe(III) chloride solution, mixed in a volume ratio of 10:1:1. The FRAP reaction solution was prepared and heated to 37 °C in a water bath prior to its use. A total of 100 μL of the diluted sample was added to 4 mL of the FRAP reagent, and the absorbance values were measured at 595 nm after 15 min, with $FeSO_4$ as the standard line; results are expressed as μmol Fe(II)·g^{-1} FW.

The ABTS assay was performed as previously described with slight modifications [46]. Briefly, 0.1 mL of the dilution solution was pipetted from the membrane into a 15 mL centrifuge tube, and 4 mL of ABTS reaction solution was added (7.4 mM ABTS + 2.6 mM $K_2S_2O_8$). Trolox was used as the standard substance, while methanol was used as the control. Concentration curves of 0 mg·L^{-1}, 200 mg·L^{-1}, 400 mg·L^{-1}, 600 mg·L^{-1}, 800 mg·L^{-1} and 1000 mg·L^{-1} of standard substance were calibrated and used as the standard line. After the reaction in the dark, the absorbance values were determined at 734 nm. The results are expressed as μmol TE·g^{-1} FW.

2.6. Enzyme Activity Detection

Enzyme activity detection kits (Solarbio, Beijing, China) were used to spectrophotometrically measure the activities of PAL, C4H, 4CL, and POD. The CAD activity was measured using an enzyme activity detection kit from League, Beijing, China. CHI activity was measured using an enzyme activity detection kit from Geruisi, Suzhou, China. Finally, the enzymatic activity of DFR was measured using a dedicated assay kit (ELISA, Mlbio, Shanghai, China). All assays were performed according to the manufacturer's instructions.

The UFGT enzyme solution was prepared as previously described [5] with minor modifications. Specifically, 1 g of frozen powder was weighed and added to 8.0 mL of extraction solution (0.2 mol·L^{-1} (pH 8.8) borate buffer, 0.005 mol·L^{-1} 2-Mercaptoethanol, 0.001 mol·L^{-1} EDTA, 0.001 mol·L^{-1} DTT, and 10% PVP). The solution was homogenized in an ice bath, then centrifuged at 12,000× *g* for 30 min at 4 °C. A total of 200 μL of the supernatant, 100 μL of 50 mmol·L^{-1} glycine buffer (pH 8.5), 15 μL of 2 mg·mL^{-1} quercetin, and 10 μL of 15 mg·mL^{-1} UDP-galactose were combined, and the reaction was carried out in a 30 °C water bath for 30 min. Finally, 75 μL of 20% trichloroacetic acid solution was added to terminate the reaction. One unit of enzyme activity was defined as a change of 0.001 per hour in OD at 350 nm.

2.7. Statistical Analysis

Basic calculations and data analysis were performed using Microsoft Excel 2010 (Microsoft Corporation, Washington, DC, USA); GraphPad Prism 8.0 (La Jolla, CA, USA) was used to plot bar charts and heat maps; and IBM SPSS Statistics 20.0 (Armonk, NY, USA) was used for analysis of variance (ANOVA) and correlation analyses. Correlation analysis was performed using Duncan's test ($p < 0.05$ and $p < 0.01$), and R 3.7.0 software was used for

correlation charting. All measurements were obtained from either three or four replicates and the error bars represent the standard deviation of the data.

3. Results

3.1. Changes in Total Phenols, Total Flavonoids, Total Anthocyanin, and Lignin Content

As shown in Figure 1, the total phenol and total flavonoid contents of pear peel exhibited similar fluctuation trends. Melatonin application significantly increased the total polyphenol and flavonoid content in the peel from the color change to the ripening period. The total phenolic and flavonoid contents of MT_{100} were 13.59% and 27.54% higher, respectively, than those of CK at fruit maturity. In addition, the anthocyanin content of the peel tended to increase and then decrease after melatonin treatment. Melatonin treatment significantly enhanced the anthocyanin content of pear peels from color change to ripening, and the maximum anthocyanin content was 6.75 $mg \cdot kg^{-1}$ after 21 days of treatment, which was 4.8 times higher than that of the control. The total anthocyanin content at maturity was 93.04% higher than that in the control, indicating that melatonin promotes the accumulation of anthocyanosides. Melatonin application also increased the lignin content in the peel from color change to ripening, and the lignin content in the peel at the ripening stage was 7.85% higher than that of the control.

