**Article**

**Exogenous Melatonin and Abscisic Acid Expedite the Flavonoids Biosynthesis in Grape Berry of Vitis vinifera cv. Kyoho**

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**Abstract:** Grape polyphenols contributing to more than half of the global polyphenol market were well studied; however, how melatonin (MLT), a potential plant hormone, and abscisic acid (ABA) affects polyphenols profile is still poorly understood. To explore whether these hormones are involved in polyphenolic biosynthesis, grape (Vitis vinifera cv. Kyoho) was exposed to MLT, ABA, and NDGA (nordihydroguaiaretic acid, an ABA biosynthesis inhibitor) treatments, and 16 polyphenols were identified from grape extracts by high performance liquid chromatography quadrupole time of flight mass spectrometry (HPLC-Q-TOF-MS). Both exogenous MLT and ABA significantly enhanced the biosynthesis of each flavonol and flavanol component, especially catechin, which was almost increased double by 200 µM of MLT treatment. Furthermore, the expression of genes involved in flavonoid biosynthesis, including 4-coumaroyl-CoA synthase, chalcone synthase, flavonoid 3′-hydroxylase, anthocyanin 3′-methyltransferase, flavonol synthase, flavonoid-3-O-glucosyltransferase, and flavonoid 3′,5′-methyltransferase were highly up-regulated as well but were down-regulated by NDGA. The present study provided new insights for improving flavonoids accumulation in agricultural production and its underlying mechanism.

**Keywords:** grape; melatonin; abscisic acid; flavonoid; Vitis vinifera

1. **Introduction**

Grapevine (Vitis vinifera L.) is a rich source of natural antioxidant compounds, mainly polyphenols, which are composed of flavonols, flavanols, and anthocyanins and contributed to more than half of the global polyphenol market [1]. Grape polyphenols are important secondary metabolites with various health-promoting effects, including anti-inflammatory, anti-cancer, anti-irradiation, anti-bacterial, preventing cardiocerebrovascular diseases, and so on [2]. Besides, Vitis vinifera L cv. Kyoho is one of the most popular cultivars due to its sweetness, juiciness, and large size, and Kyoho cultivar also contribute significantly to the world fresh table grapes [3].
Since first discovered in the Japanese morning glory, melatonin (MLT) has been widely studied in plants and plays an important role in stress resistance and antioxidation [4]. Exogenous MLT treatment delayed fruit senescence and improved postharvest commercial value, like inhibiting fruit softening, weight loss, decay rates, and respiration rate of various fruit. Also, promoted endogenous MLT biosynthesis and antioxidant system were observed in pear [5], strawberry [6], peach [7], banana [8], fruit, etc. As a functional component in wine, MLT also had synergistic health effects with polyphenols and increased the vasodilation and antioxidation activities [9]. In addition, 50 µM MLT could significantly increase the lycopene level of tomatoes by 5.8 times [10] and maintain the concentrations of total phenolics, flavonoids, and anthocyanins in litchi fruit, contributing to improved antioxidant capacity [11]. In grape berries, it was reported that pre-harvest exogenous MLT treatment significantly increased the polyphenolic content, antioxidant capacity, and related gene expressions, and improved the fruit maturity [12].

Abscisic acid (ABA) is one of the crucial plant hormones, which play vital roles in fruit ripening and development. Studies reported that exogenous ABA promoted fruit coloration, including anthocyanin and flavanol accumulation during fruit ripening in apple [13], citrus [14], grape [15], litchi [16], strawberry [17], and tomato [18]. It was also reported that the transcriptional levels of phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) genes involved in polyphenolic biosynthesis were significantly increased by exogenous ABA treatment [16]. Hu et al. observed that 25 mg L\(^{-1}\) exogenous ABA promoted flavonoid biosynthetic gene expression and maintained the color of litchi pericarp [16]. The nordihydroguaiaretic acid (NDGA), an ABA biosynthesis inhibitor, was evident to be involved in the regulation of anthocyanin biosynthesis [19]. Moreover, the effect of ABA on transcript expression of genes involved in anthocyanin and flavonoid biosynthesis has been confirmed in strawberry [20].

However, the effect of postharvest exogenous MLT, ABA, and NDGA on the performance of polyphenolic biosynthesis in grapevine is unclear. The present study was to elucidate the polyphenol profiles and try to develop its relationship with gene expression in *Vitis vinifera cv. Kyoho*, in order to provide new insights for the improvement of polyphenol accumulation.

