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Exogenous Melatonin Improves Cold Tolerance of Strawberry (*Fragaria* × *ananassa* Duch.) through Modulation of DREB/CBF-COR Pathway and Antioxidant Defense System

Faisal Hayat [1,†], Zixun Sun [1,†], Zhaojun Ni [1,†], Shahid Iqbal [1], Wuyi Xu [1], Zhihong Gao [1,*], Yushan Qiao [1], Muhammad Aammar Tufail [2], Mohammad Shah Jahan [3], Ummara Khan [4], Chunyan Wan [5] and Xianbin Gu [6]

1 Laboratory of Fruit Tree Biotechnology, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China; maken_faisal@yahoo.com (F.H.); 2017104014@njau.edu.cn (Z.S.); nizhaojun@njau.edu.cn (Z.N.); shahidiqbalpak@hotmail.com (S.I.); xwy5825@163.com (W.X.); qiaoyushan@njau.edu.cn (Y.Q.)
2 Institute for General Microbiology, Christian-Albrechts-Universität zu Kiel Am Botanischen Garten 1-9, 24118 Kiel, Germany; mtufail@ifam.uni-kiel.de
3 Department of Horticulture, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka 1207, Bangladesh; shahjahansau@gmail.com
4 Key Laboratory of Food Processing and Quality Control, College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China; khanummaraft@gmail.com
5 Zhenjiang Agricultural Science Research Institute, Jurong 212400, China; wanchuyan_423@163.com
6 Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China; xbgu_8810@163.com
* Correspondence: gzh71@njau.edu.cn; Tel.: +86-025-843-95724
† These authors contributed equally to this work.

Abstract: The strawberry (*Fragaria* × *ananassa* Duch.) is an important fruit crop cultivated worldwide for its unique taste and nutritional properties. One of the major risks associated with strawberry production is cold damage. Recently, melatonin has emerged as a multifunctional signaling molecule that influences plant growth and development and reduces adverse consequences of cold stress. The present study was conducted to investigate the defensive role of melatonin and its potential interrelation with abscisic acid (ABA) in strawberry plants under cold stress. The results demonstrate that melatonin application conferred improved cold tolerance on strawberry seedlings by reducing malondialdehyde and hydrogen peroxide contents under cold stress. Conversely, pretreatment of strawberry plants with 100 µM melatonin increased soluble sugar contents and different antioxidant enzyme activities (ascorbate peroxidase, catalase, and peroxidase) and non-enzymatic antioxidant (ascorbate and glutathione) activities under cold stress. Furthermore, exogenous melatonin treatment stimulated the expression of the DREB/CBF—COR pathways' downstream genes. Interestingly, ABA treatment did not change the expression of the DREB/CBF—COR pathway. These findings imply that the DREB/CBF-COR pathway confers cold tolerance on strawberry seedlings through exogenous melatonin application. Taken together, our results reveal that melatonin (100 µM) pretreatment protects strawberry plants from the damages induced by cold stress through enhanced antioxidant defense potential and modulating the DREB/CBF—COR pathway.

Keywords: melatonin; abiotic stress; cold tolerance; antioxidant potential; gene expression

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1. Introduction

The strawberry is an important and highly valued fruit because of its unique taste and nutritional properties [1]. Multiple environmental stresses such as salinity, drought, cold, and extreme heat directly threaten crop productivity, resulting in global food shortages [2,3]. Cold stress is a major abiotic stress that critically inhibits crop growth, production, and geographical distributions of plants [4]. Low temperatures result in large losses in strawberry production, with severe frost damage resulting in a 20–30% reduction in yield owing to the damage to

leaves and other organs [5]. However, under severe cold conditions, the internal defense system of plants is insufficient to cope with oxidative stress and its side effects. Thus, effective ways to improve the abiotic stress tolerance of strawberry plants are urgently needed.

Cold stress can affect physiological and biochemical changes such as photosynthesis, respiration, and reactive oxygen species (ROS) production [6,7]. Elevated ROS levels cause oxidative damage to DNA, proteins, and an increase in membrane lipid peroxidation, thus inhibiting cellular functions and signal transduction pathways that negatively influence the development of crops [8]. As a result, plants have evolved to be well-equipped with antioxidant defense machinery to prevent the oxidative damage induced by elevated ROS levels [9].

Melatonin is a multifunctional phytoprotectant signaling molecule involved in multiple physiological and biochemical functions in response to various environmental stresses [10]. As a free radical scavenger, melatonin can directly scavenge ROS in cellular compartments, thereby alleviating oxidative stress in plants [11]. Moreover, melatonin protects plants against a variety of environmental stresses, such as cold [12], heat [13], salinity [14], drought [15], and heavy metals [16]. These studies have highlighted that melatonin enhances plant tolerance to several environmental stresses. Although the physiological and molecular mechanisms of cold tolerance have been studied to some extent, melatonin-mediated cold tolerance in the strawberry remains elusive.

Abscisic acid (ABA) is another essential hormone that acts as a stress indicator/signal to assist plants in dealing with environmental stresses [17]. Exogenous ABA can improve antioxidant activity by preventing cell damage from reactive oxygen species [18]. Melatonin has been shown in studies to mediate ABA biosynthesis and metabolism regulation, resulting in a decrease in ABA content under stress conditions. Moreover, melatonin selectively downregulates the *MdNCED3* gene (involved in ABA biosynthesis) and upregulates *MdCYP707A1* and *MdCYP707A2* (ABA catabolic genes) in two *Malus* species under drought-stressed conditions [19]. Exogenous melatonin application increased the endogenous ABA content in *Elymus nutans* under salt stress conditions, which was significantly inhibited by fluridone (ABA synthesis inhibitor). Pretreatment with ABA or fluridone had no effect on endogenous MET concentration, implying that ABA may act as a downstream signal in the melatonin-induced cold tolerance. Interestingly, melatonin can also induce the expression of cold-responsive genes in plants, thereby increasing their resistance to cold stress in an ABA-independent manner. This implies that melatonin-induced cold tolerance might be involved in both ABA-dependent and ABA-independent pathways [20]. However, melatonin- and ABA-mediated changes in the antioxidant defense system of strawberry seedlings facing cold stress are still unknown.

C-repeat binding factors (CBFs)/dehydration-responsive element-binding proteins (DREB) encode transcription factors that play an essential role in responses to environmental stimuli, including salt, drought, fungal infection, freezing, and cold stress [21,22]. These CBF proteins can bind to the promoter regions of various cold-responsive genes, thereby controlling their expression [23]. Similarly, overexpressing *AtCBF3*, *AtCBF2*, and *AtCBF1* enhanced tolerance to drought, salt, and freezing by activating multiple downstream genes such as those that are cold-inducible and responsive to dehydration [24,25]. Over the last few years, substantial progress has been achieved in illuminating melatonin's and ABA's protective role in response to various environmental stresses in different crops [26].

To the best of our knowledge, whether melatonin can improve strawberry cold tolerance and the underlying mechanism has not been reported. Thus, this study aimed to determine the role of melatonin in improving the cold tolerance of strawberry plants. We hypothesized that melatonin treatment would protect the strawberry cultivar 'Benihoppe' from the harmful effects of cold stress, especially the oxidative damage that occurs, and improve cold stress tolerance by modulating the DREB/CBF—COR pathway and antioxidant defense system. Moreover, we aimed to explore melatonin's role and its possible interrelation with ABA in strawberry plants exposed to cold stress.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

A conventional, widely planted strawberry seedling (*Fragaria* × *ananassa* Duch.) cultivar, 'Benihoppe', was used in this study, which was provided by Zhenjiang Agricultural Science Research Institute, Jurong, Jiangsu. The seedlings were sown in a plastic pot containing a mixed substrate of garden soil and nursery substrate (1:1; *v:v*) and cultivated under greenhouse conditions (16:8 h photoperiod, 25/20 °C (day/night), the relative humidity of 65%). The pre-cultivation period was 18 days, with the intention of allowing seedlings become adapted to new conditions. The plants were drip-irrigated, but no fertilizer was applied during the study period.

2.1.1. Experiment 1

To determine the potential role of melatonin in mitigation of cold damage to strawberry plants, six-leaf-stage strawberry seedlings were treated with exogenous melatonin and subjected to cold stress treatment. The strawberry seedlings were sprayed with water or 10, 50, 100, 500, and 1000 μM melatonin solutions until the leaves dripped, once every 2 d for a total of three applications. Two days after the last melatonin application, seedlings were subjected to cold-temperature stress at 0/−4 °C (16/8 h) for 2 days. Nine additional plants were kept at 25° C and served as NT (normal temperature without melatonin application) plants. Samples for gene expression were collected at 0, 3, 6, 12, 24, 48, and 96 h of cold treatment with 100 μM melatonin, immediately flash-frozen in liquid nitrogen, and stored at −80 °C until analyzed. After 2 days of cold treatment, oxidative stress indicators, soluble sugars, non-enzymatic antioxidant concentration, and related antioxidant enzyme activities were measured.

2.1.2. Experiment 2

To determine the putative role of exogenous melatonin and ABA in cold stress resistance, strawberry seedlings were pretreated with water or ABA (100 μM), fluridone (50 μM), melatonin (100 μM), and melatonin (100 μM) + fluridone (50 μM), respectively for 3 times in a two-day interval. Two days after the last melatonin or other chemical application, plants were subjected to cold stress for two days, as described earlier. After two days of cold treatment, leaf samples were collected to perform further analysis, including both gene expression and biochemical traits.

2.2. Determination of Leaf Malondialdehyde and Hydrogen Peroxide Concentrations

Malondialdehyde (MDA) is a critical biomarker of lipid peroxidation. Two days after cold stress, the contents of MDA were measured using the thiobarbituric acid (TBA) reaction as described previously. Briefly, 0.5 g of leaf tissue was homogenized with 5 mL of 5% (*w*/*v*) trichloroacetic acid (TCA) solution followed by centrifugation at 1000× *g* for 10 min at 4 °C. One ml of supernatant was mixed with 2 mL of TCA containing a 0.5% (*w/v*) TBA solution. The mixture was boiled at 95 °C for 30 min and immediately placed on ice for cooling and centrifuged at 1000× *g* for 10 min. The absorbance of the supernatant was measured at 450, 532, and 600 nm. The MDA content was calculated as μmol g^{-1} FW.