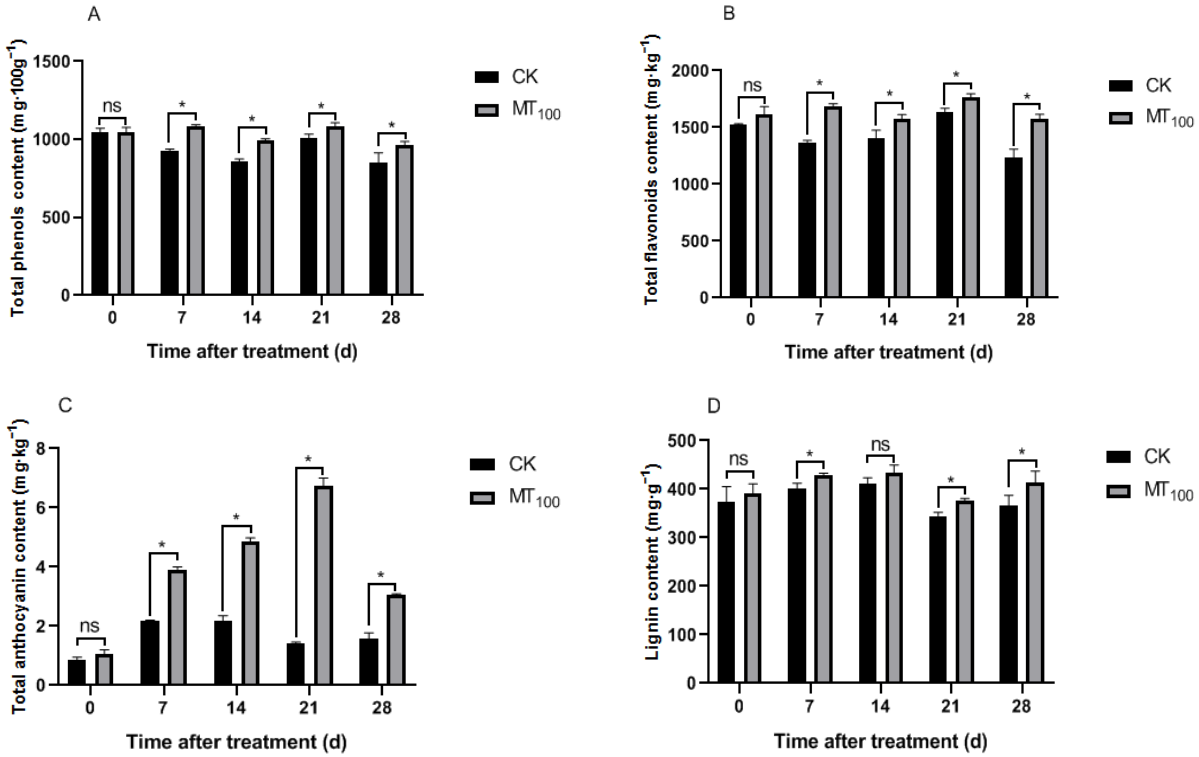

Figure 1. Changes in total phenols, total flavonoids, total anthocyanin, and lignin content at 0, 7, 14, 21, and 28 d after melatonin treatment. (**A**) Changes in the total phenol content in pear peels. (**B**) Changes in the total flavonoid content of pear peels. (**C**) Changes in the total anthocyanin content of pear peels. (**D**) Changes in the lignin content of pear peels. The error bars represent the SD across three replicates. "ns" indicates no significant difference between groups; "*" indicates significant correlation at the $p < 0.05$ level.

3.2. Effect of Melatonin on the Content of Major Phenolic Substances

A total of 23 polyphenols were detected in the peel of 'Yuluxiang' pears (Figure 2). Arbutin, chlorogenic acid, quercetin 3-O-rutinoside, isorhamnetin 3-O-rutinoside, isorhamnetin 3-O-robinioside, and epicatechin were the main polyphenols in the peel, of which the content of arbutin was the highest, ranging from 752.03 to 1105.17 $mg \cdot kg^{-1}$. Arbutin and chlorogenic acid contents gradually decreased as the fruits matured, while epicatechin,

proanthocyanidin, and flavonol contents increased. Compared with the control, the arbutin, chlorogenic acid, and epicatechin contents in the peel of 'Yuluxiang' pear on the day of MT_{100} treatment were significantly lower, while the flavonol content, such as quercetin 3-O-glucoside, quercetin 3-O-rhamnogalactoside, isorhamnetin 3-O-rutinoside, isorhamnetin 3-O-galactoside, and quercetin 3-O-rutin, in the peels pears at MT_{100} were consistently higher than those of CK from the color change to ripening stages. The arbutin content in the peels of MT_{100} was always higher than that in the control from day 7 to ripening. Chlorogenic acid, epicatechin, and proanthocyanidin contents of the MT_{100} treatment were significantly higher than those in the control on day 7. Catechin and proanthocyanidin B2 contents were significantly higher than those of CK, and with fruit age, the chlorogenic acid content was significantly lower than that of the control from days 14 to 21; however, the chlorogenic acid content in the pear peel was significantly higher than that of the control at the ripening stage. The epicatechin, proanthocyanidin B2, and catechin contents were significantly lower than those in the control from day 7 until ripening. Three anthocyanins were detected in pear peels, cyanidin-galactoside, cyanidin-arabinose, and paeoniflorin-galactoside, with cyanidin-galactoside being the most abundant. Melatonin treatment significantly increased the contents of all three substances compared to the control, with increases at the ripening stage of 91.13%, 80.00%, and 161.54%, respectively.