### 2. Results

#### 2.1. Grape Morphology and Berry TSS, TA Concentrations

In order to investigate the effects of MLT and ABA on grape acceptance, the morphology of grape bunches at harvest and after storage is shown in Figure 1a. The results showed no obvious difference in the treated grape bunches after three day (d) storage at room temperature. Total soluble solid (TSS), which is a measure for sucrose concentration was found to be non-affected with all treatments, except ABA (Figure 1b), but the total acid (TA) concentration was halved to approximately 0.3% citric acid equivalents after three d storage (Figure 1c). It was interesting to mention that exogenous MLT at a lower concentrations significantly increased the TA concentration by about 0.15% \((p < 0.05)\) compared to CT (Figure 1c).
ABA treatment. Interestingly, polyphenols, such as gallic acid, laricitrin, Kaempferol 3-O-glucoronide, proanthocyanidin trimer, and (-)-epicatechin-(4beta->8)-(-)-epicatechin/dimeric procyanidin were found to be significantly higher (< 0.05) in the concentration in nordihydroguaiaretic acid (NDGA) treated grapes compared to D0 (Table 1).

2.2. Polyphenolic Profiles

To characterize the polyphenol composition and concentration in response to exogenous treatments, the HPLC-Q-TOF-MS method was employed and a total of 18 polyphenol components were detected (Table 1). By comparison to the reported characteristic ion fragments of grape polyphenols, 16 polyphenols were identified, including four phenolic acids, three flavonols, five flavanols, and four anthocyanins. Figure 2 showed the chromatograms of these compounds at UV 280 nm, UV 320 nm, and UV 520 nm. Several mass spectra of typical components of phenolic acid, flavanol, and anthocyanin are shown in Figure 3.

Phenolic acids were found to be the most abundant polyphenolic compound in grapes (Table 1). Out of the four phenolic acids, caftaric acid was found to be the most abundant component, which accounted for more than 85% of total phenolic acid, followed by fertaric, coutaric, and gallic acid. Consistent with the total phenolic acid content, all identified phenolic acid contents were decreased during storage (Table 1-CT). Among all the treatments, MLT 200 and ABA showed better recovery of all phenolic acids compared to the control (CT). More specifically, MLT200 treatment significantly increased the gallic acid and fertaric acid contents compared to CT (< 0.05). Similarly, NDGA treated grapes showed a significantly higher concentration of gallic acid (< 0.05).

All identified flavonols and flavanols showed significantly lower content after the storage (< 0.05) (Table 1-CT). However, all exogenous treatments showed better recovery of all these components as compared to CT. Among MLT treatments at two different concentrations (100 and 200 µM), MLT200 showed significantly better recovery of all flavonol and flavanol components (< 0.05) (Table 1). More specifically, the concentrations of catechin and epicatechin and their derivatives were tremendously increased in MLT treatment as compared to CT. A similar trend was recorded in that of laricitrin under ABA treatment. Interestingly, polyphenols, such as gallic acid, laricitrin, Kaempferol 3-O-glucoronide, proanthocyanidin trimer, and (-)-epicatechin-(4beta->8)-(-)-epicatechin/dimeric procyanidin were found to be significantly higher (< 0.05) in the concentration in nordihydroguaiaretic acid (NDGA) treated grapes compared to D0 (Table 1).

Figure 1. Grape morphology (a), Total soluble solid (TSS) content (b), and Total acid content (TA) (c) of Vitis vinifera cv. ‘Kyoho’. Error bars represent the standard deviations of three replicates. Different letters (a–c) on the bars represent significant differences between treatments (p < 0.05).
### Table 1. Identified polyphenolic compounds and their comparative concentrations (mg kg⁻¹) in the berry of *Vitis vinifera* cv. ‘Kyoho’.

