MORE ON PHYTOMELATONIN: METABOLISM AND PHYSIOLOGICAL ROLES

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MORE ON PHYTOMELATONIN: METABOLISM AND PHYSIOLOGICAL ROLES

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EDITORIAL
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Editorial: More on Phytomelatonin: Metabolism and Physiological Roles

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Keywords: melatonin, plant, metabolism, physiology, stress responses

Editorial on the Research Topic

More on Phytomelatonin: Metabolism and Physiological Roles

Melatonin (N-acetyl-5-methoxytryptamine) is a tryptophan-derived indole amine molecule present in animals, plants, and microbes. It was first identified in animals in 1958 where it mediates the regulation of circadian and seasonal rhythms. Melatonin and its derivatives protect animal cells by scavenging many types of reactive oxygen and nitrogen species (ROS/RNS) and up-regulating the expression of antioxidative enzymes. It also can act as an anti-inflammatory and immunomodulator, with the potential to reduce the severity of disease-associated symptoms.

Discovered in plants a quarter of a century ago, phytomelatonin has been reported from many mono- and dicotyledonous species. Research investigating the effects of exogenous melatonin application and gene expression of melatonin-related genes indicate that the endogenous levels of phytomelatonin can modulate many aspects of plant growth and can have a protective role by reducing the negative effects of biotic and abiotic stresses.

This Research Topic focuses on phytomelatonin metabolism and physiological roles in planta and is a follow up on "Melatonin in plants." We aim to ask how melatonin alone or in association with other melatonin-derived compounds can modulate plant physiology and what other role(s) they may have. This Research Topic contains one review and 12 original research studies. The articles describe findings that further elucidate the role of phytomelatonin in biotic and abiotic stress tolerance, including some of the molecular actors and mechanism associated to phytomelatonin biosynthesis.

The review by Murch and Erland employed a systematic approach using the PRISMA protocol to highlight the current state of research published in plant physiology, growth, and metabolism since its discovery in plants in 1995. An active and exciting area of phytomelatonin research has been in enhancing understanding pathway dynamics of melatonin biosynthesis in plants with the recent discoveries of alternate pathways and biologically important melatonin isomers. Zheng et al. and Zhou et al. have characterized and identified acetylserotonin-O-methlytransferase (ASMT) and serotonin-N-acetyltransferase (SNAT) in mulberry (*Morus alba* L.) and St. John's wort (*Hypericum perforatum* L.) respectively. In mulberry, ASMT was found to be able to catalyze production of melatonin as well as two isomers described as melatonin isoform (MI)-1 and MI-2, with differential distribution of these molecules across *Morus* species and tissues surveyed. In St. John's wort an early model for phytomleatonin research two leaf specific HpSNAT are described which confer salt and drought tolerance in *Arabidopsis* transgenic plants overexpressing HpSNAT.

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Tolerance to abiotic and biotic stresses are investigated in this Research Topic and are key roles of melatonin in plants. Zhu et al. reported an enhanced resistance to *Botrytis cinerea* infection in *Arabidopsis* with overexpression of SNAT and ASMT, the final two steps in conversion of serotonin to melatonin, while susceptibility increased when silenced. This study is of particular interest as it slows for differentiation between serotonin and melatonin mediated effects. Interestingly jasmonic acid (JA) content was also increased with *SNAT* and *ASMT* expression levels in transgenics following fungal infection highlighting the role of phytomelatonin in the activation of JA signaling pathway.

Abiotic stress responses are examined for several species. In rice, Li et al. found that melatonin treatment improved seed germination at low temperature through activation of gibberellin biosynthesis, by maintaining the redox homeostasis, and endogenous melatonin biosynthesis, but prevented the buildup of abscisic acid (ABA) and hydrogen peroxide. Moreover, the authors demonstrated that melatonin acts synergistically with an ABI5-mediated signal involving the regulation of the rice *CATALASE2*. In tomato, Ding et al. found that, while the cold stress increased JA accumulation, the biosynthesis of melatonin was also increased *via* MYC2-activated SlSNAT and SlASMT therefore potentiating cold tolerance. Yang N. et al. reported that selenite treatment at low concentration improved the cold tolerance of cucumber seedlings and increased endogenous melatonin content through up-regulation of key melatonin biosynthetic genes. The research by Xing et al. on heat-resistance mediated by melatonin in chrysanthemum seedlings employed physiological and transcript analyses to reveal the underlying associated gene regulatory networks such as osmotic regulation, redox homeostasis, hormone signal transduction and the chlorophyll, flavonoid, and carotenoid metabolic pathways. In tomato, Jahan et al. found that the attenuation of heat stress induced senescence by melatonin treatment involved enhanced gibberellic acid (GA) and endogenous melatonin content but reduced abscisic acid, also evidenced using gene transcript levels. Furthermore, chemical inhibition of GA and ABA synthesis failed to produce melatonin induced heat tolerance. In tobacco, Chen, Jia et al. investigated the regulatory mechanisms by which melatonin treatment improve dehydration-induced leaf senescence in seedlings and found that foliar spraying decreased endogenous ABA content and oxidative damage. Moreover, metabolite profiling and gene expression analysis showed that melatonin induces carotenoids, inhibits chlorophyll breakdown, modulates phytohormonal biosynthesis and signaling as well as the transcriptional network underlying leaf senescence. Yang S-J. et al. reported that the high light stress response in Arabidopsis leaf was improved with exogenous melatonin application by enhancing the endogenous melatonin content, protecting the photosynthetic pigments and integrity of photosynthesis apparatus, and inhibiting ROS accumulation. In wheat, Zhang et al. compared three winter wheat varieties with contrasted responses for salt tolerance and reported the promotive effect of exogenous melatonin on germination trait under salt stress. Improved wheat germination by melatonin was found to be related to the increase in root vigor, maintenance of ion balance, decrease in H_2O_2 content, regulation of soluble protein and sugar synthesis, and changes in amino acid levels. Chen, Cao et al. found that application of melatonin on alfalfa plants submitted to high-nitrate stress improved growth and development by regulating the excess nitrogen and calcium intake, promoting nitrogen metabolism with enhanced enzyme activities, reducing ATP-utilizing system and increasing ATP regeneration.

Together this Research Topic provides readers with overview of the key roles melatonin plays in plants. It highlights innovative and growing areas in the discipline and opens new pathways of discovery that we trust readers will find inspiring.