3.3. Effects of Melatonin on the Key Enzyme Activities of Phenolics Metabolism

PAL is the first key enzyme of the phenylalanine metabolism pathway, and as shown in Figure 3, PAL enzyme activity in the peel from the color change to ripening stages generally decreased. Additionally, compared to that of the control group, melatonin treatment significantly increased PAL enzyme activity in the peels during the same period. The C4H enzyme activity in the peel showed a general trend of decreasing–increasing–decreasing as the fruit developed; MT_{100} showed higher activity on the day 14, but there was no significant difference between activities in the MT_{100} or CK groups for the rest of the period. Meanwhile, 4CL enzyme activity showed a decreasing–increasing trend, and plants treated with MT_{100} had higher enzyme activity than that of CK on the day 14. CAD and POD were the key enzymes in lignin synthesis, with the exception of day seven after treatment, the CAD enzyme activity of MT_{100} was significantly higher than that of the control throughout development, and the MT_{100} group showed higher POD enzyme activity at days 0, 7, and 21. CHI, DFR, and UFGT were the key enzymes in the process of anthocyanoside synthesis, and the activity of CHI in pear peels was significantly higher than that of the control from day 14 onwards. In summary, melatonin treatment significantly increased UFGT enzyme activity but decreased DFR enzyme activity.