For the measurement of hydrogen peroxide (H_2O_2) content, 1 mL of the first reagent was added to frozen samples of leaf tissue (0.1 g) and homogenized in an ice bath. The homogenate was placed in a 1.5 mL centrifuge tube; after centrifugation at 8000× *g* at 4 °C for 10 min, the supernatant was placed on ice for H_2O_2 measurement. The analysis was performed using (H_2O_2) Content Assay Kit (BC3595; Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions and expressed as μmol g^{-1} FW.

2.3. Determination of Soluble Sugars

The soluble sugar content was determined using the anthrone method, as described by [27]. Briefly, 0.1 g samples were added to 2 mL of 80% (*v/v*) ethanol at 80 °C for 30 min. Then, 100 μL of extracts was added with 2 mL of anthrone and boiled for 10 min. The

absorption was measured at 630 nm, and the content was quantified using a calibration curve for the sucrose standard as a reference.

2.4. Determination of Ascorbate and Glutathione Contents

For the measurement of ascorbate (AsA) contents, 1 mL of first reagent was added to frozen samples of leaf tissue (0.1 g) and homogenized in an ice bath. The homogenate was placed in a 1.5 mL centrifuge tube; after centrifugation at 8000× *g* at 4 °C for 20 min or 16,000× *g* at 4 °C for 20 min, the supernatant was placed on ice for AsA measurement. The analysis was performed using the Micro AsA Content Assay Kit (BC1235; Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

For the measurement of reduced glutathione (GSH) contents, 0.1 g of fresh leaf tissue was weighed after rinsing it twice with PBS. We used the first part of the reagent in the kit to moisten and wash the homogenizer (the homogenizer was precooled in advance). Then, 1 mL of first reagent was added, and the sample was ground on ice. The sample was centrifuged at 7100× *g* (GSH) at 4 °C for 10 min. The supernatant was collected and maintained at 4 °C. GSH contents were determined using the Micro GSH Assay Kit (BC1185; Solarbio Science & Technology Co., Ltd. Beijing, China) according to the manufacturer's instructions.

2.5. Determination of Different Antioxidant Enzymatic Activities

Leaf tissues (0.5g) were ground in liquid nitrogen with mortar and pestle and homogenized in 5 mL of extraction buffer (phosphate buffer, pH 7.5, containing 0.1 mM EDTA and 4% polyvinylpolypyrrolidone). The homogenate was centrifuged at 12,000× *g* for 20 min, and the supernatant was used for enzyme analysis [28]. The activities of antioxidant enzymes, including ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), were measured according to the methods described by Chen and Wang [29].

2.6. Determination of Total Antioxidant Capacity

The total antioxidant capacity (T-AOC) of strawberry seedlings was measured using the total antioxidant capacity Assay Kit (BC1310; Solarbio., Science & Technology Co., Ltd. Beijing, China) based on the ferric-reducing antioxidant power (FRAP) assay. The absorbance was measured at 593 nm, and the T-AOC was calculated according to the standard curve.

2.7. Determination of Endogenous Melatonin and ABA

A total of 1 g of leaf sample was homogenized in 10 mL of dichloromethane and 6 mL of H_2O–propanol (2: 1, *v/v*) solution and then ultrasonicated for 35 min at 0 °C. The homogenate was centrifuged twice at 8000× *g* for 10 min at 4 °C, and the aliquot was collected, followed by mixing with 6 mL of methanol. The liquid flowed through the C18 extraction column and dried under nitrogen gas, and the powder was dissolved in 200 µL of methanol. ABA content was determined by high-performance liquid chromatography (3200 Qtrap Ultra high-performance liquid chromatography series triple quadrupole mass spectrometry instrument, AB SCIEX): The column used was Agilent SB-C18 column (50 mm × 2.1 mm, 1.8 µm). The mobile phase A (0.5% formic acid, *v/v*) and a mobile phase B (methanol) were maintained, using a gradient program as follows: 40 to 40% B (5 min), from 40 to 50% B (5 min), and from 50 to 70% B (2 min). Column temperature: 40 °C. Injection amount: 2 µL. Melatonin content, as determined by high-performance liquid chromatography, was roughly the same as ABA. However, the mobile phase A (1% formic acid, *v/v*) and a mobile phase B (1% formic acid of acetonitrile, *v/v*) were maintained, using a gradient program as follows: 95% A and 5% B (0.5 min), 10% A and 90% B (11 min), and 95% A and 5% B (7.5 min). Column temperature 35 °C. Injection amount: 3 µL.

2.8. Extraction of Total RNA and Quantitative Real-Time PCR (qRT-PCR) Analysis

The total RNA of leaf samples was extracted using an RNA Extraction Kit (Pudi, Shanghai, China). A NanoDrop Spectrophotometer was used to analyze the quantity and purity of the RNA. The first-strand cDNA was synthesized with the PrimeScript RT reagent kit

following the manufacturer's protocol. The RT-PCR gene-specific primers were designed and synthesized by Nanjing TSINGKE Biotechnology Firm using Primer Premier 5 (Version 5.0). Table S1 displays the primer sequence list. The qRT-PCRs were performed in an Applied Biosystems 7500 Real-time PCR system (CA, USA) using the SYBR Premix Ex Taq kit described earlier [30]. The quantitative qRT-PCR conditions consisted of denaturation at 95 °C for 4 min, accompanied by 40 cycles of denaturation at 95 °C for 25 s, annealing at 60 °C for 20 s, and extension at 72 °C for 43 s. The PCR reaction was performed in a 20 µL volume with 10 µL of Master Mix (SYBR Green), 1 µL of cDNA, 8.6 µL of ddH_2O, and 0.2 µL of each primer. The relative gene expression was calculated using the comparative CT method [31].

2.9. Statistical Analysis

The statistical analysis was performed using the statistical SPSS 19.0 program (SPSS Inc., Chicago, IL, USA). Each treatment comprised 3 biological replicates, and the leaves collected from three seedlings were referred as one biological replicate. Data were analyzed by one-way analysis of variance (ANOVA), and the differences among the treatment means were compared using the least-significant difference (LSD) test at $p \leq 0.05$ [32].

3. Results

3.1. Effects of Melatonin on Phenotypes of Strawberry Leaves

Reduced growth and progressive leaf chlorosis were the most noticeable signs of cold stress in strawberry seedlings compared with plants kept at normal conditions (25 °C) (Figure 1). After melatonin treatment, growth restriction caused by cold stress was alleviated to a large extent, exhibiting a low degree of wilting. These results indicate that exogenous melatonin application improved the cold stress resistance of strawberry seedlings.

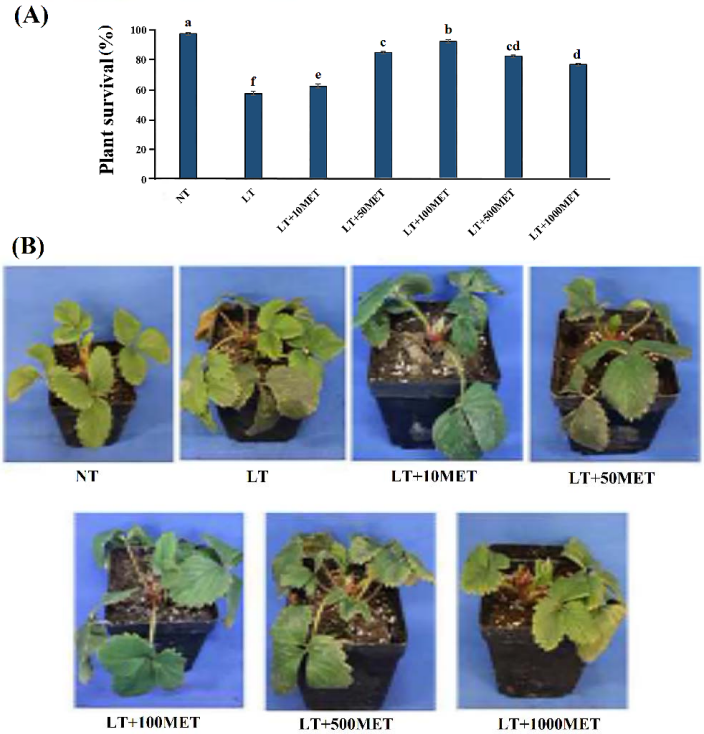

Figure 1. Effects of exogenous application of melatonin on plant survival (%) (**A**) and growth (**B**) of strawberry seedlings under cold stress. Plants treated with distilled water under the normal conditions (25 °C) served as control (NT). The strawberry seedlings were pretreated with distilled water (LT), 10 µM melatonin (LT + 10MET), 50 µM melatonin (LT + 50MET), 100 µM melatonin (LT + 100MET), 500 µM melatonin (LT + 500MET), and 1000 µM melatonin (LT + 1000MET) 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

3.2. Melatonin Application Reduces MDA and H_2O_2 Contents

For evaluating the influence of melatonin in alleviation of cold-induced oxidative stress, we measured the contents of MDA and H_2O_2 in the leaves of strawberry plants (Figure 2a,b). Cold stress significantly increased the contents of MDA and H_2O_2 in strawberry leaves, in comparison to the control, by 37% and 87%, respectively, suggesting that cold treatment caused severe oxidative stress in strawberry plants. In contrast, strawberry seedlings that were pretreated with 100 µM melatonin had profoundly alleviated stress markers (MDA and H_2O_2), which decreased in MET-treated plants by 43% and 47%, respectively, in comparison to the cold stress plants. Moreover, the higher concentration of melatonin (500–1000 µM) inhibited strawberry growth by excessive production of H_2O_2 and MDA, followed by cold stress. These findings indicate that 100 µM melatonin had the most obvious effect on reducing the MDA (about 22%) and increasing protection from lipid peroxidation. Cold stress significantly increased the accumulation of soluble sugars by 64% compared to control plants, and the content of sugars was further elevated by the melatonin pretreatment (Figure 2c).