AUTHOR CONTRIBUTIONS

FB wrote and revised the manuscript. HS, LE, MI, and JZ provided suggestions and revised the manuscript. All authors approved the manuscript and the version to be published.

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ORIGINAL RESEARCH
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Melatonin Pretreatment Confers Heat Tolerance and Repression of Heat-Induced Senescence in Tomato Through the Modulation of ABA- and GA-Mediated Pathways

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Heat stress and abscisic acid (ABA) induce leaf senescence, whereas melatonin (MT) and gibberellins (GA) play critical roles in inhibiting leaf senescence. Recent research findings confirm that plant tolerance to diverse stresses is closely associated with foliage lifespan. However, the molecular mechanism underlying the signaling interaction of MT with GA and ABA regarding heat-induced leaf senescence largely remains undetermined. Herein, we investigated putative functions of melatonin in suppressing heat-induced leaf senescence in tomato and how ABA and GA coordinate with each other in the presence of MT. Tomato seedlings were pretreated with 100 μM MT or water and exposed to high temperature (38/28°C) for 5 days (d). Heat stress significantly accelerated senescence, damage to the photosystem and upregulation of reactive oxygen species (ROS), generating *RBOH* gene expression. Melatonin treatment markedly attenuated heat-induced leaf senescence, as reflected by reduced leaf yellowing, an increased Fv/Fm ratio, and reduced ROS production. The *Rbohs* gene, chlorophyll catabolic genes, and senescence-associated gene expression levels were significantly suppressed by MT addition. Exogenous application of MT elevated the endogenous MT and GA contents but reduced the ABA content in high-temperature-exposed plants. However, the GA and ABA contents were inhibited by paclobutrazol (PCB, a GA biosynthesis inhibitor) and sodium tungstate (ST, an ABA biosynthesis inhibitor) treatment. MT-induced heat tolerance was compromised in both inhibitor-treated plants. The transcript abundance of ABA biosynthesis and signaling genes was repressed; however, the biosynthesis genes MT and GA were upregulated in MT-treated plants. Moreover, GA signaling suppressor and catabolic gene expression was inhibited, while ABA catabolic gene expression was upregulated by MT application. Taken

together, MT-mediated suppression of heat-induced leaf senescence has collaborated with the activation of MT and GA biosynthesis and inhibition of ABA biosynthesis pathways in tomato.

Keywords: leaf senescence, chlorophyll degradation, high temperature, melatonin, tomato

INTRODUCTION

Recently, high temperature has become a great threat to sessile plants; it is characterized by hastening leaf senescence (Jespersen et al., 2016) and leading to a remarkable decline in plant growth (Soltani et al., 2019). It is projected that global temperatures will increase from 1.8 to 4.0°C by 2100 (Parry and Pizer, 2007). Leaf senescence is a fine-tuned mechanism that is intensely complicated by diverse intrinsic factors, such as cell death (Ghanem et al., 2012), phytohormones (Zhang and Guo, 2018), senescence-associated genes (Li et al., 2017; Xiao et al., 2017), transcription factors (Ma et al., 2018b), and environmental factors like darkness (Weaver et al., 1998), detachment (He and Gan, 2002), drought (Lee et al., 2012), salinity or alkalinity (Yang et al., 2011; Xiao et al., 2015), and high temperature (Xu and Huang, 2007; Zheng et al., 2016). The decline in Chl content is the most prominent feature of natural or stress-induced leaf senescence (Hörtensteiner, 2006), which is important for the absorption of light and the redistribution of excitation energy in the photosynthetic electron transport chain (Grossman et al., 1995). Senescence-associated gene (SAG) expression is upregulated during the onset of senescence, while the transcripts of photosynthesis-related genes are decreased (Hörtensteiner, 2006). Leaf yellowing is manifested in senescent leaves due to the negative functioning of chlorophyll catabolic enzymes, particularly Chl a reductase (HCAR), pheophytin pheophorbide hydrolyase (PPH), non-yellow coloring 1 (NYC1), NYC1-like (NOL), and pheide a oxidase (PAO) (Barry, 2009; Hörtensteiner, 2009). The transcription of Chl catabolic genes (CCGs) is directly associated with the severity of normal or stress-induced leaf senescence in many plant species (Schelbert et al., 2009; Sakuraba et al., 2012; Zhang et al., 2016b). Another essential characteristic of leaf senescence is overaccumulation of ROS (Wu et al., 2012; Gütle et al., 2016). ROS homeostasis and the redox state regulate growth- or senescence-associated cell death. In plants, ROS are generally produced by many enzymes (Apel and Hirt, 2004). Respiratory burst oxidase homologs (Rbohs) are extensively studied ROS-creating enzymes in plants (Sagi and Fluhr, 2006; Suzuki et al., 2011). Most research findings have highlighted that Rbohs are implicated in diverse distinct signaling networks and acclimation to various stresses (Suzuki et al., 2011; Marino et al., 2012; Kaur et al., 2014).

Several phytohormones, including ABA, jasmonic acid, ethylene, and salicylic acid, promote leaf senescence; while leaf senescence is restricted by GA, auxins, cytokinins, and polyamines (Jibran et al., 2013; Kim et al., 2016; Woo et al., 2019). ABA content and ABA biosynthesis and signaling gene expression are enhanced in the course of leaf senescence (Liang et al., 2014; Mao et al., 2017). The 9-cis-epoxycarotenoid dioxynease (NCED) is the key regulatory enzyme and is considered a rate-limiting step for ABA biosynthesis (Nambara and Marion-Poll, 2005). Chl catabolic gene expression is also regulated in the presence of AREB/ABF members in Arabidopsis (Gao et al., 2016). A large number of gibberellins are found in the plant kingdom but a limited version of GAs is proactive and helpful for plant development (Yamaguchi et al., 1998). Beyond other activities, GAs are used to prolong leaf senescence (Beevers, 1966; Whyte and Luckwill, 1966; Lü et al., 2014; Xiao et al., 2019).