| Peak No. | Compound | Spectrum (nm) | RT (min) | Mw (Da) | Productions | D0 | CT | MLT100 | MLT200 | ABA | NDGA |
|---------|----------|--------------|---------|---------|-------------|----|----|--------|--------|-----|------|
|    1    | Gallic acid | 280          | 9.80    | 170     | 125         |    |    | 0.85 ± 0.02 n| 0.45 ± 0.09 n| 0.65 ± 0.03 d| 0.94 ± 0.01 b| 0.68 ± 0.03 d| 1.22 ± 0.00 a |
|    2    | Caftaric acid | 280          | 18.15   | 312     | 179,149,135 | 611.56 ± 4.70 a| 538.68 ± 17.25 b| 488.60 ± 33.41 b| 511.59 ± 8.23 c| 602.93 ± 40.67 a| 402.73 ± 2.43 d |
|    3    | Coutaric acid | 280          | 23.86   | 296     | 163         | 3.56 ± 0.04 a| 2.83 ± 0.12 c| 2.29 ± 0.07 d| 2.96 ± 0.04 c| 3.06 ± 0.03 b| 2.08 ± 0.01 e |
|    4    | Fertaric acid | 320          | 27.99   | 326     | 193,134     | 21.28 ± 0.16 b| 20.74 ± 0.43 c| 17.71 ± 0.20 d| 24.98 ± 0.15 e| 19.67 ± 0.23 d| 20.50 ± 0.16 c |
|        | Total phenolic acid content |            |         |         |             | 637.25 ± 4.92 a| 562.70 ± 17.89 b| 509.25 ± 33.71 c| 540.47 ± 8.45 b| 626.34 ± 40.96 a| 426.53 ± 2.60 d |
|    5    | Laricitrin | 280          | 13.65   | 332     | 169,125     | 3.08 ± 0.07 c| 1.96 ± 0.32 c| 3.46 ± 0.22 d| 2.71 ± 0.01 d| 3.58 ± 0.54 b| 4.11 ± 0.16 a |
|    6    | Syringetin | 280          | 14.88   | 346     | 183         | 1.50 ± 0.00 a|           |           | 1.16 ± 0.00 b| 0.73 ± 0.00 d| 0.88 ± 0.00 c |
|    7    | Kaempferol 3-O-glucoside | 280          | 34.27   | 462     | 415,311,149 | 1.94 ± 0.22 b| 1.38 ± 0.04 b| 1.34 ± 0.10 b| 1.97 ± 0.12 a| 1.50 ± 0.12 b| 2.16 ± 0.10 a |
|        | Total flavonol content |            |         |         |             | 6.52 ± 0.29 b| 3.34 ± 1.73 c| 4.80 ± 0.32 d| 5.84 ± 0.13 c| 5.81 ± 0.66 b| 7.45 ± 0.26 a |
|    8    | Proanthocyanidin trimer | 280          | 13.26   | 866       | 866,865,695, 575,451,407, 287,243,125 | 2.43 ± 0.05 b| 1.34 ± 0.01 f| 1.38 ± 0.03 e| 2.01 ± 0.01 e| 1.45 ± 0.01 d| 2.98 ± 0.01 a |
|        | (-)-epicatechin-(4βa,βb)- | | | | | | | | | | |
|        | (+)-epicatechin/dimeric procyanidin | | | | | | | | | | |
|    9    | Catechin | 280          | 18.42   | 578     | 425,407,289,245 | 10.94 ± 0.77 a| 6.05 ± 0.58 d| 5.42 ± 0.36 d| 8.25 ± 0.51 b| 8.32 ± 0.00 b| 7.23 ± 0.16 b |
|    10   | (+)-epicatechin-(4βa,βb)- | | | | | | | | | | |
|        | (+)-epicatechin/dimeric procyanidin | | | | | | | | | | |
|    11   | Epicatechin | 280          | 20.91   | 290     | 245         | 13.57 ± 0.29 a| 4.20 ± 0.02 e| 5.60 ± 0.43 e| 8.24 ± 0.15 c| 7.13 ± 0.28 d| 8.64 ± 0.18 b |
|    12   | Total flavonol content |            |         |         |             | 44.66 ± 1.82 a| 20.63 ± 1.00 f| 23.44 ± 1.46 e| 32.80 ± 1.25 c| 28.36 ± 0.67 d| 35.61 ± 1.18 b |
|    13   | Peonidin 3-O-glucoside | 520          | 23.35   | 463     | 301         | 4.27 ± 0.06 d| 5.52 ± 0.10 b| 3.19 ± 0.25 f| 6.10 ± 0.07 a| 4.57 ± 0.09 c| 3.62 ± 0.03 c |
|    14   | Malvidin 3-O-glucoside | 520          | 25.50   | 493     | 331         | 0.32 ± 0.18 c| 0.83 ± 0.11 a| 0.48 ± 0.05 b| 0.57 ± 0.05 b| 0.57 ± 0.04 b| 0.45 ± 0.04 c |
|    15   | Malvidin 3-O-(6’-O-coumaroyl)-glucoside-5-O-glucoside | 520          | 57.32   | 801     | 639,493,331 | 0.99 ± 0.01 b| 1.00 ± 0.04 e| 0.55 ± 0.02 d| 1.03 ± 0.02 a| 0.84 ± 0.00 c| 1.03 ± 0.06 ab |
|        | Peonidin 3-O-(6’-O-coumaroyl)-glucoside-5-O-glucoside | | | | | | | | | | |
|    16   | Total anthocyanin content |            |         |         |             | 8.25 ± 0.31 c| 9.48 ± 0.25 b| 5.42 ± 0.37 e| 10.05 ± 0.25 a| 8.20 ± 0.17 c| 7.11 ± 0.15 d |
|    17   | Unknown | 280          | 7.49    | 370     | 207         | 6.50 ± 0.01 a| 4.49 ± 0.19 d| 4.58 ± 0.09 d| 6.19 ± 0.12 b| 4.82 ± 0.08 c| 6.44 ± 0.04 a |
|    18   | Unknown | 280          | 12.86   | 452     | 323,89      | 2.52 ± 0.21 a| 1.36 ± 0.05 d| 1.28 ± 0.09 d| 2.02 ± 0.06 b| 1.79 ± 0.01 b| 1.69 ± 0.18 c |
|        | Total polyphenol content |            |         |         |             | 705.70 ± 7.56 a| 602.00 ± 21.11 b| 548.77 ± 36.04 e| 597.37 ± 10.27 b| 675.32 ± 42.55 a| 484.83 ± 4.41 d |