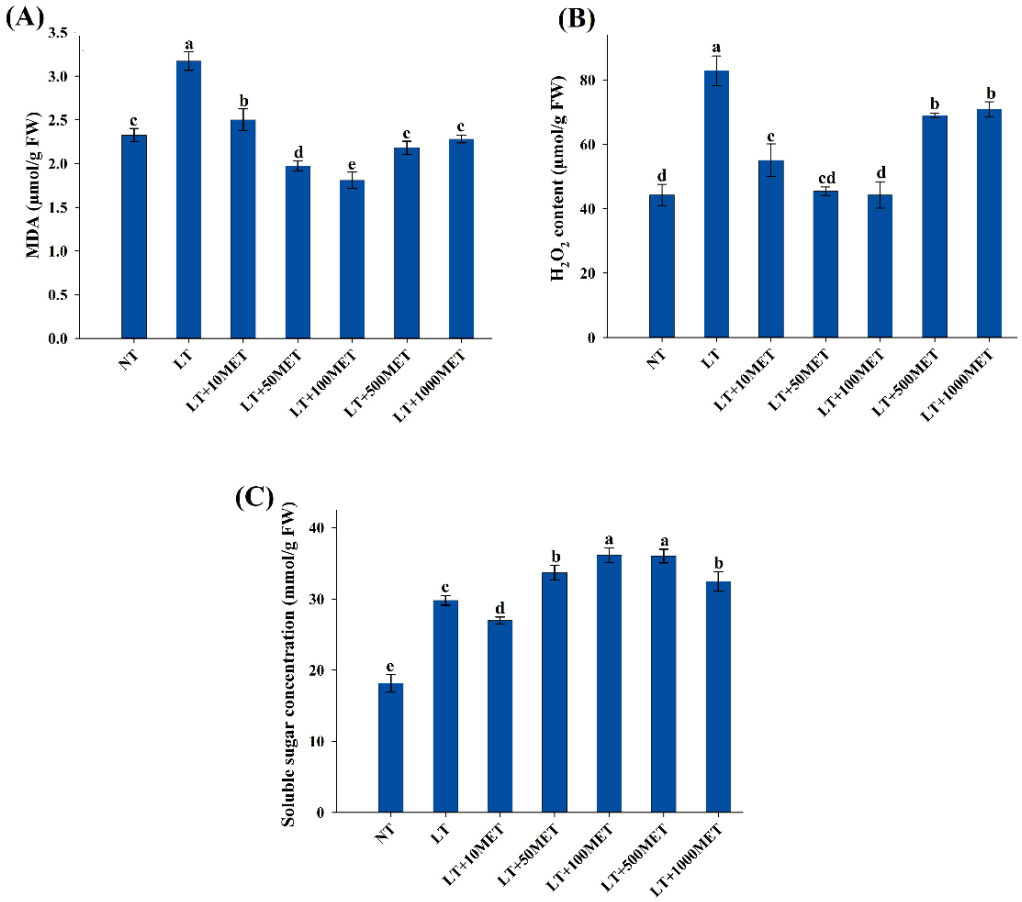

Figure 2. Effects of exogenous melatonin on MDA (**A**), H_2O_2 (**B**), and soluble sugar concentrations (**C**) in strawberry seedlings under cold stress. Plants treated with distilled water under the normal conditions (25 °C) served as control (NT). The strawberry seedlings were pretreated with distilled water (LT), 10 µM melatonin (LT + 10MET), 50 µM melatonin (LT + 50MET), 100 µM melatonin (LT + 100MET), 500 µM melatonin (LT + 500MET), and 1000 µM melatonin (LT + 1000MET) 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

3.3. Effects of Melatonin on Nonenzymatic Antioxidants

Non-enzymatic antioxidants (AsA and GSH) were measured in the leaves of strawberry plants (Figure 3). The AsA content was increased when the strawberry seedlings were exposed to cold stress by 32% compared with NT plants. In addition, AsA content was further increased by (111%) in 100 µM melatonin-pretreated cold-stressed seedlings compared to the NT seedlings (Figure 3a). Likewise, when strawberry seedlings were exposed to LT, the GSH content was also increased in LT plants by 9% compared with NT plants (Figure 3b). Furthermore, the 100 µM melatonin concentration significantly increased GSH content by 47% than NT plants (Figure 3b). In contrast, the higher concentration of melatonin (500 or 1000 µM) decreased the GSH content.

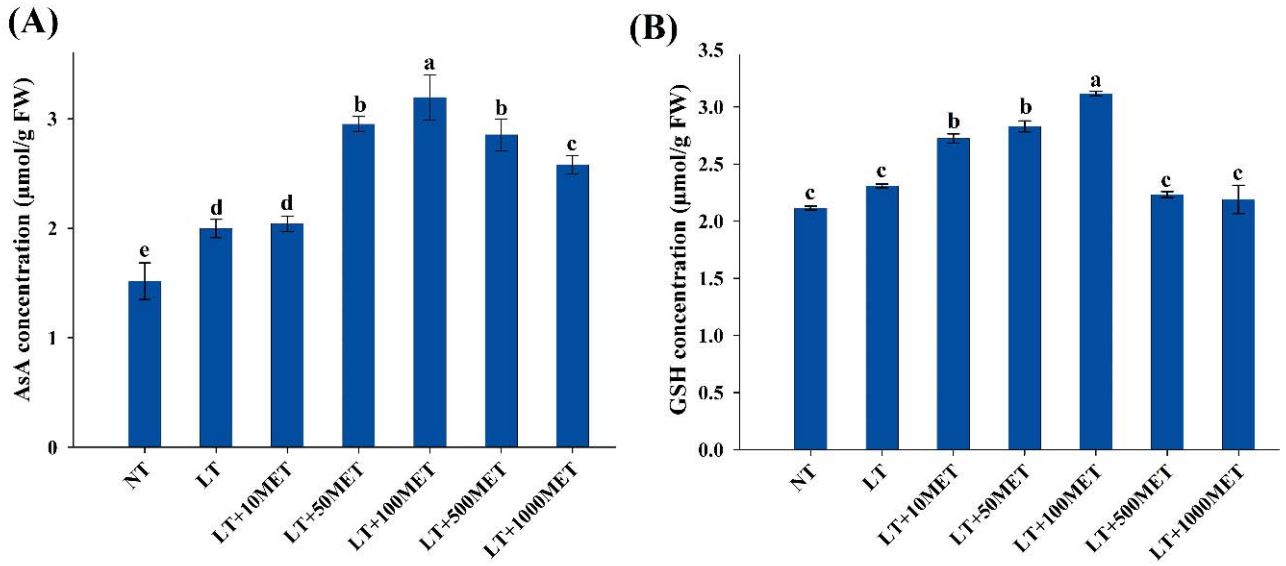

Figure 3. Effects of exogenous melatonin on non-enzymatic antioxidants (AsA (**A**) and GSH (**B**)) in strawberry seedlings under cold stress. Plants treated with distilled water under the normal conditions (25 °C) served as control (NT). The strawberry seedlings were pretreated with distilled water (LT), 10 µM melatonin (LT + 10MET), 50 µM melatonin (LT + 50MET), 100 µM melatonin (LT + 100MET), 500 µM melatonin (LT + 500MET), and 1000 µM melatonin (LT + 1000MET) 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

3.4. Effects of Melatonin on Antioxidant Enzyme Activities

Antioxidant enzyme activities were markedly enhanced in the leaves of strawberry seedlings by exogenous melatonin under cold stress conditions compared with untreated plants (Figure 4). Furthermore, the different melatonin treatments showed distinct behavior for increasing antioxidant enzyme activities. Melatonin applications enhanced APX activity compared with the control, and the highest activity was found in 100 µM-melatonin-treated plants, which accounted for 93% (Figure 4a). The CAT activity revealed an increasing trend similar to SOD activity. However, CAT activity in 100 µM- and 500 µM-melatonin-treated seedlings was considerably higher than in other concentrations at 70% and 59%, respectively, more than only-LT plants (Figure 4b). Similarly, POD activity showed an increasing pattern with increasing melatonin concentration and reached a maximum level at 100 µM melatonin and was 81% higher than in LT plants (Figure 4c). SOD activity was shown to increase with increasing melatonin concentrations under cold stress except for higher levels (500 and 1000 µM) of melatonin (Figure 4d), and the highest activity was observed in 100 µM-treated plants, and it was 61% higher than in NT plants.

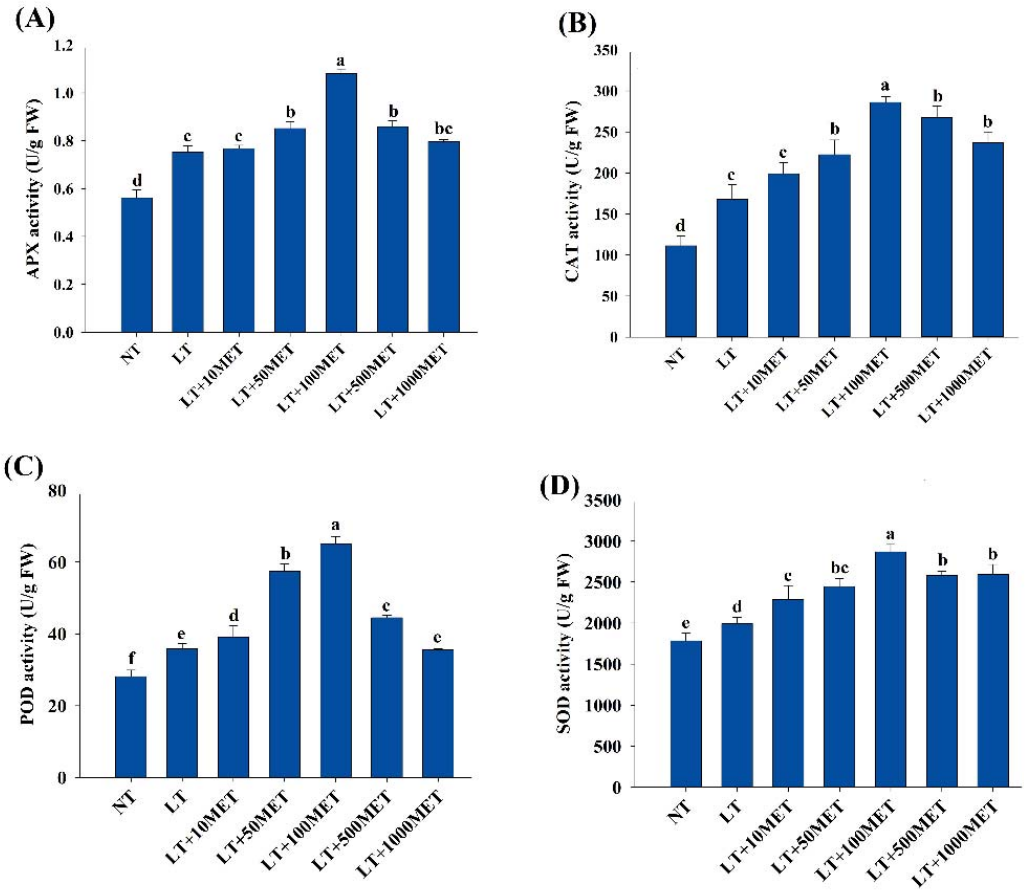

Figure 4. Effects of exogenous melatonin on the activities of antioxidant enzymes (APX (**A**) CAT (**B**), POD (**C**), and SOD (**D**)) in strawberry seedlings under cold stress. Plants treated with distilled water under the normal conditions (25 °C) served as control (NT). The strawberry seedlings were pretreated with distilled water (LT), 10 μM melatonin (LT + 10MET), 50 μM melatonin (LT + 50MET), 100 μM melatonin (LT + 100MET), 500 μM melatonin (LT + 500MET), and 1000 μM melatonin (LT + 1000MET) for 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