ABA and GA participate in diverse as well as antagonistic roles in plant development processes, flowering, and regulate various environmental stimuli from the physiological to the molecular level (Weiss and Ori, 2007; Golldack et al., 2013; Liu and Hou, 2018). Heat treatment decreases GA and increases ABA content in Arabidopsis during seed germination (Toh et al., 2008). The increased ABA content in germinating seeds during heat stress causes upregulation of ABA biosynthesis genes; by contrast, a lower GA content in imbibed seeds leads to the downregulation of GA biosynthesis gene expression (Toh et al., 2008). The key seed development dimer FUS3 and ABA metabolic genes are activated during seed germination, whereas GA catabolic gene expression is restricted under heat stress, leading to delayed germination (Chiu et al., 2012). Correspondingly, the DELLA proteins RGA or GAI, as well as *ABI3* and *ABI5*, distinctly induce small ubiquitin-related modifiers (SOMs) that modulate GA and ABA biosynthesized genes under heat stress in Arabidopsis (Lim et al., 2013).

Melatonin acts as an essential antioxidant that leads to prolonged leaf senescence under stress environments (Arnao and Hernández-Ruiz, 2015). Exogenous application of melatonin on tryptophan decarboxylase (*TDC*), serotonin N-acetyltransferase (*SNAT*), tryptamine 5-hydroxylase (*T5H*) and caffeic acid O-methyltransferase (*COMT*) transgenic plants (Byeon et al., 2015) enhanced melatonin content (Zhang N. et al., 2014), which inhibit chlorophyll reduction and downregulation of CCE and SAG gene expression under diverse stresses (Wang et al., 2012; Liang et al., 2015; Shi et al., 2015b; Ma et al., 2018a). In addition, melatonin is a well-known ROS scavenger and excellent antioxidant that scavenges excess ROS (Li et al., 2012; Ahammed et al., 2018; Jahan et al., 2020) and inhibits the stress-induced senescence mechanism in plants. Melatonin efficacy in terms of inhibition of senescence-induced damage has been reported in some previous studies, including Arabidopsis, kiwi, grapes, rice, barley, Chinese flowering cabbage and ryegrass (Arnao and Hernández-Ruiz, 2009; Wang et al., 2013; Liang et al., 2015; Zhang et al., 2016a; Liang et al., 2018; Shi et al., 2019; Tan et al., 2019). Melatonin inhibits senescence-related gene expression during drought-induced leaf senescence in apple trees (Wang et al., 2013). A recent

experiment showed that melatonin prolongs senescence in kiwifruit leaves via enhancement of the antioxidant defense system and upregulation of flavonoid biosynthesis (Liang et al., 2018). In addition, melatonin is involved in eliminating Chl degradation by suppressing Chl degradation enzymes (Weeda et al., 2014). Melatonin application led to enhanced drought stress-induced leaf senescence, resulting in decreased ABA production and ABA biosynthesis gene expression (Li et al., 2015). Interestingly, melatonin treatment in Chinese flowering cabbage prolonged storage-induced leaf senescence through restricted ABA production and lowered Chl reduction associated with ABA signaling transcription factors, i.e., *BrABF1*, *BrABF4* and *BrABI5* (Tan et al., 2019). Arnao and Hernández-Ruiz (2009) showed that both melatonin and cytokinin treatment effectively reduced dark-induced Chl loss in barley leaves, and the effects were more pronounced than those of cytokinin treatment alone. The inherent ability of melatonin could help to mitigate diverse stresses through linking with other phytohormones (Arnao and Hernández-Ruiz, 2014). Despite ample documentation of the roles of melatonin in terms of stress tolerance mechanism, melatonin-mediated heat-induced leaf senescence with other hormones is still not fully understood, and it is unclear how melatonin interacts with GA and/or ABA signaling networks to mitigate senescence. In the present experiment, we demonstrated that melatonin functioned synergistically with GA while acting antagonistically with ABA in their biosynthesis and signaling pathways to prolong heat-induced leaf senescence in tomato.

MATERIALS AND METHODS

Planting Materials and Growing Conditions

Tomato (*Solanum lycopersicum* Cv. Hezuo 903) seeds were used as the test material for this experiment. Sterilized seeds were incubated for germination on moistened filter papers in a dark place at 28 ± 1°C for 30 h. After germination, seeds were placed in plastic trays filled with organic substrates (peat and vermiculite: 2:1, v:v) in an artificial climate growth chamber. The following growth environmental conditions were maintained: temperature: 28/19 ± 1°C (day/night), relative humidity: 65–75%, and 12 h photoperiods (PAR 300 μmol m^{-2} s^{-1}). When the second leaves were fully expanded, seedlings were shifted into the same growth substrate mixtures, and every alternate day, they were irrigated with nutrient solution.

Treatment Application and Sample Collection

When the seedlings attained the fourth leaf stage, half of the seedlings were foliar sprayed with melatonin at a concentration of 100 μM every 2 days and continued for seven (7) days, while the other half of the seedlings were hydrosprayed with distilled water. One week after treatments, melatonin and water-treated seedlings were subjected to high-temperature stress at 38/28°C (16/8 h) for 5 days. Leaves sampled (third leaf from the top to bottom) were collected at different time points for further biochemical analysis.

We applied ABA and GA inhibitors to verify the function of GA and ABA in MT-mediated heat tolerance. One week after foliar spraying with melatonin or water in the abovementioned volume, seedlings underwent different inhibitor treatments. The plants were foliar sprayed with 1 mM paclobutrazol (PCB, a GA biosynthesis inhibitor) and 1 mM sodium tungstate (ST, an ABA biosynthesis inhibitor) before 12 h of heat stress at 38/28°C (16/8 h) for 24 h, after which leaf samples were collected for endogenous GA and ABA measurement.

Evaluation of Leaf Senescence

Plant physiological attributes, including chlorophyll fluorescence, gas exchange parameters, chlorophyll content, relative electrolytic leakage (REL), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2), were applied for the assessment of leaf senescence. Approximately 0.50 g of composite leaf tissue was extracted in 80% cold acetone to determine the chlorophyll contents and the extraction was centrifuged to collect the supernatant, and the chlorophyll content was determined spectrophotometrically (Arnon, 1949). Two essential fluorescence attributes net photosynthetic rate (P_n) and stomatal conductance (G_s), were measured with a portable photosynthesis system (Li-6400; LI-COR, Inc., Lincoln, NE, United States) from 10.00 am to 11.00 am. The cuvette conditions were maintained as follows: 25°C temperature, 70% relative humidity, 800 μmol photons $m^{-2}s^{-1}$ PPFD (photosynthetic photon flux density), and 380 ± 10 μmol mol^{-1} external CO_2 concentration (Ahammed et al., 2020a; Hasan et al., 2020).