*Data are shown in mean ± standard deviation (n = 3). D0, at harvest; CT, control; MLT100, 100 µM melatonin treatment; MLT200, 200 µM melatonin treatment; ABA abscisic acid treatment; NDGA, nordihydroguaiaretic acid treatment. Different letters (a–e in the table) of the same compound under different treatments represent statistically significant differences (p < 0.05).*
Figure 2. Identification of individual polyphenols in the berry of *Vitis vinifera* cv. ‘Kyoho’. HPLC chromatograms recorded at 280, 320, and 520 nm of the grape extract. The numbered identified peaks are listed in Table 1. To avoid confusion caused by overlapping pictures, only data of D0 (at harvest), CT (control), MLT200 (200 µM melatonin treatment), and ABA (abscisic acid) groups with better effects on polyphenolic biosynthesis promotion were presented in this figure.
Four anthocyanin compounds were determined, primarily consisted of malvidin and peonidin (Table 1). In agreement with the profiles of total anthocyanin content, the lower concentration of exogenous MLT significantly decreased the content of individual anthocyanins ($p < 0.05$). Furthermore, the peonidin-3-O-glucoside compound, which accounted for more than half of the total anthocyanins in the ‘Kyoho’ berry, was found to significantly increase in MLT200 treatment as compared to CT ($p < 0.05$).

2.3. Total Phenolic Acid, Flavonol, Flavanol, and Anthocyanin Contents

Polyphenolic content, reflecting the antioxidant capacity of grape berry, is critical to its quality evaluation, particularly to juices and wines. Our results revealed that total phenolic acid content was declined to 562.70 mg kg$^{-1}$ in control after three day (d) storage. Almost all exogenous treatments except ABA was found to significantly decrease the total phenolic acid in grapes ($p < 0.05$) (Table 1). Moreover, since total polyphenol content was mainly determined by phenolic acid, its trend was basically consistent with that of phenolic acid (Table 1). Table 1 showed that the total flavanol wshelf-lost after the storage; however, in the exogenous treated grapes, the contents were significantly recovered ($p < 0.05$) and highest were recorded in NDGA (35.61 ± 1.18 mg kg$^{-1}$) followed by MLT200 (32.80 ± 1.25 mg kg$^{-1}$) treated grapes. The total content of flavonol varied similar to that of flavanol. Anthocyanin content is mainly responsible for the color of the fruit and played a major role in wine formulation. Total anthocyanin content was found to be increased in the control group as compared to D0, which was maintained in ABA treated grapes (Table 1). It was interesting to report that MLT200 showed significantly ($p < 0.05$) higher concentration of total anthocyanin content (10.05 ± 0.25 mg kg$^{-1}$) compared to control and all other treatments.
2.4. Expression of Genes Involved in Polyphenolic Biosynthesis

To better understand how MLT and ABA-induced polyphenol anabolism, RT-qPCR was performed to investigate the gene expression profiles of enzymes involved in polyphenolic biosynthetic pathways (Figure 4). The results indicated that the flavonoid biosynthesis in the ‘Kyoho’ berry was suppressed by the down-regulation of gene expression levels after storage (CT), which was inconsistent with the change in total polyphenol content. Furthermore, almost all gene expression levels were up-regulated by exogenous ABA treatment, except for anthocyanidin 3-O-glucosyltransferase (3GT) and anthocyanidin reductase (ANR); and the upstream flavonoid biosynthetic genes, including 4-coumaroyl-CoA synthase (4CL), flavonoid 3′,5′-hydroxylase (F3′,5′H), LDOX, anthocyanin 3′-methyltransferase (OMT), and flavonoid 3′,5′-methyltransferase (AOMT), showed higher transcript levels. On the contrary, most gene expressions in the NDGA group were down-regulated in comparison with ABA. Additionally, most gene expression levels were increased by exogenous MLT, especially OMT, flavanol synthase (FLS), and AOMT genes, which were in agreement with the accumulation of laricitrin and syringetin compounds. And the expression of other genes like CHI, flavone 3β-hydroxylase (F3H), DFR, LDOX, and ANR were inhibited by both MLT100 and MLT200 treatments.