3.5. Effects of Melatonin on Expression Analysis of Cold-Related Genes

We assayed the transcriptional levels of cold-stress-related genes in strawberry seedlings (Figure 5). These findings indicate that the expression of cold-responsive genes (*COR*) was triggered in response to cold stress. After 12 and 48 h of cold treatment, the relative expression of the *COR47* gene was 1.49- and 11.20- fold higher in plants treated with μM melatonin compared with LT plants. After 96 h of cold treatment, expression of *COR47* was 26.58-fold higher in melatonin-pretreated plants than in control plants. The expression of *COR1* showed a similar pattern to *COR47*, which had higher expression levels after 96 h of cold treatment. The expression of *COR3* was affected by melatonin-treated plants after 0 h of cold treatment; the relative expression level of *COR3* was significantly higher in melatonin-treated plants than in control plants. After 3, 6, 12, and 48 h of cold treatment, the relative expression of the *HSF* gene was considerably higher in melatonin-treated plants compared with control plants, indicating that melatonin has the positive effect of an increase in cold tolerance. Compared with non-treated plants, the relative expression of *CBF4*, *DREB1B*, *DREB1E*, and *DREB2A* was significantly higher in the melatonin-pretreated plants after 48 h of cold stress. However, the expression of *CBF4*, *DREB1B*, *DREB1E*, and *DREB2A* was not affected by melatonin application after 96 h of cold treatment. Taken together, this study indicates that exogenous melatonin also conferred improved resistance

on cold stress in strawberry seedlings by activating the DREB/CBF-COR signaling pathway in cold-treated strawberry seedlings.

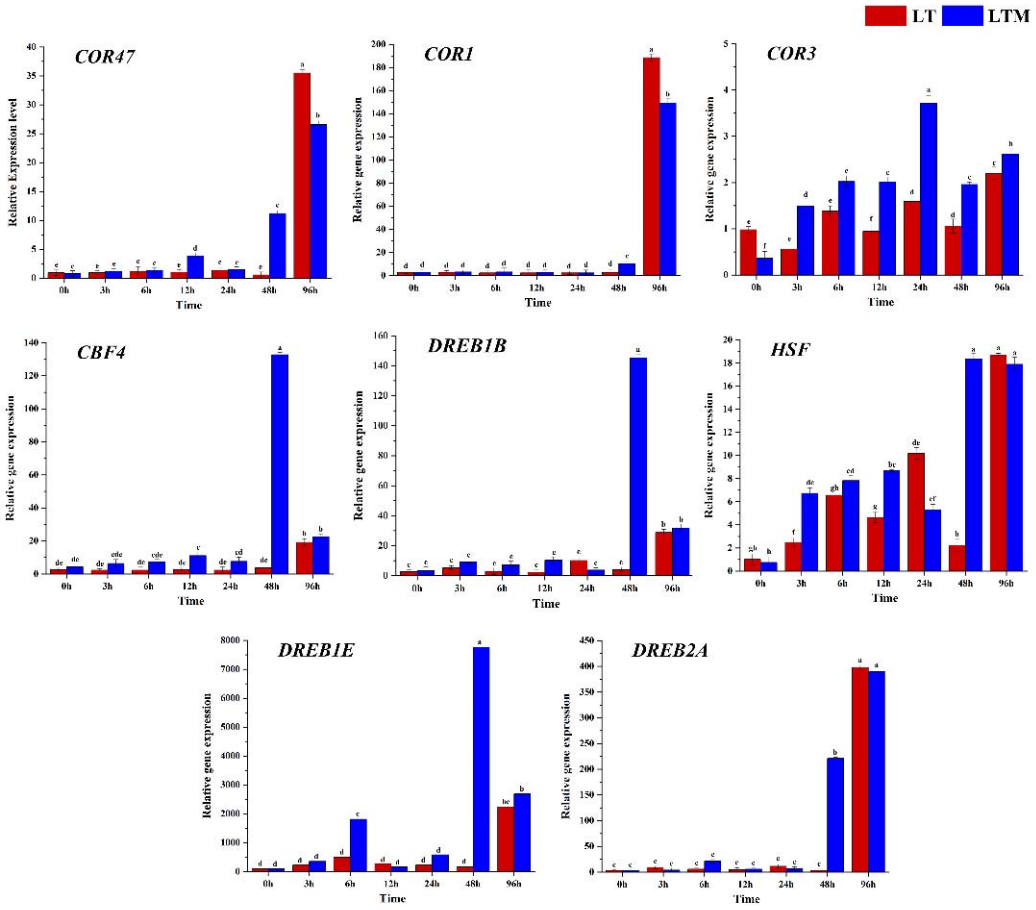

Figure 5. Expression of cold-related genes in leaves of strawberry under cold stress. The strawberry seedlings were pretreated with distilled water (LT) or 100 μM melatonin (LTM) 3 times in a two-day interval and then exposed to cold stress for 2 days. Samples were taken at 0, 3, 6, 12, 24, 48, and 96 h after cold treatment. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

3.6. ABA Involved in Melatonin-Mitigated Oxidative Damage Caused by Cold Stress

To examine the putative role of ABA in melatonin-mitigated oxidative damage caused by cold stress, an inhibitor of endogenous ABA biosynthesis was used (fluridone). Exogenously applied treatments such as melatonin and ABA significantly impacted the cold tolerance of strawberry seedlings (Figure 6). Two days after cold stress, the MDA concentration of strawberry seedlings was decreased ($p < 0.05$) by the application of exogenous melatonin and ABA treatments by 49% and 50%, respectively, compared to LT plants (Figure 6a). Moreover, the concentration of H_2O_2 displayed a similar pattern to that observed for MDA, with minor differences among treatments (Figure 6b).

Next, we assayed the activities of SOD, CAT, POD, and APX in the leaves of strawberry seedlings to verify the positive influence of ABA and melatonin under cold stress (Figure 7a–d). After 2 days of cold stress, 50 μM of exogenously applied fluridone decreased APX, POD, SOD, CAT, and T-AOC concentrations compared to other treatments. These antioxidant concentrations decreased when melatonin and fluridone were combined (Figure 7a–d). On the other hand, the enzymatic activities, including CAT, POD, APX, and T-AOC, were higher in melatonin-pretreated plants than in other treatments under cold stress conditions. However, pretreatment with fluridone alone did not enhance the enzymatic and non-enzymatic antioxidant concentrations under cold stress conditions.

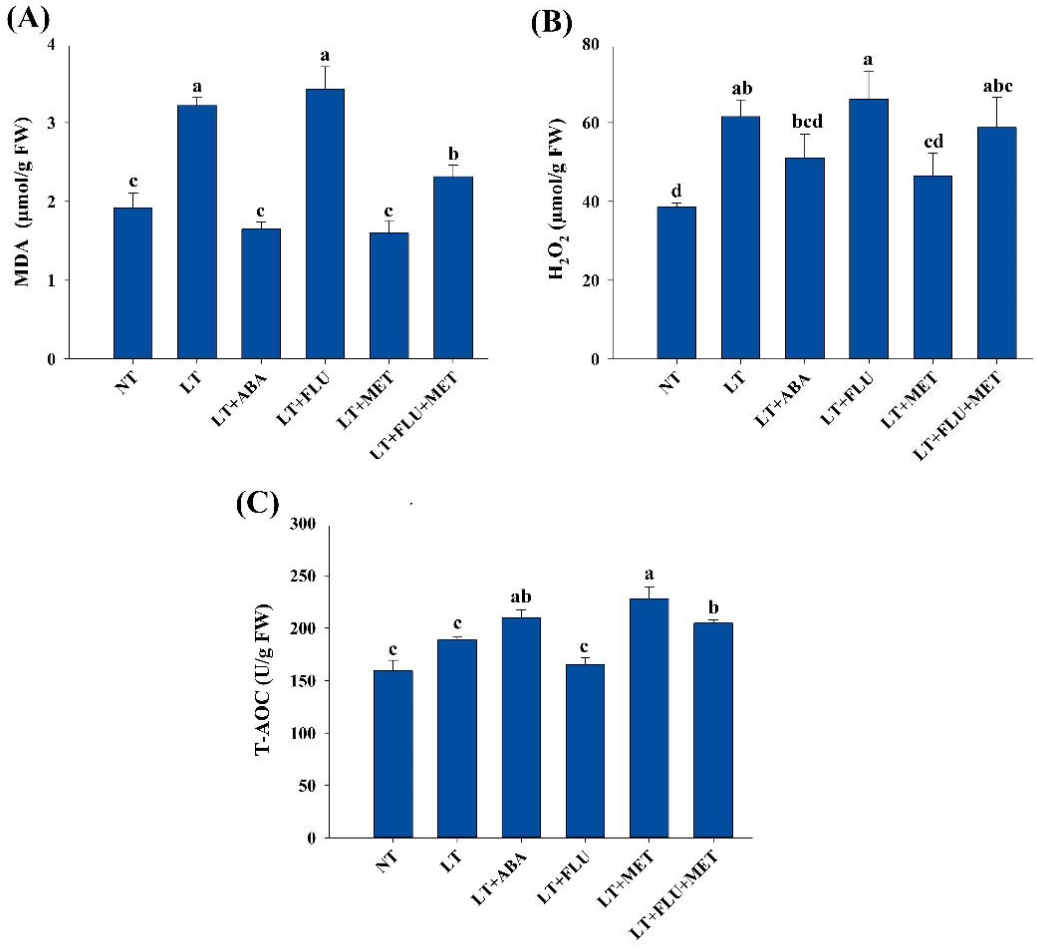

Figure 6. Effects of exogenous melatonin and ABA on melatonin on MDA (**A**), H_2O_2 (**B**), and total antioxidant capacity (T-AOC) (**C**) in strawberry seedlings under cold stress. Plants treated with distilled water under the normal conditions (25 °C) served as control (NT). The strawberry seedlings were pretreated with distilled water (LT), 100 μM ABA (LT + ABA), 50 μM fluridone (LT + FLU), 100 μM melatonin (LT + MET), and 50 μM fluridone + 100 μM melatonin (LT + FLU + MET) 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

3.7. Endogenous Melatonin, ABA Concentration, and Expression Analysis of ABA Metabolism Genes

After being exposed to cold stress, the levels of endogenous melatonin and ABA were measured in the leaves of strawberry seedlings to establish the relationship between melatonin and ABA concentrations. Increased endogenous ABA levels were observed after exogenous ABA treatment; however, decreased endogenous ABA levels were observed after exogenous melatonin treatment (Figure 8a). Seedlings treated with exogenous melatonin + fluridone (MET + FLU) had considerably increased endogenous melatonin content under cold stress (Figure 8b). Additionally, the relative expression of ABA metabolic genes was quantified. The expression of the *NCED1* and *CYP707A2* genes in the leaves of strawberry seedlings was higher in exogenous ABA-pretreated seedlings (Figure 8c,f). On the other hand, exogenous melatonin did not induce significant changes in ABA content (Figure 8b).