The maximum PSII quantum yield (Fv/Fm) was monitored as described by Maxwell and Johnson (2000), and an IMAGING-PAM chlorophyll fluorescence analyzer (Heinz Walz, Effeltrich, Germany) was used Fv/Fm measurement. Images were taken using a charge-coupled device (CCD) at the emitted fluorescence.

According to Khan et al. (2017) and Jahan et al. (2019b), we calculated the relative electrolyte leakage of the stressed leaves, and the REL was estimated using the following formula:

$$\text{REL } (\%) = \frac{\text{EC1} - \text{EC0}}{\text{EC2} - \text{EC0}} \times 100$$

The MDA (malondialdehyde) content was determined following the instructions of Heath and Packer (1968). The concentration of H_2O_2 in stressed tomato leaves was measured according to Velikova et al. (2000) instructions.

Determination of Melatonin Content

The melatonin content of tomato leaves was extracted using a commercial melatonin ELISA Kit (Qingdao Sci-tech Innovation Quality Testing Co., Ltd., Qingdao, China) following the company's instructions. Briefly, 0.10 g of composite leaf sample was homogenized in 150 μL of 1 × stabilizer and 750 μL of ethyl acetate followed by proper vortexing. The homogenate was then extracted and evaporated to dryness, and the pellet was dissolved in a stabilizer solution. For the enzyme-linked

immunosorbent assay, 100 μL of melatonin extract and 50 μL of 1 × melatonin antibody were kept in the microplate and incubated at 25°C on a plate shaker at 500 rpm for 1 h. Melatonin content was assessed by a microplate reader (Pow-erWaveX, Bio-Tek, United States), and on the basis of the standard curve, the concentration was computed using the reading of the absorbance at 450 nm.

Quantification of Endogenous ABA Content

Approximately 500 mg of composite fresh tomato leaves was granulated in liquid nitrogen and then blended in ice-cold 80% methanol (v/v) extraction solution. The extracts were centrifuged at 12,000 g for 15 min at 4°C. The whole supernatant was run through a Sep-Pak C18 cartridge (Waters, Milford, MA, United States) to reduce the extraneous materials. According to the manufacturer's protocols, endogenous ABA was estimated with an ABA ELISA Kit (Qingdao Sci-tech Innovation Quality Testing Co., Ltd., Qingdao, China).

Analysis of Endogenous GA Content

Approximately 0.50 g of fresh composite tomato leaf sample was blended in 10 mL of ice-cold 80% methanol (v/v) extraction solution, including 1 mM butylated hydroxytoluene. The extraction solution was incubated at 4°C for 4 h, and the supernatant was transferred to a 10 mL centrifuge tube. Afterward, the supernatant was centrifuged for 8 min at 3,500 g. After incubation for 4 h at 4°C, the mixture was transferred to a 10 mL centrifuge tube and then centrifuged at 3,500 g for 8 min. The whole supernatant was run through a Sep-Pak C18 cartridge (Waters, Milford, MA, United States) to reduce the extraneous materials. Subsequently, the remaining residues were dissolved in 0.01 mL^{-1} PBS (phosphate buffer solution). The final endogenous GA concentration was estimated using a GA ELISA Kit (Qingdao Sci-tech Innovation Quality Testing Co., Ltd., Qingdao, China).

RNA Extraction and Gene Expression Assays

Total RNA was extracted from 0.1 g of composite tomato leaves using an RNAsimple Total RNA Kit (Tiangen, Beijing, China, DP419) as per the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to generate cDNA using the SuperScript First-strand Synthesis System (Takara, Tokyo, Japan). qRT-PCR (quantitative real-time PCR) analyses were executed employing ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China) and the qPCR run in the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The gene-specific primers were made based on the cDNA sequences, and the reference gene *Actin* was used (**Supplementary Table S1**). The relative gene expression was determined according to Shen et al. (2019).

Statistical Analysis

The whole experiment repeated at least three independent biological replicates for the analysis of each component. Data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS 21.0 software (SPSS Inc., Chicago, IL, United States), and the significance of mean differences between treatments was analyzed with Tukey's honestly significant difference test (HSD) at $P < 0.05$.

RESULTS

Exogenous Application of Melatonin Delays Heat-Induced Leaf Senescence in Tomato Seedlings

As presented in **Figure 1A**, after 3 d of heat stress, the seedling leaves started to become yellow, which was prominent at day 5. However, MT-treated tomato leaves still had greener leaves than the heat-stressed seedlings on both days (**Figure 1A**). An analogous pattern was observed for non-invasive chlorophyll fluorescence (**Figure 1B**). As expected, senescence-related physiological attributes, in particular, the maximum PSII quantum yield (Fv/Fm) ratio and the total chlorophyll pigment content declined significantly following stress progression, and their values were significantly elevated in MT-treated leaves, which were approximately 1.26- and 1.51-fold those of the heat-stressed leaves, respectively, at 5 d of heat stress treatment (**Figures 2A,D,E**). However, the maximum chlorophyll contents were preserved in the MT-treated plants compared with melatonin-free plants. In response to heat stress, the net photosynthetic rate (P*n*) and stomatal conductance (G*s*) decreased throughout the experimental period; the rate of decline was more pronounced in non-treated seedlings than in melatonin-treated plants (**Figures 2B,C**).

Melatonin Reduces Oxidative Damage and Modulates the Expression of the Rbohs Gene Under Heat Stress

We monitored relative electrolyte leakage (REL), malondialdehyde (MDA), and H_2O_2 contents to investigate the oxidative damage of heat-stressed seedlings. As shown in **Figure 2**, along with the progression of stress duration, the content of the abovementioned stress markers obviously increased in heat-stressed seedlings, while prior spraying of 100 μM MT profoundly alleviated these stress markers (REL, MDA, and H_2O_2 decreased in MT-treated leaves by 27.72, 26.78, and 19.48%, respectively, relative to their melatonin-free counterparts at 5 d of heat treatment), indicating that MT-treated leaves accumulated lower amounts of ROS (**Figures 2G–I**). Genes encoding the ROS-forming enzyme RBOH have been widely documented to be induced under stress conditions, and the relative expression of *RbohB*, *RbohC,* and *RbohD*-like was markedly elevated throughout the stress duration (**Figure 2**), reaching approximately 5. 57-, 27. 67-, and 7.92-fold from the initial time to 5 d of stress, respectively. In contrast, MT-treated seedlings showed downregulation of the expression of the same genes compared to heat-stressed seedlings, accounting for 1. 37-, 1. 58-, and 2.00-fold lower expression at 5 days of stress, respectively (**Figures 2J–L**).