Figure 4. Heatmap of gene expression profiles involved in polyphenolic biosynthesis in the berry of Vitis vinifera cv. ‘Kyoho’. The log2 of relative expression levels are shown, measured by RealTime Quantitative Polymerase Chain Reaction (RT-qPCR). CT was set as control.

3. Discussion

Grapes are known to be one of the richest sources of various health-promoting compounds, especially polyphenols [1,21]. Vitis vinifera cv. ‘Kyoho’ variety is known for its easy skin peeling effect and considered to possess higher polyphenolic content, thus widely used in wine formulation. In general, our present research results revealed positive changes in polyphenolic content after various postharvested exogenous treatments of grape.

For fresh-eating, it is essential to check the level of TSS and TA contents before harvesting. TSS and TA are important criteria which determine the maturity and taste. All exogenous treatments in the present study showed different levels of TSS and TA contents. Among all exogenous treatments, MLT maintained the TSS level in postharvested grape, while only a lower concentration of MLT showed
significant increase in TA content as compared to control ($p < 0.05$, Figure 1). This might be due to the fact that melatonin inhibited grape respiration, slowed down citric acid circulation, and reduced the degradation of soluble acid, which was confirmed in sweet cherry [22]. The color indicates the ripening stage and berry development. A noticeable change in the berry color was recorded in the control group, while in other exogenous treated groups, it was less predominant. It was previously reported that MLT spraying reduced the under-ripeness and over-ripeness during grape pre-veraison, promoting the berry ripening synchronicity and wines made with it were more fruity, spicy, and sweet [23].

Hilbert et al. [24] identified and detected structures of 18 flavanols from six different Vitis varieties using liquid phase mass spectrometry (LC-MS) and liquid chromatography-nuclear magnetic resonance spectroscopy (LC-NMR), respectively. In order to elucidate the effect of these exogenous treatments on polyphenolic profiles of in *Vitis vinifera* cv. ‘Kyoho’ berry during storage, we detected a total of 18 polyphenolic compounds and identified 16 by HPLC-Q-TOF-MS, and determined their relative quantitatively concentrations by HPLC, and investigated the gene expressions involved in the polyphenolic biosynthetic pathway. Moreover, the exogenous melatonin was widely applied in fruit to improve its quality. For instance, grape treated with MLT at pre-veraison stage possessed higher levels of catechins, epicatechins, and peonidin derivatives after maturation [25]. This is consistent with our finding that MLT significantly promoted catechin content ($p < 0.05$). Moreover, the fruit quality and yield of tomato were improved by exogenous MLT, and the contents of phenolics and flavonoids in green-mature tomato were increased by 14.29% and 30.77%, respectively, which were almost twice than that in the red-mature tomato [26]. These results suggested that the polyphenolic biosynthesis was slowed down during fruit senescence, in accordance with the decrease of total polyphenol content in CT after storage (Table 1). In the present study, the polyphenolic contents (phenolic acids, flavonols, and anthocyanins) showed differences among different treatments and within polyphenolic contents (Table 1). For example, MLT 200 was found to maintain the total phenolic acid content, while ABA treatment showed an increase in its concentration. However, in the case of total flavonol and flavanol content, NDGA was found to be the best followed by MLT200 and ABA. Anthocyanin, which is mainly responsible for the fruit color, was found to be up-regulated during MLT200 treatment. MLT at lower concentrations was not found much effective in regulating polyphenolic content as compared to a higher concentration. Overall, the total polyphenol content was found to be declined after postharvested storage condition (CT), possibly due to the oxidation process, but maintained and up-regulated by exogenous treatment, especially MLT200 and ABA, respectively (Table 1). This might be attributed to the improved antioxidant capacity in ‘Kyoho’ berry by MLT or ABA, which was further evident in litchi [11] and strawberry [6].