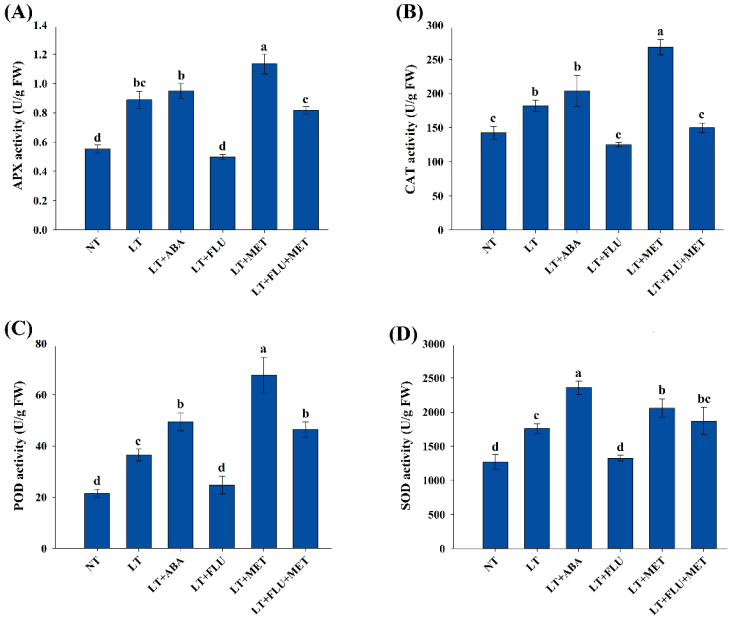

Figure 7. Effects of exogenous melatonin and ABA on the activities of antioxidant enzyme (APX (**A**), CAT (**B**), POD (**C**), and SOD (**D**)) in strawberry seedlings under cold stress. Plants treated with distilled water under the normal conditions (25 °C) served as control (NT). The strawberry seedlings were pretreated with distilled water (LT), 100 µM ABA (LT + ABA), 50 µM fluridone (LT + FLU), 100 µM melatonin (LT + MET), and 50 µM fluridone + 100 µM melatonin (LT + FLU + MET) 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

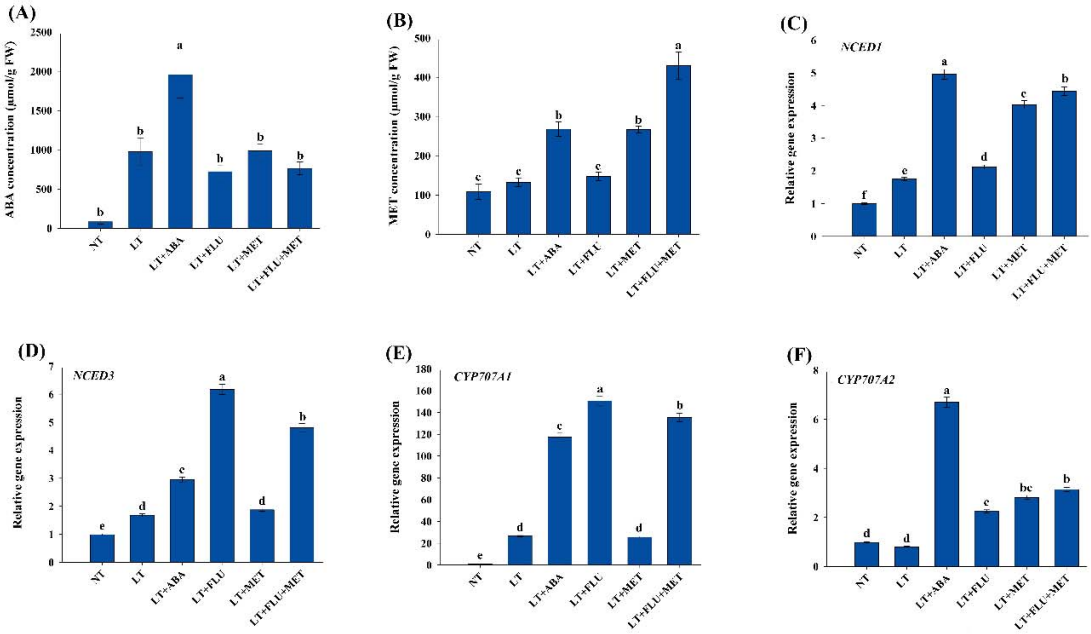

Figure 8. Effects of exogenous melatonin and ABA treatment on the endogenous ABA concentration (**A**), MET concentration (**B**), relative expressions of *NCED1* (**C**), *NCED3* (**D**), *CYP707A1* (**E**), and *CYP707A2* genes (**F**) in strawberry seedlings under cold stress. Plants treated with distilled water under the normal conditions (25 °C) served as control (NT). The strawberry seedlings were pretreated with distilled water (LT), 100 µM ABA (LT + ABA), 50 µM fluridone (LT + FLU), 100 µM melatonin (LT + MET), and 50 µM fluridone + 100 µM melatonin (LT + FLU + MET) 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

3.8. Effects of Melatonin and ABA on Expression Analysis of Cold-Related Genes

Next, we analyzed the exogenous ABA effect on the expression of DREB/CBF-COR-related genes in response to cold stress (Figure 9). Compared with exogenous ABA treatment, melatonin significantly upregulated the expression of *CBF4*, *DREB1B*, *DREB1E*, and *DREB2A* genes. Conversely, ABA treatment did not change the DREB/CBF-COR pathway expression, indicating that the improved cold tolerance of strawberries under cold stress after ABA treatment was not related to the DREB/CBF-COR pathway. Taken together, this investigation suggests that exogenous melatonin enhanced the cold tolerance of strawberry seedlings, and the upregulation of DREB/CBF expression may be involved in the melatonin-mediated cold response in strawberry seedlings.

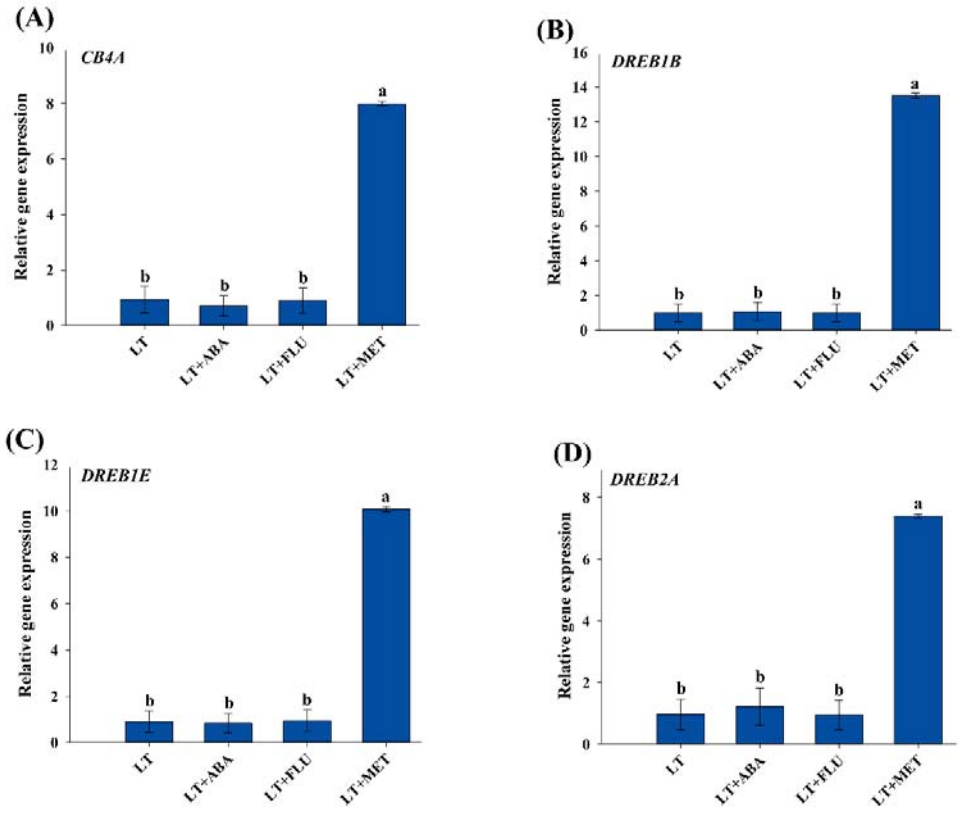

Figure 9. Effects of exogenous melatonin and ABA treatment on cold-related genes; transcript levels of *CBF4A* (**A**), *DREB1B* (**B**), *DREB1E* (**C**), and *DREB2A* (**D**) in strawberry seedlings under cold stress. The strawberry seedlings were pretreated with distilled water (LT), 100 μM ABA (LT + ABA), 50 μM fluridone (LT + FLU), 100 μM melatonin (LT + MET), and 50 μM fluridone + 100 μM melatonin (LT + FLU + MET) 3 times in a two-day interval and then exposed to cold stress for 2 days. Graphs represent the mean of three biological replicates. Values labeled with different letters indicate significant differences among treatments at ($p \leq 0.05$) by LSD.