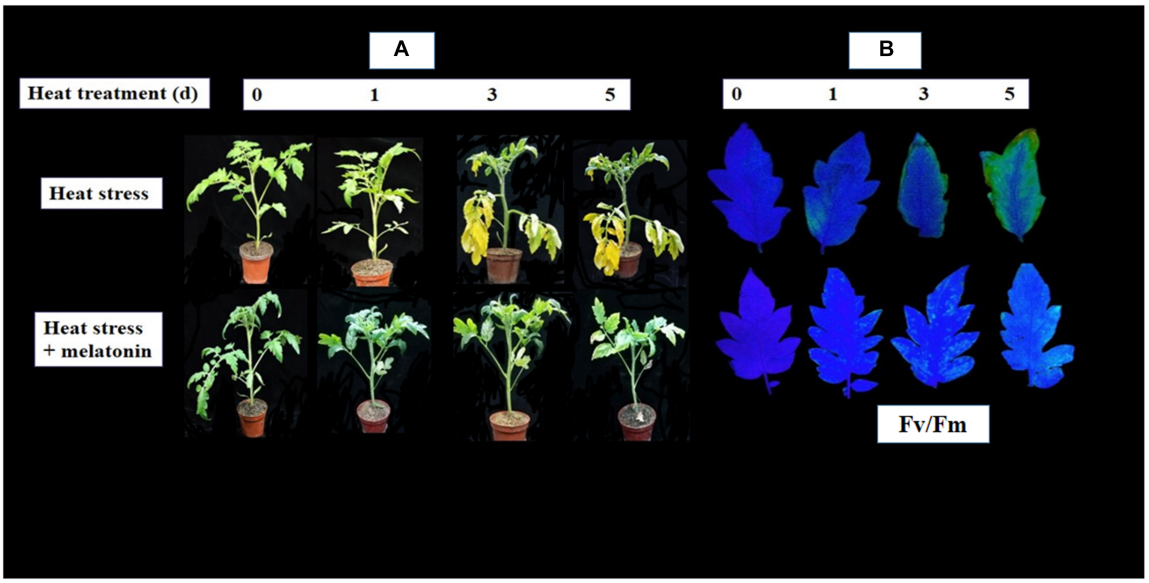

FIGURE 1 | Exogenous application of melatonin (100 μM) delays heat induced (38/28°C for 5 days) leaf senescence in tomato. **(A)** Phenotypic appearance of tomato leaves during heat stress in presence or absence of melatonin treatment and **(B)** Chlorophyll fluorescence imaging with Fv/Fm of tomato leaves during heat stress in presence or absence of melatonin treatment.

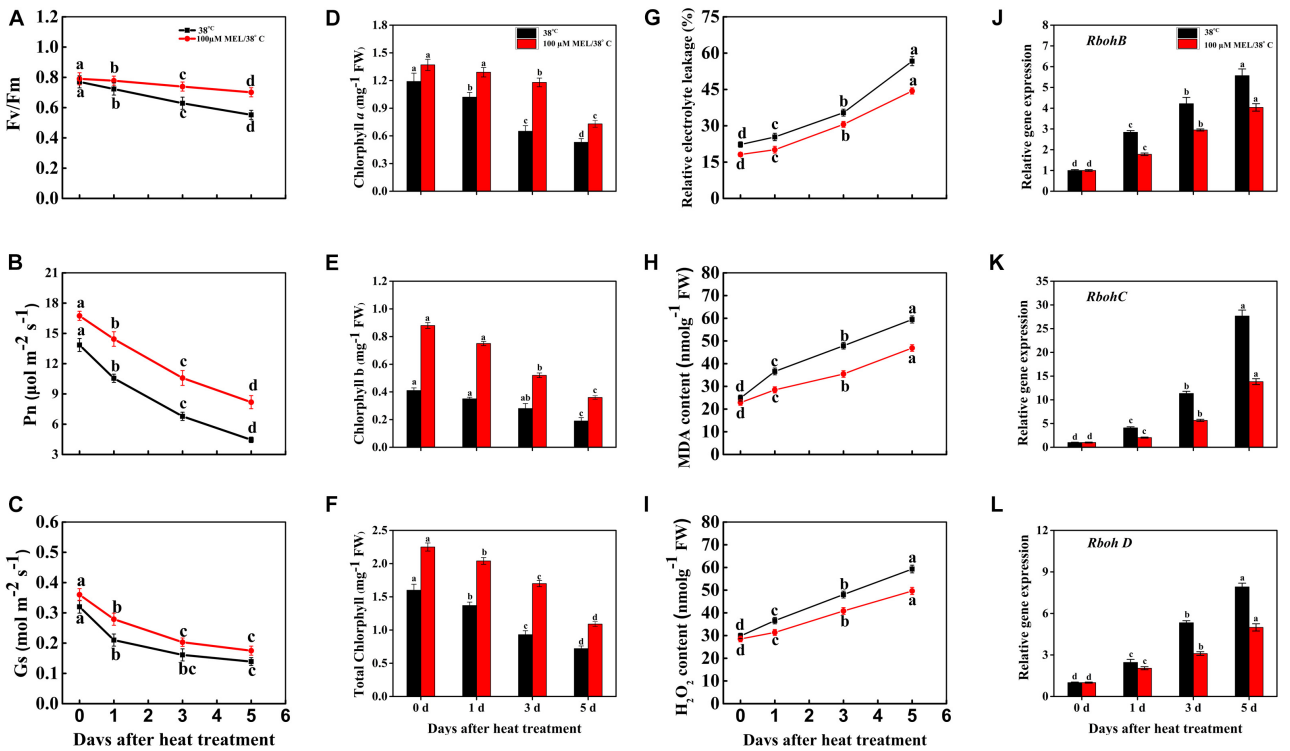

FIGURE 2 | Effects of exogenous melatonin treatment (100 μM) on senescence associated physiological attributes in tomato under heat stress (38/28°C for 5 days). **(A)** Changes of Fv/Fm value, **(B)** net photosynthetic rate (*Pn*), **(C)** stomatal conductance (*Gs*), **(D–F)** changes of Chlorophyll content, **(G)** relative electrolyte leakage, **(H)** malondialdehyde (MDA), **(I)** hydrogen per oxide (H_2O_2) content, **(J–L)** relative expression of Rbohs genes during heat stress with or without of melatonin treatment. Different letters denote the significant variations between the treatments and the average values were measured by Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$. Data represented as the mean ± standard error of triplicate biological replicates.