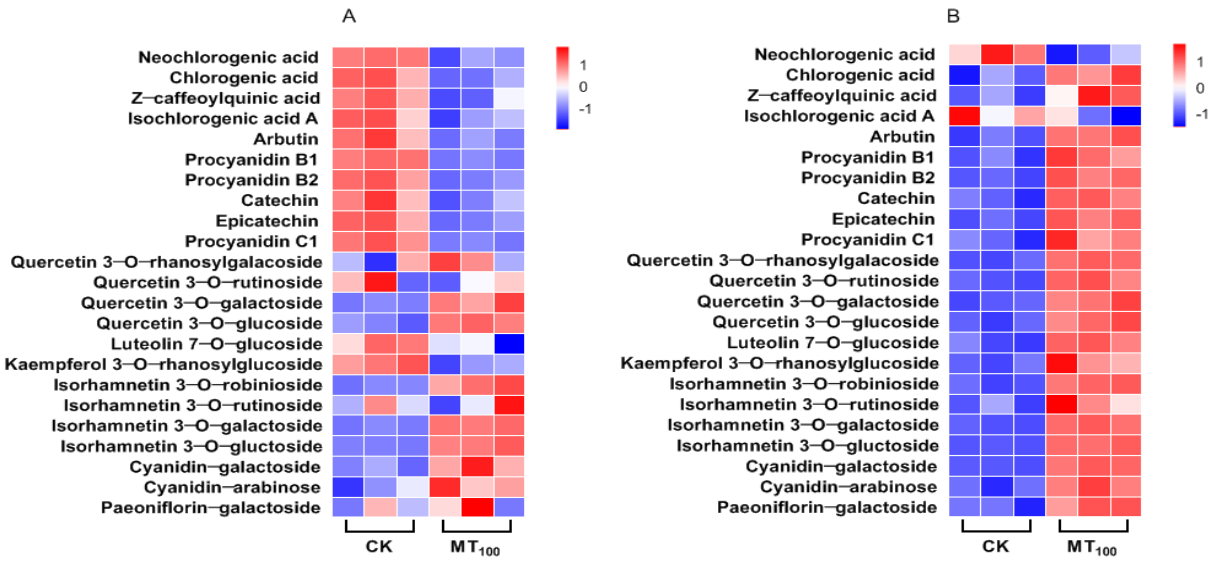

Figure 2. *Cont.*

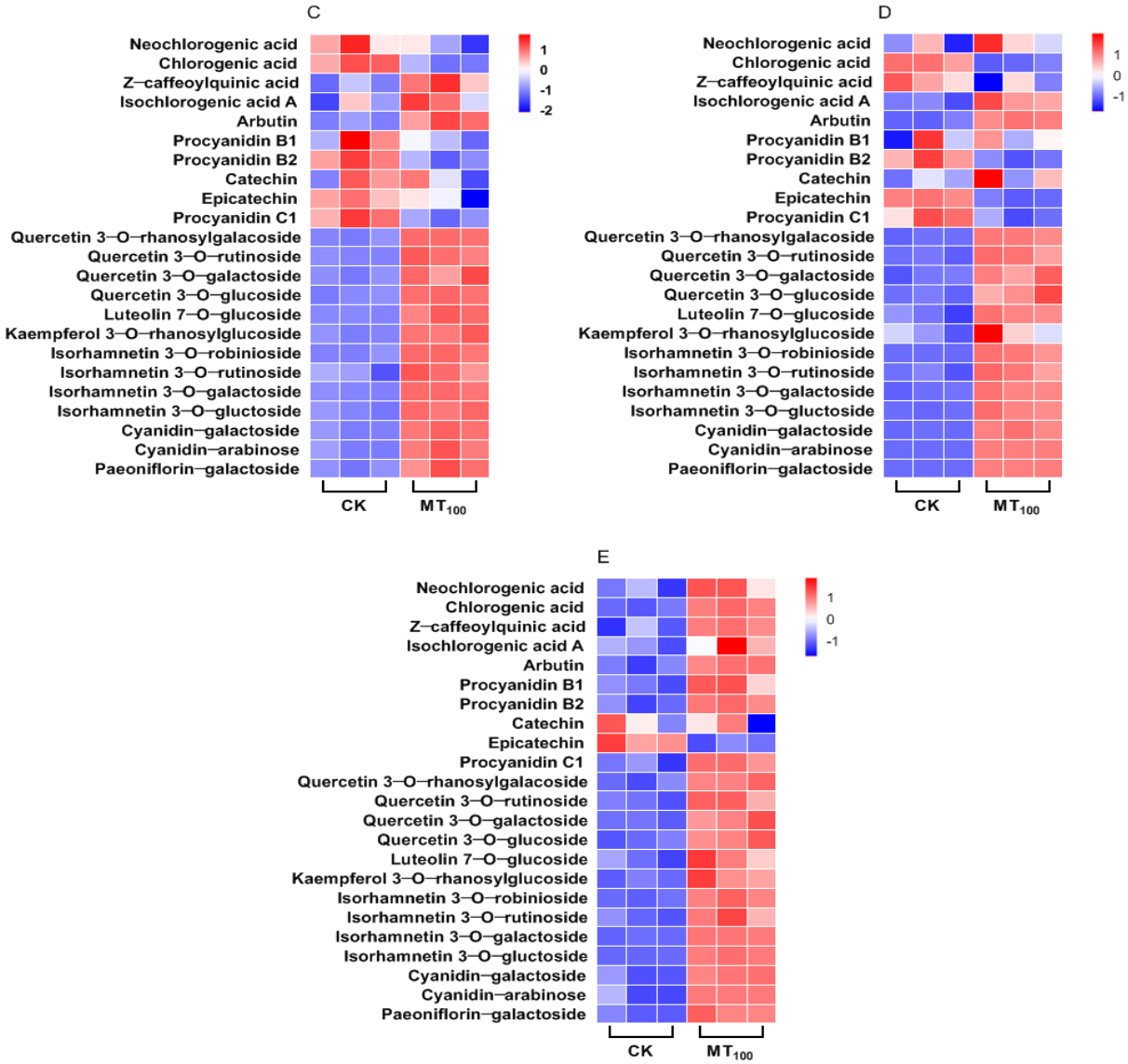

Figure 2. Changes in major monophenol contents after melatonin treatment. (**A**–**E**) represent the changes in individual phenols at 0, 7, 14, 21, and 28 days after melatonin treatment, respectively. The red and blue colors indicate the upregulation and downregulation of metabolites, respectively. Three replicates were assessed for both CK and MT_{100}.

3.4. Effects of Melatonin on Antioxidant Capacity

As shown in Figure 4, the DPPH, FRAP, and ABTS values of the peel showed similar slight fluctuations from the color change to the ripening stage. Specifically, from days 7 to 28, the ABTS of MT_{100} was significantly higher than that of the control. The DPPH and FRAP of the peel showed similar increasing trends. The DPPH, FRAP, and ABTS activities were higher in the peels of fruit in the MT_{100} group than those in the CK group at the ripening stage, evidenced by increases of 15.11%, 14.28%, and 21.83%, respectively. In summary, melatonin can enhance the antioxidant capacity of the peel of pear fruits from the color change to the ripening stage.