4. Discussion

Cold stress is one of the major environmental factors that negatively impact plant growth and production and ultimately reduce crop yield [33]. Therefore, plants have developed various defensive strategies to respond to stresses. Melatonin is a broad-spectrum antioxidant and highly effective free radical scavenger that contributes to the detoxification of excess ROS, leading to enhanced stress tolerance [34,35]. During our study, the protective role of melatonin and its potential interrelation with ABA were investigated in strawberry seedlings under cold stress. Plants can protect themselves from the oxidative stress caused by cold stress by increasing their antioxidant capacity. However, if their antioxidant capacity is weak, applying several exogenous compounds with high-antioxidant properties

could help them increase stress tolerance [36]. The use of plant growth regulators and biostimulators such as "melatonin" could be an efficient approach to achieve this objective.

Our present study indicated that the growth of strawberry seedlings was markedly inhibited after the plants were exposed to cold stress. However, the application of melatonin reduced the severity of cold-induced growth inhibition (Figure 1B); our results further confirmed the findings of the previous reports that application of melatonin alleviated cold stress and positively affected plant growth in *Solanum lycopersicum* [37], *Arabidopsis thaliana* [38], *Cynodon dactylon* [27], and *Cucumis sativus* [39].

Cold-induced oxidative stress adversely affected cell membranes and enzymatic activities of plants. MDA content is primarily believed to be a reliable indicator of cellular damage [40]. In this study, the oxidative damage levels, as determined by H_2O_2 and lipid peroxidation levels, were increased in strawberry seedlings subjected to cold stress. Conversely, exogenous application of melatonin effectively protected plant cells from oxidative damage by lowering the accumulation of H_2O_2 and lipid peroxidation levels (evident from lower MDA contents), suggesting that melatonin protects strawberry plants from cold-induced oxidative stress (Figure 2a). This is in agreement with previous reports that melatonin decreased MDA contents and conferred cold tolerance on watermelon [41], tomato [37], *Elymus nutans* [20], and tea plants (*Camellia sinensis*) [42]. Moreover, exogenous melatonin application led to strikingly declined MDA levels in watermelon plants [43]. Our study found that the higher concentration of melatonin (500–1000 uM) reduced leaf growth compared with 100 uM melatonin. Our findings are in accordance with previous studies stating that a higher melatonin concentration suppressed leaf growth in Arabidopsis by reducing cell size and cell number [44].

Osmotic regulation is a key self-defense mechanism enacted by plants under stress. Plants produce high concentrations of osmotic regulators (e.g., soluble sugars) to increase cytoplasmic solute concentrations, reduce water potential, and reduce cell water loss [45]. In the current study, the soluble sugar content increased under cold stress, which could be further promoted by applications of melatonin (except 10 μM treatment) (Figure 2c), probably because melatonin promoted the synthesis of stress proteins in strawberry seedlings and protected proteins from damage, indicating that melatonin participated in the osmotic adjustment of strawberry seedlings. Similary, Shi et al. [27] reported that exogenous melatonin improves abiotic stress resistance in bermudagrass by activating the antioxidant enzyme system and increasing secondary metabolites such as sugar, alcohol, amino acid and organic acids [27], which was confirmed in the current work. Furthermore, the application of melatonin noticeably enhanced soluble sugar concentration, which helps plants regulate their osmotic balance and alleviates stress-induced maize growth inhibition [46].

Plants protect their cells from oxidative damage by eliminating excess ROS through an efficient antioxidant defense mechanism under stress conditions [47]. According to Mittler [48], increased antioxidant enzyme activities under various stresses lead to potential and specific ROS scavenging. Melatonin is a broad-spectrum antioxidant because it strengthens plants' antioxidant defense system and improves tolerance mainly by detoxifying excess ROS [10]. Likewise, melatonin could enhance salt resistance by regulating the corresponding genes encoding antioxidant enzymes in cucumbers under high salinity [49]. In the current study, the activities of antioxidant enzymes (APX, CAT, POD, and SOD) were lower in untreated control plants (LT) under cold stress. However, exogenous treatment with 100 μM melatonin significantly enhanced several antioxidant enzymatic activities of strawberry leaves under cold stress conditions, certifying the antioxidant function of melatonin in the strawberry against cold stress (Figure 4a–d). Several studies have found that melatonin treatments mitigated the negative effects of cold stress by stimulating the antioxidant defense system and enhances tolerance [41,50]. Our results are in accordance with the previous finding that melatonin-deficient tomatoes were less protective against abiotic stress than wild-type tomatoes, whereas melatonin-rich plants had a better ability to confront specific stresses [51].

Moreover, two essential nonenzymatic antioxidants, GSH and ASA, play an essential role in ROS scavenging in the ASA-GSH cycle, thereby reducing damage to plants from unfavorable conditions [52]. In the present study, the AsA content of strawberry seedling leaves increased significantly under cold stress, which could be further promoted by 50 to 500 μM-melatonin application (Figure 3a). Moreover, 100 μM melatonin application significantly enhanced the GSH content in strawberry seedlings compared with other treatments (Figure 3b). Thus, melatonin alleviated oxidative stress caused by cold stress depending on the relationship between melatonin and its applied concentrations. These findings are consistent with earlier studies that exogenous melatonin markedly enhanced the activity of non-enzymatic antioxidants under different stresses by improving the scavenging capacity of H_2O_2 and $O_2 \cdot -$ and reducing oxidative stress [10,13].

Many studies have demonstrated that ABA plays an important role in regulating plant growth, development, and responses to several environmental stresses [53,54]. The present study found that exogenous ABA treatment efficiently mitigated cold-induced oxidative damage in strawberry seedlings, as evidenced by a lower MDA content and decreased H_2O_2 accumulation (Figure 6a,b). Moreover, pretreatment with ABA enhanced SOD, APX, CAT, and T-AOC activities under cold stress. Similar results were observed in *Capsicum annuum* and *Elymus nutans* under ABA and low-temperature conditions [20,55]. The previous study demonstrated that ABA interacted with other essential signaling molecules, such as Ca^{2+}, NO, and H_2O_2, regulating responses to cold tolerance [20]. However, it is unclear whether ABA is involved in melatonin-mediated cold tolerance in strawberry seedlings. To further investigate the roles of ABA signaling in response to cold stress and exogenous melatonin treatment, fluridone, an inhibitor of endogenous ABA biosynthesis, was used. After being exposed to cold stress, pretreatments with fluridone in the presence of melatonin inhibited the positive effect of melatonin on cell membranes, as indicated by increases in MDA concentration, H_2O_2 levels, and a decline in antioxidant enzymes (APX, CAT, POD, SOD, and T-AOC activities (Figure 7a–d). These findings suggest that ABA is involved in the melatonin-induced antioxidant defense in response to cold stress.

The CBF/COR signaling pathway is the most important part of the cold stress response [56]. The C-repeat/dehydration element-binding protein (CBF/DREB1) gene is rapidly expressed during cold stress and contributes to plant cold tolerance [57]. The relative expression of the *COR47* gene was significantly higher in 100 μM-melatonin-pretreated plants after 12 and 48 h of cold treatment (Figure 5). Similarly, the expression level of *COR3* was also significantly higher in melatonin-pretreated plants at different time intervals than non-treated plants. Additionally, overexpression of a *CBF* gene, e.g., in barley, potato, apple, and poplar, improves freezing tolerance. The improved freezing tolerance in transgenic plants is associated with the induction of *COR* genes [58].

Melatonin treatment showed an increased expression of *COR3* and *COR47* compared with untreated plants under cold stress. In *Elymus nutans*, it has been reported that exogenous melatonin increased cold resistance and expression of several *CBF* and *COR* genes, revealing the possibility of the melatonin enhancement of low-temperature resistance by activating downstream cold-responsive genes [20]. Compared with the non-treated control plants, the relative expression of *CBF4*, *DREB1B*, *DREB1E*, and *DREB2A* was significantly higher in melatonin-pretreated plants after 48 h of cold stress (Figure 5). Overexpression of *CBF1* activated the downstream *COR* genes and increased freezing stress tolerance in Arabidopsis [59]. *TaDREB2*- and *TaDREB3*-transgenic wheat and barley plants significantly improved cold tolerance and increased expression of the stress-responsive *LEA/COR/DHN* genes [60]. Subsequently, melatonin application improves freezing tolerance in plants exposed to freezing temperatures [61]. These changes in gene expression suggest that melatonin enhanced strawberry tolerance of cold stress by regulating the genes of the DREB/CBF-COR pathway.

It is thought that DREB/CBF proteins improve stress tolerance by binding to the DRE/CRT cis-acting element of target genes in an ABA-independent manner [62]. Furthermore, ABA treatment did not induce a higher expression of CBF and COR genes in

Elymus nutans [20]. In the current study, the positive effects of ABA on the cold resistance of strawberry plants were verified, and it was found that pretreatment with melatonin did not increase the content of endogenous ABA. However, at the same time, ABA treatment significantly affects the content of endogenous melatonin. Furthermore, melatonin affects partial gene expression of the ABA biosynthesis and catabolism processes, and ABA treatment does not cause changes in *DREB/CBF* gene expression. Therefore, it is speculated that the response of melatonin to low-temperature stress in the strawberry is different from the ABA-dependent pathway, but cross-talks may exist between two signal pathways. However, the specific relationship between the two pathways is still uncertain. ABA is an important phytohormone that controls multiple physiological and biochemical processes. It has a crucial role in biotic and abiotic stresses, including cold resistance during plant growth and development [63]. *NCED* and *CYP707A* are critical genes in ABA metabolism [64]. In the present study, the relative expressions of *NCED1* and *CYP707A2* genes in the leaves of strawberry seedlings were noticeably higher in exogenous ABA-pretreated seedlings. Conversely, exogenous melatonin did not induce significant changes in ABA content (Figure 8a). These results suggest that melatonin could affect ABA biosynthesis, and the expressions of *NCED1* and *CYP707A2* genes were increased due to melatonin.