Melatonin Treatment Inhibited the Expression of Chlorophyll Degradation and Senescence Marker Genes During Heat Stress

Leaf yellowing is the most apparent sign of senescence, resulting in degradation of leaf chlorophyll mediated by chlorophyll catabolic genes (CCGs). The transcript abundance of chlorophyll degradation-related genes (*SGR1*, *SGR2*, *NYC*, *NOL*, *PPH*, *PAO*, and *RCCR*) and senescence marker genes (*SAG12*) were checked as seedlings sprayed with melatonin or without melatonin under heat stress. The transcript abundance of all CCGs and senescence marker genes was significantly upregulated throughout the treatment period (**Figure 3**). Compared to all other CCGs, the highest transcript abundance was observed for the *SGR1* and *SGR2* genes, as evidenced by 11.79- and 18.82-fold higher transcripts at 5 d of heat treatment relative to the early stage of treatment (0 h). Conversely, pretreatment with MT significantly repressed the expression of those genes at 5 d in comparison with melatonin-free heat-treated plants, as evidence by 22.03% lower

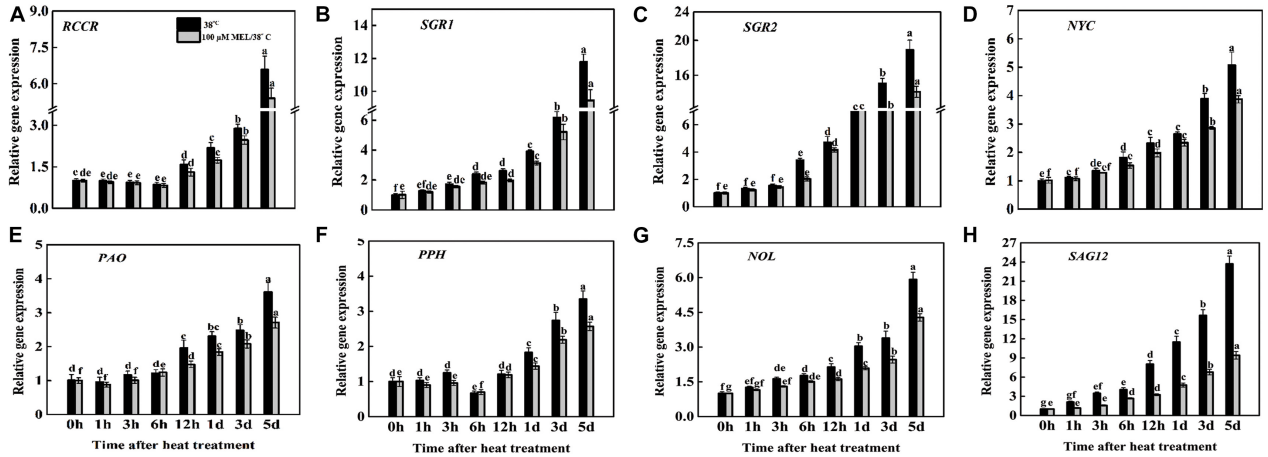

FIGURE 3 | Effects of exogenous melatonin treatment on the transcript abundance of **(A–G)** chlorophyll catabolic genes (*RCCR, SGR1*, *SGR2*, *NYC*, *PAO*, *PPH*, and *NOL*) and **(H)** senescence associated gene (*SAG12*) during heat stress with or without of melatonin treatment. Different letters denote the significant variations between the treatments and the average values were measured by Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$. Data represented as the mean ± standard error of triplicate biological replicates.

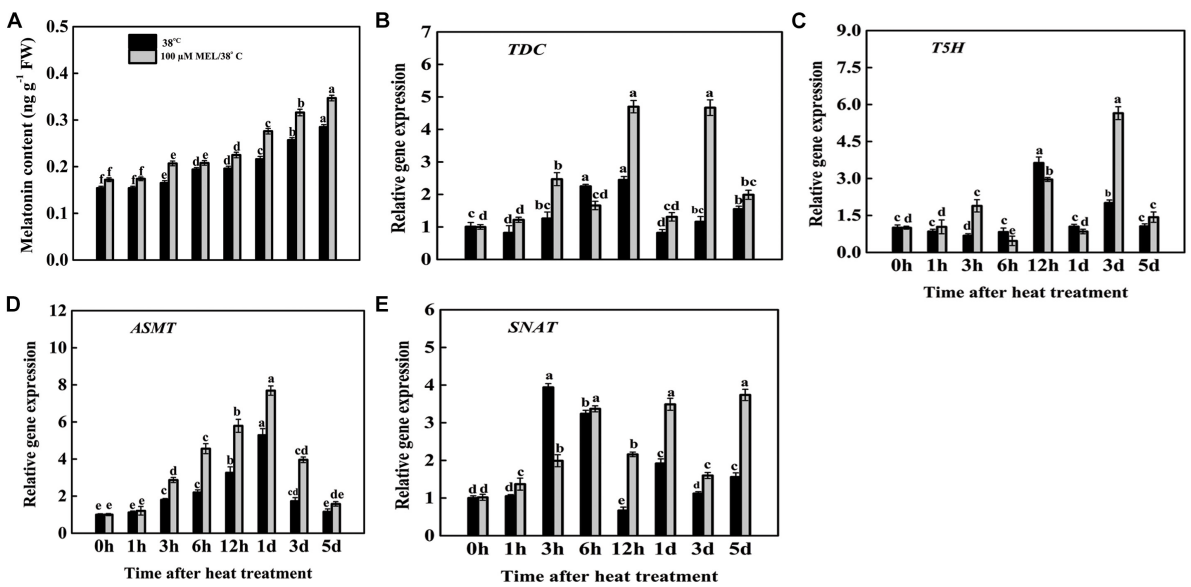

FIGURE 4 | Effects of exogenous melatonin treatment on the **(A)** endogenous melatonin content and **(B–E)** transcript abundance of melatonin biosynthesis genes (*TDC, T5S, ASMT,* and *SNAT*) during heat stress with or without of melatonin treatment. Different letters denote the significant variations between the treatments and the average values were measured by Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$. Data represented as the mean ± standard error of triplicate biological replicates.