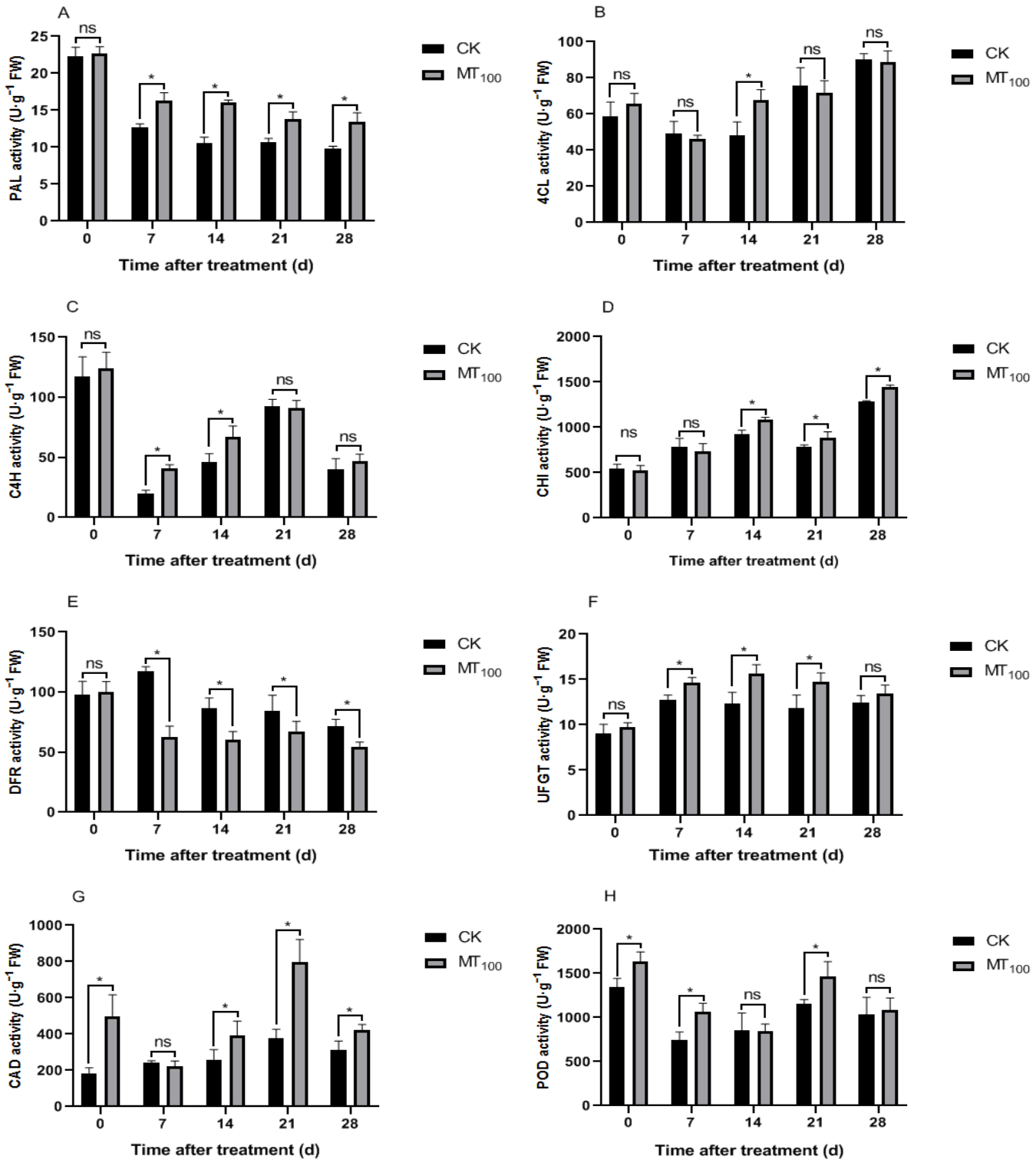

Figure 3. Changes in the activities of key enzymes in phenolic metabolism at different fruit developmental stages. Changes in the enzyme activities of (**A**) PAL, (**B**) 4CL, (**C**) C4H, (**D**) CHI, (**E**) DFR, (**F**) UFGT, (**G**) CAD, and (**H**) POD in pear peels at 0, 7, 14, 21, and 28 d after treatment. Error bars indicate standard deviations of three replicates. "ns" indicates no significant difference between groups; "*" indicates significant correlation at the $p < 0.05$ level.