Metabolomics and transcriptomic analysis of melatonin-treated bermudagrass revealed melatonin led to transcriptional regulation of genes involved in nitrogen, carbohydrate, and hormone metabolism, along with the changes in the content of sucrose, glucose, and galactose. In *Arabidopsis thaliana,* Zhao et al. [65] reported that melatonin regulates carbohydrates' metabolism and activity of cell wall invertase improves cellulose, xylose, and galactose for cell wall reinforcement and callose deposition. So, we speculate that exogenous melatonin participated in multiple signaling pathways during the response of strawberries to cold stress. Though melatonin did not participate in the ABA-dependent pathway, it indirectly affected other metabolic synthesis pathways and led to the intermittent influence on ABA metabolic genes.

5. Conclusions

In the present study, we found that low-temperature stress affected strawberry growth. Melatonin application improves the cold tolerance of strawberry seedlings. The most effective concentration was 100 μM, and at this concentration, biomass production was noticeably increased. The application of melatonin in strawberry seedlings improved the antioxidant defense system by decreasing MDA and H_2O_2 contents under cold stress conditions. Moreover, we noticed that both exogenous melatonin and ABA could ameliorate oxidative damage caused by cold stress. Further investigations revealed that exogenous melatonin played an important role in enhancing cold tolerance by stimulating the expression of downstream genes in the DREB/CBF-COR pathway. However, ABA treatment failed to participate in this pathway. Overall, these findings indicate that the ABA-independent pathway may contribute to melatonin-induced cold tolerance in strawberry seedlings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8030194/s1, Table S1: Primer sequences used for qRT-PCR.

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References

1. Fierascu, R.C.; Temocico, G.; Fierascu, I.; Ortan, A.; Babeanu, N.E. Fragaria genus: Chemical composition and biological activities. *Molecules* **2020**, *25*, 498. [CrossRef] [PubMed]
2. Takahashi, F.; Kuromori, T.; Urano, K.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Drought stress responses and resistance in plants: From cellular responses to long-distance intercellular communication. *Front. Plant Sci.* **2020**, *11*, 1407. [CrossRef] [PubMed]
3. Waqas, M.A.; Wang, X.; Zafar, S.A.; Noor, M.A.; Hussain, H.A.; Azher Nawaz, M.; Farooq, M. Thermal Stresses in Maize: Effects and Management Strategies. *Plants* **2021**, *10*, 293. [CrossRef]
4. Hayat, F.; Ma, C.; Iqbal, S.; Huang, X.; Omondi, O.K.; Ni, Z.; Shi, T.; Tariq, R.; Khan, U.; Gao, Z. Rootstock-Mediated Transcriptional Changes Associated with Cold Tolerance in *Prunus mume* Leaves. *Horticulturae* **2021**, *7*, 572. [CrossRef]
5. Huang, C.; Qin, N.; Sun, L.; Yu, M.; Hu, W.; Qi, Z. Selenium improves physiological parameters and alleviates oxidative stress in strawberry seedlings under low-temperature stress. *Int. J. Mol. Sci.* **2018**, *19*, 1913. [CrossRef] [PubMed]
6. John, R.; Anjum, N.A.; Sopory, S.K.; Akram, N.A.; Ashraf, M. Some key physiological and molecular processes of cold acclimation. *Biol. Plant.* **2016**, *60*, 603–618. [CrossRef]
7. Wang, D.Z.; Jin, Y.N.; Ding, X.H.; Wang, W.J.; Zhai, S.S.; Bai, L.P.; Guo, Z.F. Gene regulation and signal transduction in the ICE–CBF–COR signaling pathway during cold stress in plants. *Biochemistry* **2017**, *82*, 1103–1117. [CrossRef]
8. Zafar, S.A.; Uzair, M.; Khan, M.R.; Patil, S.B.; Fang, J.; Zhao, J.; Singla-Pareek, S.L.; Pareek, A.; Li, X. DPS1 regulates cuticle development and leaf senescence in rice. *Food Energy Secur.* **2021**, *10*, e273.
9. Yu, D.; Liu, X.; Cui, Y.; Bi, Q.; Zhao, Y.; Li, D.; Yu, H.; Wang, L. Comparative transcriptome combined with morpho-physiological analyses revealed candidate genes potentially for differential cold tolerance in two contrasting apricot (*Prunus armeniaca* L.) cultivars. *Trees* **2020**, *34*, 1205–1217. [CrossRef]
10. Altaf, M.A.; Shahid, R.; Ren, M.; Mora-Poblete, F.; Arnao, M.B.; Naz, S.; Anwar, M.; Altaf, M.M.; Shahid, S.; Shakoor, A. Phytomelatonin: An overview of the importance and mediating functions of melatonin against environmental stresses. *Physiol. Plant.* **2021**, *172*, 820–846. [CrossRef]
11. Reiter, R.J.; Mayo, J.C.; Tan, D.; Sainz, R.M.; Alatorre-Jimenez, M.; Qin, L. Melatonin as an antioxidant: Under promises but over delivers. *J. Pineal Res.* **2016**, *61*, 253–278. [CrossRef] [PubMed]
12. Li, J.; Arkorful, E.; Cheng, S.; Zhou, Q.; Li, H.; Chen, X.; Sun, K.; Li, X. Alleviation of cold damage by exogenous application of melatonin in vegetatively propagated tea plant (*Camellia sinensis* (L.) O. Kuntze). *Sci. Hortic.* **2018**, *238*, 356–362. [CrossRef]
13. Jahan, M.S.; Shu, S.; Wang, Y.; Chen, Z.; He, M.; Tao, M.; Sun, J.; Guo, S. Melatonin alleviates heat-induced damage of tomato seedlings by balancing redox homeostasis and modulating polyamine and nitric oxide biosynthesis. *BMC Plant Biol.* **2019**, *19*, 414. [CrossRef] [PubMed]
14. Liu, X.; Xia, H.; Yuan, X.; Lv, X.; Liang, D. Effects of exogenous melatonin on the physiological characteristics of kiwifruit hayward under salt stress. In *IOP Conference Series: Earth and Environmental Science, Proceedings of the 4th International Conference on Advances in Energy Resources and Environment Engineering, Chengdu, China, 7–9 December 2018*; IOP Publishing: Bristol, UK, 2019; Volume 237, p. 52076.
15. Liang, D.; Ni, Z.; Xia, H.; Xie, Y.; Lv, X.; Wang, J.; Lin, L.; Deng, Q.; Luo, X. Exogenous melatonin promotes biomass accumulation and photosynthesis of kiwifruit seedlings under drought stress. *Sci. Hortic.* **2019**, *246*, 34–43. [CrossRef]
16. Kaya, C.; Okant, M.; Ugurlar, F.; Alyemeni, M.N.; Ashraf, M.; Ahmad, P. Melatonin-mediated nitric oxide improves tolerance to cadmium toxicity by reducing oxidative stress in wheat plants. *Chemosphere* **2019**, *225*, 627–638. [CrossRef]
17. Zhao, J.; Zhao, L.; Zhang, M.; Zafar, S.A.; Fang, J.; Li, M.; Zhang, W.; Li, X. Arabidopsis E3 ubiquitin ligases PUB22 and PUB23 negatively regulate drought tolerance by targeting ABA receptor PYL9 for degradation. *Int. J. Mol. Sci.* **2017**, *18*, 1841. [CrossRef]
18. Neill, S.; Barros, R.; Bright, J.; Desikan, R.; Hancock, J.; Harrison, J.; Morris, P.; Ribeiro, D.; Wilson, I. Nitric oxide, stomatal closure, and abiotic stress. *J. Exp. Bot.* **2008**, *59*, 165–176. [CrossRef]
19. Li, C.; Tan, D.-X.; Liang, D.; Chang, C.; Jia, D.; Ma, F. Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behaviour in two *Malus* species under drought stress. *J. Exp. Bot.* **2015**, *66*, 669–680. [CrossRef]
20. Fu, J.; Wu, Y.; Miao, Y.; Xu, Y.; Zhao, E.; Wang, J.; Sun, H.; Liu, Q.; Xue, Y.; Xu, Y. Improved cold tolerance in *Elymus nutans* by exogenous application of melatonin may involve ABA-dependent and ABA-independent pathways. *Sci. Rep.* **2017**, *7*, 39865. [CrossRef]
21. Shi, H.; Qian, Y.; Tan, D.; Reiter, R.J.; He, C. Melatonin induces the transcripts of CBF/DREB1s and their involvement in both abiotic and biotic stresses in Arabidopsis. *J. Pineal Res.* **2015**, *59*, 334–342. [CrossRef]
22. Wu, J.; Folta, K.M.; Xie, Y.; Jiang, W.; Lu, J.; Zhang, Y. Overexpression of Muscadinia rotundifolia *CBF2* gene enhances biotic and abiotic stress tolerance in Arabidopsis. *Protoplasma* **2017**, *254*, 239–251. [CrossRef]