RCCR, 24.76% reduction in *SGR1*, 30.78% reduction in *SGR2*, 31.00% decline in *NYC*, 33.21% decline in *PAO*, 30.35% reduction in *PPH*, and 38.31% lower *NOL* (**Figure 3**). The expression pattern of the senescence marker gene (*SAG12*) showed the same trend as the chlorophyll degradation genes. The transcript abundance of *SAG12* was upregulated with the progression of treatment duration both in MT-treated and MT-free plants, but its expression was remarkably lower (2.51-fold from only heat-stressed plants at day 5) in MT-treated tomato leaves from the initial treatment to the end of the experiment (**Figure 3H**).

Exogenous Melatonin Application Induces Endogenous Melatonin and Upregulates Melatonin Synthesis Genes Under Heat Stress

Endogenous melatonin content was measured at 0, 1, 3, 6, 12 h, 1, 3, and 5 days after heat stress in both melatonin-treated and melatonin-free seedlings (**Figure 4**). Melatonin content was elevated under heat stress, and with the progression of treatment duration, its content was increased, and the highest melatonin content was recorded at 5 d of heat treatment, at 1.85-fold higher than the initial time (0 h) of treatment. In contrast, melatonin addition further led to marked elevation in endogenous melatonin content from the beginning to the last day of stress. The endogenous melatonin content in MT-treated heat-stressed seedlings at 5 d reached 0.347 ng g^{-1} FW, which was 2.01-fold higher than that at the initial time of treatment (0 h) and 1.21-fold greater than that in seedlings subjected to only heat stress at 5 d of treatment (**Figure 4A**).

Melatonin-treated heat-stressed tomato seedlings significantly upregulated the transcript abundance of melatonin synthesis genes, namely, *TDC*, *T5H*, *SNAT*, and *ASMT* (**Figures 4B–E**). The transcript level of *TDC* gradually increased after 1 h of treatment, reached a peak at 12 h, and then decreased its expression. *TDC* expression again peaked at 12 h in tissues that received melatonin, and it was 4.67-fold higher than that in the early stage of treatment (0 h). Conversely, only heat-treated seedlings suppressed *TDC* expression from the early stage to the end of treatment and showed higher expression after 12 h of heat stress. *TDC* expression in melatonin-treated seedlings was 90.61% higher than that in only heat-stressed seedlings at 12 h of treatment (**Figure 4B**). The transcript abundance of *T5H* in both heat-stressed seedlings with or without melatonin treatment fluctuated, and obviously, the expression was higher in pretreated melatonin-stressed tissues. The transcript level of *T5H* in melatonin-treated seedlings reached a peak at 3 d after treatment, and it was 5.65-fold higher than that in the initial stage of treatment, while the expression in melatonin-free treated seedlings was 181.09% lower than that in melatonin-treated plants at 3 d of heat stress (**Figure 4C**). The transcript levels of *ASMT* and *SNAT* in the untreated tomato plants were lower from the initial stage to the final stage of stress. *ASMT* and *SNAT* expression peaked at 1 and 5 days of treatment and was 7.69- and 3.74-fold higher, respectively, than expression at the initial time (0 h) (**Figures 4D,E**). In summary, the imposition of high temperature repressed melatonin biosynthesis genes due to the inhibition of melatonin production during the stress period. As expected, melatonin pretreatment enhanced these aforementioned synthesis genes more intensely throughout the treatment period.

Effects of Melatonin on Endogenous ABA Content and Its Biosynthesis Pathways

ABA is an effective modulator that accelerates leaf senescence (Liang et al., 2014; Mao et al., 2017). To assess whether the addition of melatonin modifies the endogenous production of ABA, the ABA concentration in tomato leaves under stressed conditions was determined. We observed a substantial increase in ABA volume both in the presence and absence of melatonin-treated plants with the progression of the heat stress period, but the ABA concentration declined in melatonin-treated plants. The lowest amount of accumulation was seen on day 3 and was approximately 51.69% lower in tissues treated with melatonin than in tissues treated with heat stress alone (**Figure 5A**). The highest melatonin accumulation was found in only heat-stressed seedlings at day 5, and it was recorded as 36.34% higher than that in seedlings that received melatonin. In contrast, the endogenous ABA content was markedly reduced in the ST treatment but was higher than that in the control plants. Melatonin plus ST treatment further decreased ABA accumulation under heat stress, implying that melatonin controls ABA production under heat stress (**Figure 7A**). In addition to justifying whether the repression of senescence by melatonin has been correlated with the modulation of ABA biosynthesis or signaling, the relative transcripts of the core genes associated with ABA biosynthesis and signaling have been investigated (**Figure 5**). The mRNA levels of ABA biosynthetic genes *NCED1*, *NCED2,* and *AAO3* were upregulated in heat-stressed leaves to varying degrees throughout the treatment duration. The transcript levels of *NCED1*, *NCED2,* and *AAO3* in heat-treated seedlings peaked at 12, 6 h, and 1 days after stress treatment and were 11. 25-, 5. 9-, and 3.74-fold higher than those in the initial stage of treatment (0 h), respectively (**Figures 5B–D**), and the expression of these genes was inhibited by melatonin treatment. In the ABA signaling pathway, *ABI3* and *ABI5* encode essential transcription factors, which increased their transcript abundance under high-temperature conditions to varying magnitudes, but the expression of these two genes was repressed in MT-treated plants throughout the stress period, and *ABI3* and *ABI5* decreased 37.74 and 61.14%, respectively, at 5 d of treatment compared to the heat-stressed seedlings (**Figures 5E,F**). In addition, to further confirm the contribution of melatonin to ABA modification under high-temperature conditions, we also quantified the mRNA levels of two ABA catabolism genes, *CYP707A1* and *CYP707A2*. As expected, the relative transcripts of these two genes were significantly upregulated to varying extents in melatonin-treated tissues compared with heat-stressed seedlings. The highest expression of *CYP707A1* and *CYP707A2* was observed in melatonin-pretreated tissues 6 h after treatment and increased 162.33 and 160.76%, respectively, relative to melatonin-free heat-stressed seedlings (**Figures 5G,H**). Altogether, the above findings imply that melatonin addition