Additionally, both the polyphenol accumulation and PAL expression were observed to be promoted by pre-harvest MLT [12]. In agreement with former studies, our RT-qPCR results showed that 4CL, CHS, F3′H, OMT, FLS, UDPG and AOMT genes involved in flavonoid biosynthesis were more expressed in different degrees under MLT treatment (Figure 5). In tomato, ABA significantly promoted carotenoid and flavonoid biosynthesis via up-regulating the expression of related genes by 2.08 to 35 times, initiating polyphenol accumulation 2–4 d earlier than control [27]. It was reported that exogenous treatment of ABA in strawberry accelerated flavonoid and anthocyanin biosynthesis, as well as the up-regulated CHS, CHI, F3H, flavonoid 3′-hydroxylase (F3′H), and DFR expression [20]. And Olivares et al. [28] found that exogenous ABA significantly promoted grape coloration and advanced the harvest time by 37 d. In the present study, total polyphenol content, especially phenolic acid, flavonols, and flavanols were also increased by ABA treatment (Table 1). Particularly, it was revealed that all individual flavonoid compounds except anthocyanins exhibited higher content in ABA and NDGA groups than control (Table 1). Accordingly, 4CL, CHS, OMT, FLS, and AOMT genes involved in polyphenolic biosynthesis showed higher expression in ABA and NDGA groups than control (Figure 5). Expression levels of genes such as CHI, F3H, F3′H, F3′5′H, DFR, LDOX, leucoanthocyanidin reductase (LAR), and flavanoid-3-O-glucosyltransferase (UDPG) were significantly up-regulated in response to exogenous ABA; however significantly and adversely lower in response to NDGA ($p < 0.05$) (Figure 5).
A similar outcome was also found in MLT groups, in which not all genes involved in flavonoid biosynthesis had higher expression levels (Figure 4). This paradoxical phenomenon might be due to the profiles of accumulated flavonoids, which were determined from not only the involvement of key genes in the biosynthesis pathway, such as 4CL, CHS, OMT, F3′H, and AOMT; but also, from the blocking of the ABA biosynthesis pathway by NDGA. It was reported that the polyphenolic biosynthesis and the internal browning in postharvest pineapple were inhibited by the application of 380 µM ABA, due to the improved activity of antioxidant enzymes [29].

![Figure 5. Polyphenolic biosynthesis pathway in berry of Vitis vinifera cv. Kyoho. Red represents up-regulation, while blue represents down-regulation, and white indicates no effect. The deeper the color, the larger the change. And the left reveals MLT200 effect, whereas the right indicates ABA effect. The square and cloud shapes indicate the change in gene expression and component content, respectively.](image)

It was notable to mention that both MLT and ABA promoted flavonoid biosynthesis; therefore, is there any possible interaction between the influences resulted from exogenous MLT and ABA? Changes of polyphenol contents and gene expressions of vital enzymes involved in polyphenol biosynthesis under ABA and MLT treatments were presented in Figure 5. ABA has been widely recognized as a promoter to polyphenolic biosynthesis, and the increase in the expression of ABA receptor VPYL1 led to the accumulation of anthocyanin and a series of ABA-responsive gene transcripts in grape berries [30]. The expression of key enzymes in the anthocyanin biosynthesis pathway, including PAL, C4H, CHS, F3′H, and DFR, was up-regulated by exogenous ABA in litchi [16] and strawberry [20]. Moreover, the transcription factor MYB could be activated by ABA, then bound to bHLH and WD40 to form a protein complex, which increased anthocyanin biosynthesis [31]. In addition, FLS and UDPG in the flavonoid biosynthesis pathway were higher expressed by ABA as well [18]. Our results from the present study further confirmed the positive role of ABA in fruit flavonoid biosynthesis (Figure 5). Additionally, the first discovery of MLT receptor in Arabidopsis thaliana suggested that MLT was involved in the regulation of physiological attributes, including fruit development and ripening through receptor-mediated signaling cascades [32]. Xu et al. also published that MLT might promote grape ripening by increasing levels of ABA and ethylene [33]. However, very few studies were carried out on the MLT effect on fruit flavonoid biosynthesis so far [12,34]. Our study effectively contributes to narrow down this gap, particularly on grape berries and found that MLT significantly improved flavonoid biosynthesis ($p < 0.05$) via increasing gene expressions of 4CL,
CHS, FLS, AOMT, and UDPG (Figure 5). Enhanced flavonoid contents contribute to higher antioxidant activities, thus contributed to the senescence inhibition, quality maintenance of postharvest fruit. Therefore, we postulated a molecular model on the roles of ABA and MLT in polyphenolic biosynthesis in Vitis vinifera cv. ‘Kyoho’ berry (Figure 6). Briefly, exogenous ABA promoted the transcription of key enzymes in the main route of polyphenolic biosynthesis pathways, which was then diverged into two roads, leading to higher levels of flavonoids, including anthocyanins. And MLT enhanced flavonoid biosynthesis as well, mainly in a later stage of the pathway. ABA also up-regulated the expression of the MYBA1 (v-myb avian myeloblastosis viral oncogene homolog A1) transcription factor, which further forms a protein complex with transcription factors bHLH and WDR1 to facilitate anthocyanin biosynthesis in grapevine [35]. Increased flavonoid contents contributed to enhanced antioxidant activities and inhibited grape senescence [11]. Our findings provided new insights and made a deeper understanding of flavonoid biosynthesis and gene expression in Vitis vinifera cv. ‘Kyoho’ berry under exogenous ABA and MLT. Moreover, since ABA and MLT showed similar effects on promoting the flavonoids biosynthesis, their potential interaction will be further explored in our follow-up study.