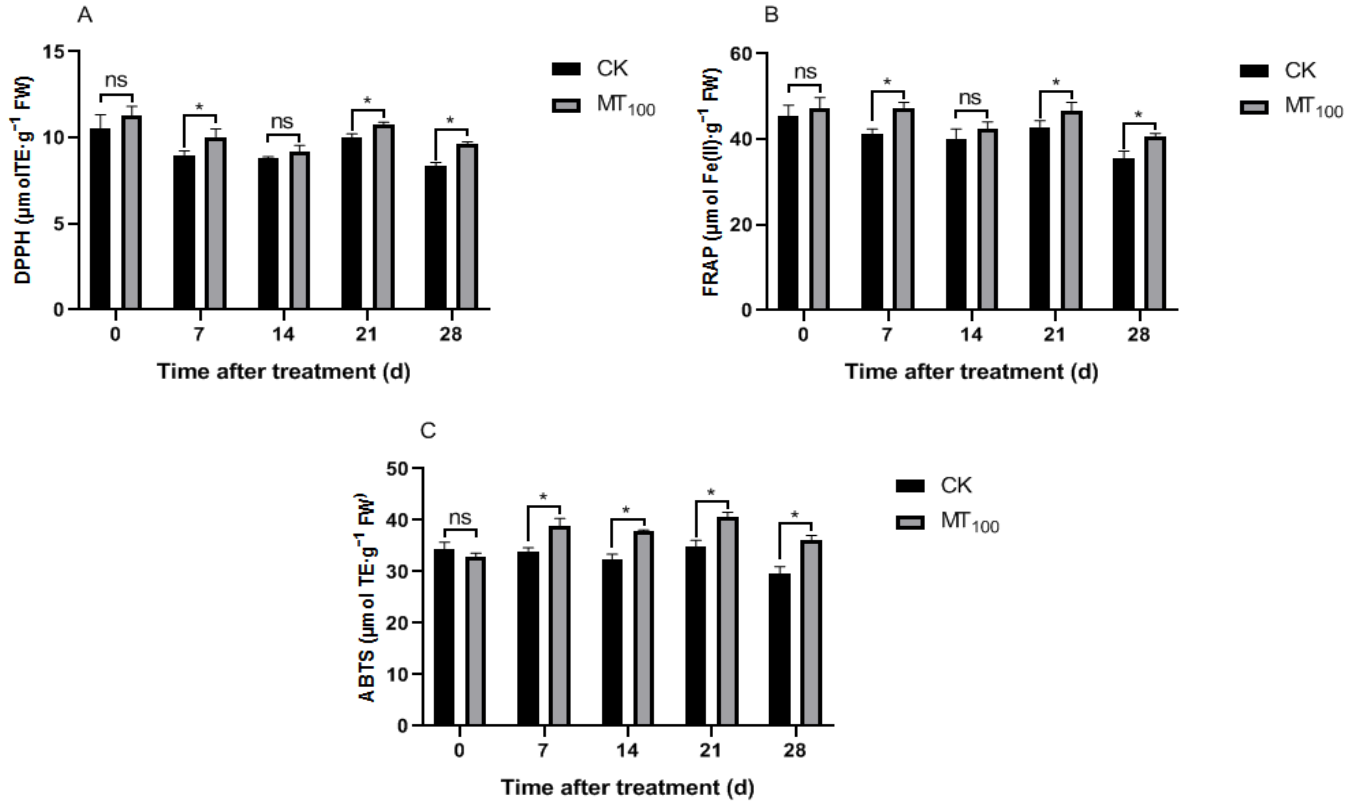

Figure 4. Changes in in vitro antioxidant capacity of pear peels treated with melatonin (MT_{100}) or water (CK). Changes in the contents of (**A**) DPPH, (**B**) FRAP, and (**C**) ABTS in pear peels at 0, 7, 14, 21, and 28 d after treatment. Error bars indicate standard deviations of three replicates. "ns" indicates no significant difference between groups; "*" indicates significant correlation at the $p < 0.05$ level.

3.5. Correlation Analysis between Antioxidant Capacity and Major Phenolics

As shown in Figure 5, analysis of antioxidant capacity and correlation with total phenolics, total flavonoids, total anthocyanosides, and major monomeric phenolics showed that DPPH was significantly positively correlated with total phenolics and total flavonoids ($p < 0.01$), with correlation coefficients of 0.88 and 0.78, respectively. FRAP was highly significantly positively correlated with total phenolics and total flavonoids (correlation coefficient: 0.93; $p < 0.01$ and correlation coefficient: 0.84; $p < 0.01$, respectively) and significantly positively correlated with arbutin content (correlation coefficient: 0.67; $p < 0.05$). The ABTS of the peel was positively correlated with the total phenolic, flavonoid, and anthocyanin contents, with correlation coefficients of 0.73 ($p < 0.01$), 0.84 ($p < 0.01$), and 0.85 ($p < 0.01$), respectively. The ABTS radical-scavenging activity of pear peels was significantly positively correlated with arbutin and epicatechin (correlation coefficient: 0.72; $p < 0.05$ and correlation coefficient: 0.70; $p < 0.05$, respectively). The total phenolics content was positively correlated with total flavonoid content, with a highly significant positive correlation coefficient of 0.90 ($p < 0.01$). There was a significant positive correlation between epicatechin and the contents of total flavonoids and anthocyanins, but negatively correlated with isorhamnetin 3-O-rutinoside content, with correlation coefficients of 0.63, 0.69, and −0.65, respectively ($p < 0.05$).