23. Gilmour, S.J.; Zarka, D.G.; Stockinger, E.J.; Salazar, M.P.; Houghton, J.M.; Thomashow, M.F. Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J.* **1998**, *16*, 433–442. [CrossRef] [PubMed]
24. Cook, D.; Fowler, S.; Fiehn, O.; Thomashow, M.F. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 15243–15248. [CrossRef] [PubMed]
25. Thomashow, M.F. Molecular basis of plant cold acclimation: Insights gained from studying the CBF cold response pathway. *Plant Physiol.* **2010**, *154*, 571–577. [CrossRef] [PubMed]
26. Tiwari, R.K.; Lal, M.K.; Kumar, R.; Chourasia, K.N.; Naga, K.C.; Kumar, D.; Das, S.K.; Zinta, G. Mechanistic insights on melatonin-mediated drought stress mitigation in plants. *Physiol. Plant.* **2021**, *172*, 1212–1226. [CrossRef] [PubMed]
27. Shi, H.; Jiang, C.; Ye, T.; Tan, D.-X.; Reiter, R.J.; Zhang, H.; Liu, R.; Chan, Z. Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L). Pers.] by exogenous melatonin. *J. Exp. Bot.* **2015**, *66*, 681–694. [CrossRef] [PubMed]
28. Zafar, S.A.; Hameed, A.; Ashraf, M.; Khan, A.S.; Li, X.; Siddique, K.H.M. Agronomic, physiological and molecular characterisation of rice mutants revealed the key role of reactive oxygen species and catalase in high-temperature stress tolerance. *Funct. Plant Biol.* **2020**, *47*, 440–453. [CrossRef]
29. Chen, J.X.; Wang, X.F. *Experimental Guide for Plant Physiology*; South China Science and Technology University Press: Guangzhou, China, 2002; pp. 120–121.
30. Fang, J.; Guo, T.; Xie, Z.; Chun, Y.; Zhao, J.; Peng, L.; Zafar, S.A.; Yuan, S.; Xiao, L.; Li, X. The URL1-ROC5-TPL2 transcriptional repressor complex represses the *ACL1* gene to modulate leaf rolling in rice. *Plant Physiol.* **2021**, *185*, 1722–1744. [CrossRef]
31. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef]
32. Steel, R.G.D.; Torrie, J.H.; Dickey, D.A. *Principles and Procedures of Statistics: A Biometrical Approach*, 3rd ed.; McGraw Hill Book Co. Inc.: New York, NY, USA, 1997.
33. Chinnusamy, V.; Zhu, J.-K.; Sunkar, R. Gene regulation during cold stress acclimation in plants. In *Plant Stress Tolerance*; Springer: Berlin/Heidelberg, Germany, 2010; pp. 39–55.
34. Nawaz, M.A.; Huang, Y.; Bie, Z.; Ahmed, W.; Reiter, R.J.; Niu, M.; Hameed, S. Melatonin: Current status and future perspectives in plant science. *Front. Plant Sci.* **2016**, *6*, 1230. [CrossRef]
35. Altaf, M.A.; Shahid, R.; Ren, M.X.; Naz, S.; Altaf, M.M.; Qadir, A.; Anwar, M.; Shakoor, A.; Hayat, F. Exogenous melatonin enhances salt stress tolerance in tomato seedlings. *Biol. Plant.* **2020**, *64*, 604–615. [CrossRef]
36. Altaf, M.A.; Shahid, R.; Ren, M.; Naz, S.; Altaf, M.M.; Khan, L.U.; Tiwari, R.K.; Lal, M.K.; Shahid, M.A.; Kumar, R. Melatonin Improves Drought Stress Tolerance of Tomato by Modulation Plant Growth, Root Architecture, Photosynthesis, and Antioxidant Defense System. *Antioxidants* **2022**, *11*, 309. [CrossRef]
37. Ding, F.; Liu, B.; Zhang, S. Exogenous melatonin ameliorates cold-induced damage in tomato plants. *Sci. Hortic.* **2017**, *219*, 264–271. [CrossRef]
38. Bajwa, V.S.; Shukla, M.R.; Sherif, S.M.; Murch, S.J.; Saxena, P.K. Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. *J. Pineal Res.* **2014**, *56*, 238–245. [CrossRef] [PubMed]
39. Zhao, H.; Ye, L.; Wang, Y.; Zhou, X.; Yang, J.; Wang, J.; Cao, K.; Zou, Z. Melatonin increases the chilling tolerance of chloroplast in cucumber seedlings by regulating photosynthetic electron flux and the ascorbate-glutathione cycle. *Front. Plant Sci.* **2016**, *7*, 1814. [CrossRef] [PubMed]
40. Hasanuzzaman, M.; Bhuyan, M.H.M.; Zulfiqar, F.; Raza, A.; Mohsin, S.M.; Al Mahmud, J.; Fujita, M.; Fotopoulos, V. Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator. *Antioxidants* **2020**, *9*, 681. [CrossRef]
41. Li, H.; Dong, Y.; Chang, J.; He, J.; Chen, H.; Liu, Q.; Wei, C.; Ma, J.; Zhang, Y.; Yang, J. High-throughput microRNA and mRNA sequencing reveals that microRNAs may be involved in melatonin-mediated cold tolerance in *Citrullus lanatus* L. *Front. Plant Sci.* **2016**, *7*, 1231. [CrossRef]
42. Li, J.; Yang, Y.; Sun, K.; Chen, Y.; Chen, X.; Li, X. Exogenous melatonin enhances cold, salt and drought stress tolerance by improving antioxidant defense in tea plant (*Camellia sinensis* (L.) O. Kuntze). *Molecules* **2019**, *24*, 1826. [CrossRef]
43. Li, H.; Chang, J.; Zheng, J.; Dong, Y.; Liu, Q.; Yang, X.; Wei, C.; Zhang, Y.; Ma, J.; Zhang, X. Local melatonin application induces cold tolerance in distant organs of *Citrullus lanatus* L. via long distance transport. *Sci. Rep.* **2017**, *7*, 40858. [CrossRef]
44. Wang, T.; Ye, C.; Wang, M.; Chu, G. Identification of cold-stress responsive proteins in *Anabasis aphylla* seedlings via the iTRAQ proteomics technique. *J. Plant Interact.* **2017**, *12*, 505–519. [CrossRef]
45. Verbruggen, N.; Hermans, C. Proline accumulation in plants: A review. *Amino Acids* **2008**, *35*, 753–759. [CrossRef] [PubMed]
46. Ahmad, S.; Kamran, M.; Ding, R.; Meng, X.; Wang, H.; Ahmad, I.; Fahad, S.; Han, Q. Exogenous melatonin confers drought stress by promoting plant growth, photosynthetic capacity and antioxidant defense system of maize seedlings. *PeerJ* **2019**, *7*, e7793. [CrossRef] [PubMed]
47. Wang, L.Y.; Liu, J.L.; Wang, W.X.; Sun, Y. Exogenous melatonin improves growth and photosynthetic capacity of cucumber under salinity-induced stress. *Photosynthetica* **2016**, *54*, 19–27. [CrossRef]
48. Mittler, R. ROS are good. *Trends Plant Sci.* **2017**, *22*, 11–19. [CrossRef] [PubMed]

49. Zhang, H.; Zhang, N.A.; Yang, R.; Wang, L.; Sun, Q.; Li, D.; Cao, Y.; Weeda, S.; Zhao, B.; Ren, S.; et al. Melatonin promotes seed germination under high salinity by regulating antioxidant systems, ABA and GA 4 interaction in cucumber (*Cucumis sativus* L.). *J. Pineal Res.* **2014**, *57*, 269–279. [CrossRef]
50. Zhang, Y.P.; Xu, S.; Yang, S.J.; Chen, Y.Y. Melatonin alleviates cold-induced oxidative damage by regulation of ascorbate–glutathione and proline metabolism in melon seedlings (*Cucumis melo* L.). *J. Hortic. Sci. Biotechnol.* **2017**, *92*, 313–324. [CrossRef]
51. Okazaki, M.; Higuchi, K.; Aouini, A.; Ezura, H. Lowering intercellular melatonin levels by transgenic analysis of indoleamine 2, 3-dioxygenase from rice in tomato plants. *J. Pineal Res.* **2010**, *49*, 239–247. [CrossRef]
52. Bybordi, A. Effect of Ascorbic Acid and Silicium on Photosynthesis, Antioxidant Enzyme Activity, and Fatty Acid Contents in Canola Exposure to Salt Stress. *J. Integr. Agric.* **2012**, *11*, 1610–1620. [CrossRef]
53. Fujita, Y.; Fujita, M.; Satoh, R.; Maruyama, K.; Parvez, M.M.; Seki, M.; Hiratsu, K.; Ohme-Takagi, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. *Plant Cell* **2005**, *17*, 3470–3488. [CrossRef]
54. Karimi, R.; Ershadi, A. Role of exogenous abscisic acid in adapting of 'Sultana'grapevine to low-temperature stress. *Acta Physiol. Plant.* **2015**, *37*, 151. [CrossRef]
55. Guo, W.L.; Chen, R.G.; Gong, Z.H.; Yin, Y.X.; Ahmed, S.S.; He, Y.M. Exogenous abscisic acid increases antioxidant enzymes and related gene expression in pepper (*Capsicum annuum*) leaves subjected to chilling stress. *Genet. Mol. Res* **2012**, *11*, 4063–4080. [CrossRef]
56. Knight, M.R.; Knight, H. Low-temperature perception leading to gene expression and cold tolerance in higher plants. *New Phytol.* **2012**, *195*, 737–751. [CrossRef]
57. Guo, J.; Ren, Y.; Tang, Z.; Shi, W.; Zhou, M. Characterization and expression profiling of the ICE-CBF-COR genes in wheat. *PeerJ* **2019**, *7*, e8190. [CrossRef]
58. Park, S.; Lee, C.; Doherty, C.J.; Gilmour, S.J.; Kim, Y.; Thomashow, M.F. Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. *Plant J.* **2015**, *82*, 193–207. [CrossRef]
59. Jaglo-Ottosen, K.R.; Gilmour, S.J.; Zarka, D.G.; Schabenberger, O.; Thomashow, M.F. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **1998**, *280*, 104–106. [CrossRef]
60. Morran, S.; Eini, O.; Pyvovarenko, T.; Parent, B.; Singh, R.; Ismagul, A.; Eliby, S.; Shirley, N.; Langridge, P.; Lopato, S. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant Biotechnol. J.* **2011**, *9*, 230–249. [CrossRef]
61. Shi, H.; Chan, Z. The cysteine2/histidine2-type transcription factor ZINC FINGER OF ARABIDOPSIS THALIANA 6-activated C-REPEAT-BINDING FACTOR pathway is essential for melatonin-mediated freezing stress resistance in Arabidopsis. *J. Pineal Res.* **2014**, *57*, 185–191. [CrossRef]
62. Agarwal, P.K.; Jha, B. Transcription factors in plants and ABA dependent and independent abiotic stress signalling. *Biol. Plant.* **2010**, *54*, 201–212. [CrossRef]
63. Kim, Y.-H.; Choi, K.-I.; Khan, A.L.; Waqas, M.; Lee, I.-J. Exogenous application of abscisic acid regulates endogenous gibberellins homeostasis and enhances resistance of oriental melon (*Cucumis melo* var. L.) against low temperature. *Sci. Hortic.* **2016**, *207*, 41–47. [CrossRef]
64. Kushiro, T.; Okamoto, M.; Nakabayashi, K.; Yamagishi, K.; Kitamura, S.; Asami, T.; Hirai, N.; Koshiba, T.; Kamiya, Y.; Nambara, E. The Arabidopsis cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: Key enzymes in ABA catabolism. *EMBO J.* **2004**, *23*, 1647–1656. [CrossRef]
65. Zhao, H.; Xu, L.; Su, T.; Jiang, Y.; Hu, L.; Ma, F. Melatonin regulates carbohydrate metabolism and defenses against Pseudomonas syringae pv. tomato DC 3000 infection in *Arabidopsis thaliana*. *J. Pineal Res.* **2015**, *59*, 109–119. [CrossRef]