Figure 6. Possible model depicting the mechanism of abscisic acid (ABA) and melatonin (MLT) promoted flavonoid biosynthesis in the berry of Vitis vinifera cv. ‘Kyoho’ based on previous and present studies.

4. Materials and Methods

4.1. Plant Materials and Experimental Design

Three independent biological replications of grape (Vitis vinifera cv. ‘Kyoho’) in August with homogenous size were hand-harvested at the same maturity from an organic vineyard in Jinhua city, Zhejiang province, China. For each biological replication, grape bunches were collected in thermocol boxes and delivered to the laboratory in 1.5 h. After acclimatizing at 25 °C for 2 h in the laboratory, the grape bunches were randomly and evenly divided into five groups (three bunches in each group) (Figure 7). Each group was immersed for 20 min in different exogenous chemicals at varied concentrations, i.e., CT (distilled water, control), MLT100 (100 μM melatonin, Macklin Biochemical Company, Ltd., Shanghai, China), MLT200 (200 μM melatonin), ABA (1 mM abscisic acid, Macklin Biochemical Company, Ltd., Shanghai, China) and NDGA (0.5 mM nordihydroguaiaretic acid, Tokyo Chemical Industry, Ltd., Shanghai, China) (Figure 7). Afterward, the grapes were air-dried and stored at room conditions (i.e., 22 ± 3 °C temperature and 60 ± 5% relative humidity) for three d. Berries were randomly removed for determination of total soluble solids (TSS) and titratable acidity (TA), and other berries, including the skin, the flesh, and seeds were ground, mixed, and frozen in liquid nitrogen and stored at −80 °C for further analysis (Figure 7).
4.2. Total Soluble Solids (TSS) and Titratable Acidity (TA) Determination

TSS and TA of the fruit juice were measured by a Brix-Acidity Meter (Model PAL-BX/ACID F5, Atago, Japan). Three independent replicates were carried out.

4.3. Polyphenols Extraction and Separation

Polyphenols in berry (approximately 2.0 g) were extracted with 1.0 mL of 1% HCl-methanol, at dark 4 °C for 12 h. The resulting supernatant obtained after centrifugation at 10,000×g for 15 min was filtered through the 0.22 µm microporous membrane. Polyphenolic compounds were separated by injection into the HPLC system (LC-20AD, SHIMADZU incorporation, Japan) with an AQ-C18 column (water-based, 5 µm, 4.6 × 250 mm, Welch Materials incorporation, Shanghai, China). The elution parameters were as follows: Injection volume, 10 µL; column temperature, 40 °C; Solvent A, 1% phosphoric acid; solvent B, acetonitrile; solvent flow rate, 1.0 mL min⁻¹; and the gradient: 0–10 min, 2% B; 10–55 min, 10% B; 55–65 min, 18% B; 65–68 min, 50% B; 68–69 min, 80% B; 69–79 min, 2% B. The signal was monitored at 280, 320 and 520 nm.

4.4. Identification and Quantification of Polyphenols by HPLC and HPLC-Q-TOF-MS

Polyphenols including phenolic acids, flavonoids (including flavones, flavonols, flavanols, flavanones, anthocyanins and so on) were identified using HPLC (as mentioned before) coupled to quadrupole time-of-flight mass spectrometry (Q-TOF-MS) (TripleTOF™ 5600+, SCIEX incorporation, United States) with an electrospray ionization source (ESI) system. Data were generated in negative ion mode with a scan range of 100–1500 m/z and the source voltage at −4.5 kV, the source temperature at 550 °C. The pressure of Gas 1 (Air) and Gas 2 (Air) was 50 psi, whereas that of curtain gas (N2) was 35 psi. The molecular formula proposed by PeakView software version 1.2 for different signals were compared with previously reported polyphenolic compounds, especially in Vitis vinifera.

The polyphenolic compounds were quantified by comparison of peak areas with standard calibration curves of the gallic acid (Macklin Biochemical Company, Ltd., Shanghai, China) and epicatechin (Aladdin, Shanghai, China) at 280 and 320 nm, and the cyanidin-3-O-glucoside.
(Macklin Biochemical Company, Ltd., Shanghai, China) at 520 nm. The total concentrations of phenolic acid, flavonol, flavanol, and anthocyanin were obtained by the addition of each identified component concentrations.