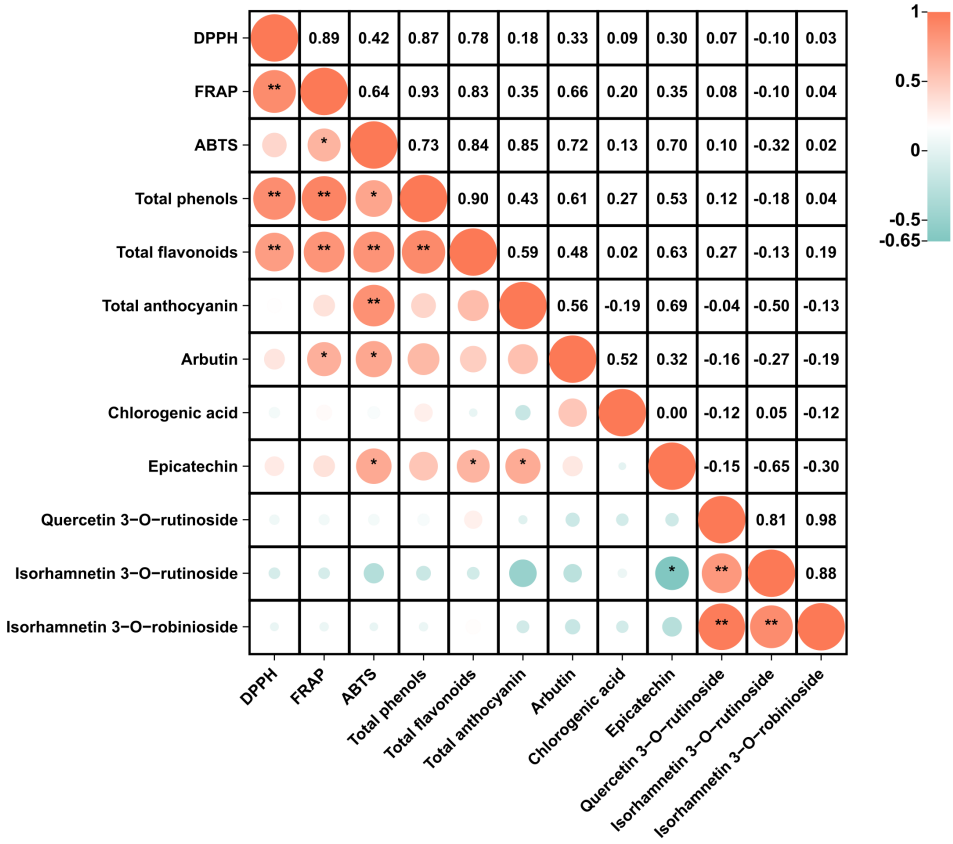

Figure 5. Correlation analysis of the antioxidant capacity of peels with major phenolic compounds. "*" indicates significant correlation at the $p < 0.05$ level; "**" represents significant correlation at the $p < 0.01$ level. Positive correlations are shown in red, negative correlations are shown in green, and the size of the circles corresponds to the size of the correlation coefficients.

4. Discussion

4.1. The Regulatory Effects of Melatonin on Fruit Coloring

Anthocyanidin metabolism is an important pathway for phenylpropanoids and clearly affects fruit coloration during development. This study found that the spraying of melatonin significantly increased the content of anthocyanidin in the peel, which is consistent with the results of several studies; however, some researchers obtained the opposite result in a study on Arabidopsis plants [47], which concluded that melatonin inhibited the synthesis of dihydrocannabinol and reduced anthocyanin accumulation in plants. Further, Li et al. found that melatonin could inhibit the accumulation of anthocyanin in Arabidopsis seeds [48], suggesting that the mechanism of melatonin regulation of anthocyanidin metabolism is different depending on plant genus and/or species.

PAL initiates the phenylalanine metabolic pathway. In this study, PAL enzyme activity was significantly higher than that of CK in the same period after melatonin treatment, which can provide more prerequisite substances for the synthesis of anthocyanidins and thus facilitate the synthesis of anthocyanidins. A previous study found that an increase in the activity of PAL enzymes is favorable for the accumulation of anthocyanidins synthesis [49]. In addition, we found that melatonin treatment resulted in higher CHI and UFGT enzyme activities but lower DFR enzyme activity than those in the control. In contrast, Murray and Hackett [50] suggested that the lack of DFR activity was a limiting factor for anthocyanoside accumulation, and Miranda [51] also found that the upregulation of DFR-related genes favored anthocyanoside synthesis. We surmise that dihydroquercetin is a common substrate in DFR-catalyzed anthocyanoside and flavonol synthesis pathways. Melatonin induces the production of flavonols, leading to a greater flux of dihydroquercetin into the flavonol

pathway; the significant increase in the content of flavonol phenols in the results of the present study also supports this conclusion. We speculate that the DFR enzyme is not the dominant factor in the melatonin-regulated anthocyanin synthesis of the 'Yuluxiang' pear.

In the current investigation, it was observed that the total anthocyanin content of melatonin-treated plants first increased and then decreased. We found that there may be two reasons for this. First, this pattern may be related to the partial degradation of anthocyanosides in the early ripening stage of the fruit, as melatonin treatment promotes early ripening of the fruits [5]. The second explanation is the gradual decrease in the rate of anthocyanoside synthesis during the later stages of fruit development as the fruit grows. Therefore, the specific mechanism through which melatonin affects anthocyanin content requires further verification.

4.2. Relationship between Melatonin and Lignin Metabolism

Lignin, an important product of the phenylpropane pathway, is widely distributed in higher plants, and is mainly deposited in the cell wall [52]. Lignin has a profound effect on plant growth and development and is widely involved in vascular transport, cellular mechanical support, pathogen responses, and environmental stress [53]. Studies have shown that the *PAL* gene is closely related to plant lignin content and that the activity of the *PAL* gene promoter is thought to be an intermediate for lignin synthesis in xylem vessels [54]. The *PAL2* promoter contains three regulatory motifs known as AC elements. These AC elements are also found in the promoters of other genes encoding enzymes involved in lignin biosynthesis, such as *C4H* and *4CL* [55].

In our study, the PAL, C4H, and 4CL enzyme activities of MT_{100} were significantly higher than those of CK near maturity, which, to some extent, also reflects the positive regulatory effect of the three enzymes on lignin synthesis. CAD is a fundamental enzyme of lignin synthesis. Its function is to reduce aldehydes to alcohols, which is a crucial stage within the lignin biosynthesis pathway [56]. Some studies have shown that *CAD* is expressed at higher levels than *PAL* and *POD* during the lignification process in pathogen-infected plants [57]. In this study we found that the trend of changes in the CAD enzyme and lignin content was consistent, which reflects its importance in lignin synthesis. In addition, POD enzymes also play an important role in the lignification process of plants by positively regulating lignin synthesis [58]. This pattern was observed in this study as well, since MT_{100} had higher POD enzyme activity than that of the control early and midway through melatonin treatment. In conclusion, preharvest melatonin can effectively increase the activity of key enzymes in the lignin synthesis pathway to promote lignin synthesis, but postharvest melatonin treatment inhibits the lignification of fruits during cold storage [59], which suggests that there is a difference in the regulatory mechanisms exerted by melatonin at different stages of fruit development. These findings suggest that the regulatory effect of melatonin on peel lignin is closely related to the timing of its application.

4.3. Changes in Melatonin and Phenolics Content and Antioxidant Capacity

Polyphenols exert strong antioxidant effects after injury as free radical scavengers in vitro [60]. As the first protective barrier of the fruit, enhancing the antioxidant capacity of the pear peels plays a crucial role in damage resistance. In our study, we found that MT_{100} had higher PAL enzyme activity than that of CK from the color change to maturity development stages. The enhancement in PAL enzyme activity, the first rate-limiting enzyme in phenylpropane metabolism, significantly promoted an increase in total phenolic and flavonoid contents. Additionally, the antioxidant capacity of pear peels was significantly and positively correlated with the contents of total phenolics, total flavonoids, total anthocyanins, arbutin, and epicatechin, with total phenolics and total flavonoids contributing the most. Our findings are consistent with the results of several other studies [61]. The concentration of phenolic compounds, including chlorogenic acid, arbutin, rutin, epicatechin, proanthocyanidins, and others, have been accurately measured (Figure S1). We found that the levels of epicatechin, catechin, and proanthocyanidin were lower than those in

the control from 7 d to 21 d after the second melatonin treatment, while anthocyanidins showed the opposite trend, which is in agreement with the results of other studies [17], possibly because melatonin preferentially activates the enzymes of anthocyanidin synthesis and promotes the synthesis of substances in the anthocyanidin pathway rather than the flavanol synthesis pathway.

Our findings in this study provide preliminary evidence which indicate that melatonin has an important effect on phenylpropanoid metabolism in pear peels (Figure 6). However, the mechanisms through which melatonin influences phenylpropanoid metabolism via primary metabolism, and the identities of the key regulatory enzymes or genes that respond to the action of melatonin, should be a focus of future studies.

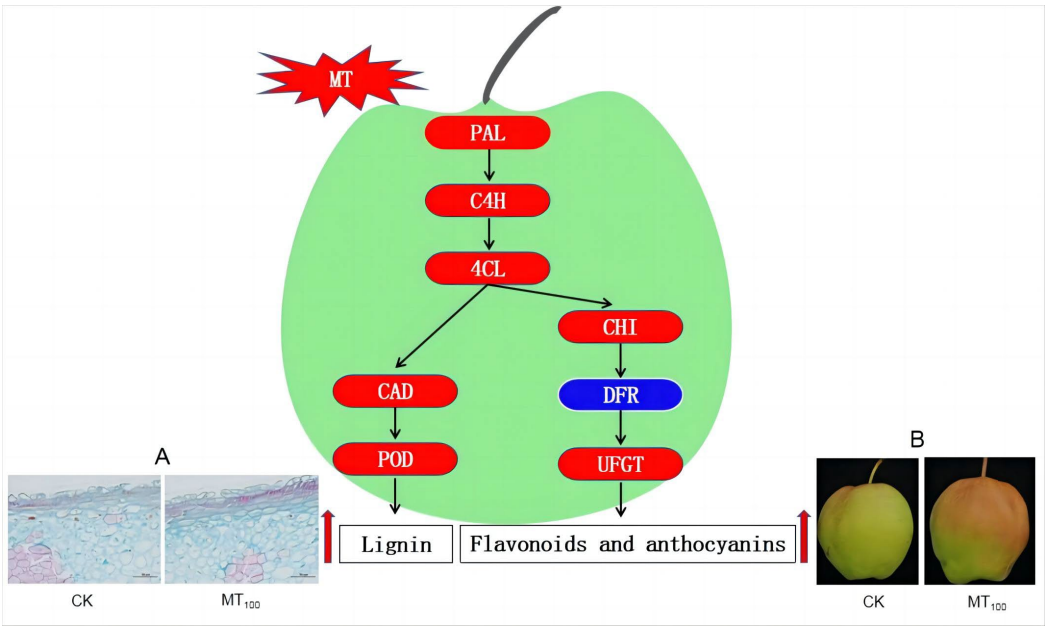

Figure 6. A schematic representation of the melatonin-mediated regulation of pericarp phenylpropane metabolism. Red and blue shading indicate an up and down regulation of enzyme activity, respectively. Panel (**A**) shows a paraffin section of the peel stained with m-trihydroxybenzene, whereas Panel (**B**) presents images showing changes in pear peel coloration. Red arrows indicate an increase in substance content.

5. Conclusions

In this study, we demonstrated that melatonin application can enhance the accumulation of lignin in the peel of pears by promoting increases in the activities of PAL, C4H, 4CL, CAD, and POD enzymes, which contribute to enhancing the pressure resistance of the peel. Furthermore, melatonin can intensify CHI and UFGT enzyme activities, facilitating the accumulation of anthocyanins, thereby leading to the production of redder fruit. In addition, melatonin stimulates phenylpropanoid metabolism, resulting in significant increases in the levels of both total phenolic compounds and total flavonoids in pear peels, thereby enhancing their antioxidant capacity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13122898/s1, Figure S1: Chromatograms of several phenols.

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