4.5. Real-Time Quantitative PCR (RT-qPCR)

Genes involved in the polyphenolic biosynthesis pathway were selected according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg/pathway.html). The primers of these genes used in this study were listed in Appendix A Table A1. Total RNAs were extracted from powdered grape samples using cetyl trimethyl ammonium bromide (CTAB) protocol described by Gambino et al. [36] and quantified with microplate reader (TECAN, Spark®, Männedorf, Switzerland), followed by reverse-transcription to cDNA with a PrimeScript™ RT Reagent Kit (code DRR047 A, TaKaRa, Japan). RT-qPCR was conducted on TB Green™ Premix Ex Taq™ (RR420A, TaKaRa, Japan) according to the instructions mentioned in the product. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a calibrator to calculate the relative transcriptional levels of genes by the $2^{-\Delta\Delta CT}$ method. Three independent replicates were carried out. And the results were presented as a heat map using the Heml 1.0 software [37].

4.6. Statistical Analysis

Experiments were completely randomly designed. All data analysis was performed using SPSS V20.0 (IBM Corp, Armonk, NY, USA) software and presented as mean ± standard deviation with analysis of variance (ANOVA) and Tukey test at a significant level of $p < 0.05$.

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Abbreviations

PAL phenylalanine ammonia-lyase  
C4H cinnamate-4-hydroxylase  
PCH p-coumarate 3-hydroxylase  
COMT caffeic acid 3-O-methyltransferase  
4CL 4-coumaroyl-CoA synthase  
CHS chalcone synthase  
CHI chalcone isomerase  
F3′H flavonoid 3′-hydroxylase  
F3′5′H flavonoid 3′5′-hydroxylase  
F3H flavanone 3β-hydroxylase  
DFR dihydroflavonol 4-reductase  
LDOX leucoanthocyanidin dioxygenase  
3GT anthocyanidin 3-O-glucosyltransferase  
OMT anthocyanin 3′-methyltransferase  
UGT75C1 anthocyanin 5-O-glucosyltransferase  
3AT anthocyanidin 3-O-glucoside 6′-O-acyltransferase  
CYP75A flavonoid 3′,5′-hydroxylase  
AOMT flavonoid 3′,5′-methyltransferase  
FLS flavonol synthase  
UDPG flavonoid-3-O-glucosyltransferase  
LAR leucoanthocyanidin reductase  
ANR anthocyanidin reductase
Appendix A

Table A1. Primers used in RT qPCR.

| Genes | Forward Primers (5′-3′) | Reverse Primers (5′-3′) |
|-------|-------------------------|------------------------|
| 4CL   | ACCACCTCCCTCCTCCACAC    | GCTCCGAGAAAGGAGACG     |
| CHS   | ACCACCTGGATTCTCTGAGA    | GAAGGCTTCCACAAAGCTC    |
| CHI   | TTTGTTGTTTCCCTTGTTCGAC  | GCAGACGAATCTTATCCATAG  |
| F3H1  | CCAATCATAGGACAGTCTGCC   | TGACAGGATAACGGTGAGCC   |
| F3H2  | CTGTGGTGAACTCCGACTGC    | CAAATGTTATGGGCTCTCC    |
| F3′H  | GAGATCAACGGCTACCACATC   | CTTGACGAGACCTCGTGG     |
| DFR   | TGTTAATGTGCAATGGCC      | CAGTGAGGCAGAGCCCTTG    |
| LDOX  | ACCCTATCCCTCCACACATC    | AGTAGAGGCGCTTGCTCCT    |
| 3GT   | TCAAGAAGACCAGACCCCTA    | TGTTCTACTACGGGCTCTA    |
| OMT   | AGAGAAGAAGGCTACCAAAG    | GCTATGGTATGGGCTGAGTAG  |
| LAR   | CGTTTGGTAGCATAAGAGGTTTC | GAGATGAGGCGAGATGGGT    |
| ANR   | TTGATGGGACAGGCTTCGTGG   | AGTGTCCTAGGCGAGCATGAGC |
| FLS   | CCAGCCAATCTCCAAACTC     | TTGGCCATTTGCAATGTTGA   |
| UDPG  | TGCTACCTAAGGCAGCTG      | GCTGGATTTGAGAATGTTG    |
| AOMT  | AGGTGTGGACATGACATCA     | TTATCGTAAGCTATGCCC     |

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**Sample Availability:** Samples of the compounds are not available from the authors.

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