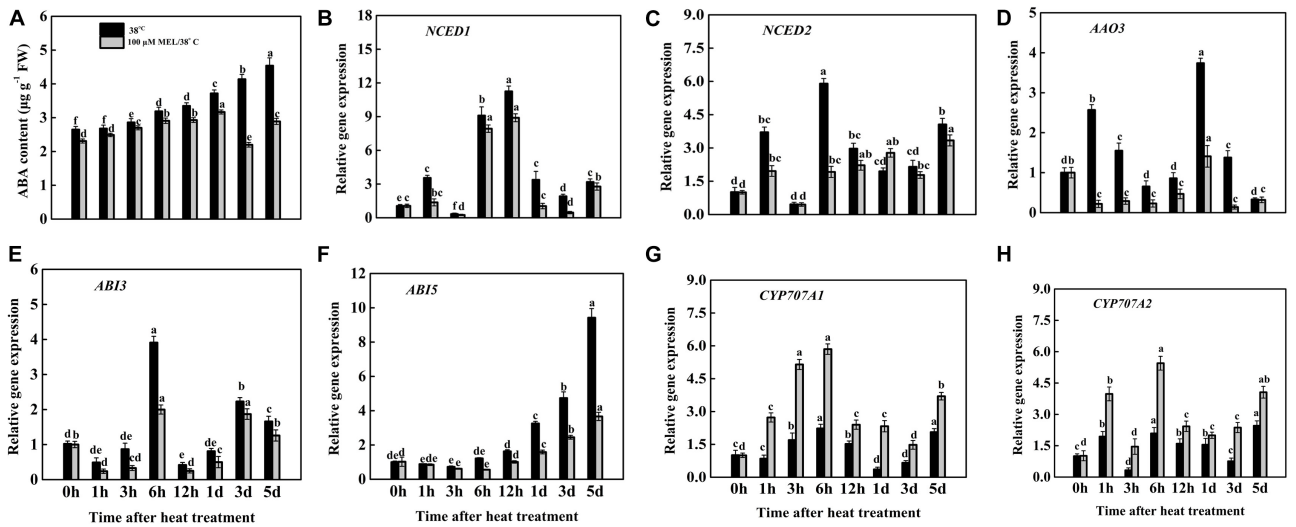

FIGURE 5 | Effects of exogenous melatonin treatment on the **(A)** endogenous ABA content and **(B–D)** transcript abundance of ABA biosynthesis genes (*NCED1, NCED2,* and *AAO3*), **(E,F)** ABA signaling genes (*ABI3* and *ABI5*), **(G,H)** ABA of catabolic genes (*CYP707A1,* and *CYP707A2*) during heat stress with or without of melatonin treatment. Different letters denote the significant variations between the treatments and the average values were measured by Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$. Data represented as the mean ± standard error of triplicate biological replicates.

delays heat-induced leaf senescence in tomato, and it might be closely associated with reduced endogenous ABA production along with regulation of the ABA metabolic pathway.

Effects of Exogenous Melatonin on Endogenous GA Accumulation and Its Biosynthesis Pathways

To determine the interaction of GA mediating MT-induced heat tolerance, we estimated the endogenous GA accumulation of tomato plants. However, heat stress resulted in decreases in the GA content throughout the treatment period to varying degrees. Exogenous melatonin pretreatment significantly elevated the GA content under heat stress. The maximum GA accumulation was found 1 d after heat stress, and it was 1.24-fold higher than the initial time (0 h) of heat treatment and 1.35-fold higher on the same day compared to heat-stressed leaves (**Figure 6A**). The endogenous GA content profoundly decreased in the PCB treatment under heat stress and was lower than that in control, while treatment with melatonin plus PCB under heat stress slightly increased the GA content, suggesting that MT-mediated heat tolerance is associated with GA (**Figure 7B**). In addition to verifying whether the inhibition of senescence by MT has been associated with the regulation of GA biosynthesis pathways, the mRNA levels of the core genes associated with GA synthesis, signaling, and catabolism were investigated (**Figure 6**). We assumed that the relative expression of GA biosynthesis genes (*GA20ox1* and *GA20ox2*) might be modulated by melatonin treatment under heat stress. As displayed in **Figure 6**, mRNA level analysis revealed that the transcript abundances of *GA20ox1* and *GA20ox2* were downregulated under only heat stress conditions, while the addition of melatonin throughout the heat treatment duration reversed the downregulation of the expression of these genes. The transcription levels of *GA20ox1* and *GA20ox2* in melatonin-treated plants reached their peaks after treatment for 3 h and 1 d; their expression was 1.35- and 4.02-fold higher, respectively, than heat-stressed tissues at the same time points (**Figures 6B,C**). DELLA proteins (GAI and RGA) coordinate with key regulatory elements, modifying downstream genes transcriptionally to suppress plant growth, whereas GA enhances plant growth and development by suppressing DELLA inhibition to stimulate GA (Davière and Achard, 2013). To obtain more insight into how GA and melatonin interact to mitigate heat-induced leaf senescence, we also quantified the GA signaling repressor gene *GAI*. As shown in **Figure 6D**, with the progression of heat stress duration, the abundance of *GAI* transcripts was significantly upregulated until the end of the experiment; however, exogenous application of melatonin constantly suppressed this gene expression. On day 5, the transcript level of *GAI* in melatonin-treated tissue was 50.06% lower than that in only heat-stressed seedlings (**Figure 6D**), indicating that melatonin might play a key role in inhibiting DELLA production by suppressing *GAI* expression, which helps to delay heat-induced leaf senescence. The key synthesized catabolic bioactive GA enzyme is GA2ox, which encodes for the negative regulation of GA metabolism. We also checked the expression of two crucial GA2ox-encoding genes (*GA2ox1* and *GA2ox2*); our qRT-PCR results showed that the overall transcription levels of these two genes were upregulated under heat-stressed tomato leaves; however, the expression of these genes was significantly suppressed by melatonin treatment under high temperature (**Figures 6E,F**). Accordingly, our results indicate that heat-induced leaf senescence is suppressed in tomato seedlings with melatonin treatment, which may be strongly interlinked with endogenous GA content as well as GA biosynthesis and signaling pathways.

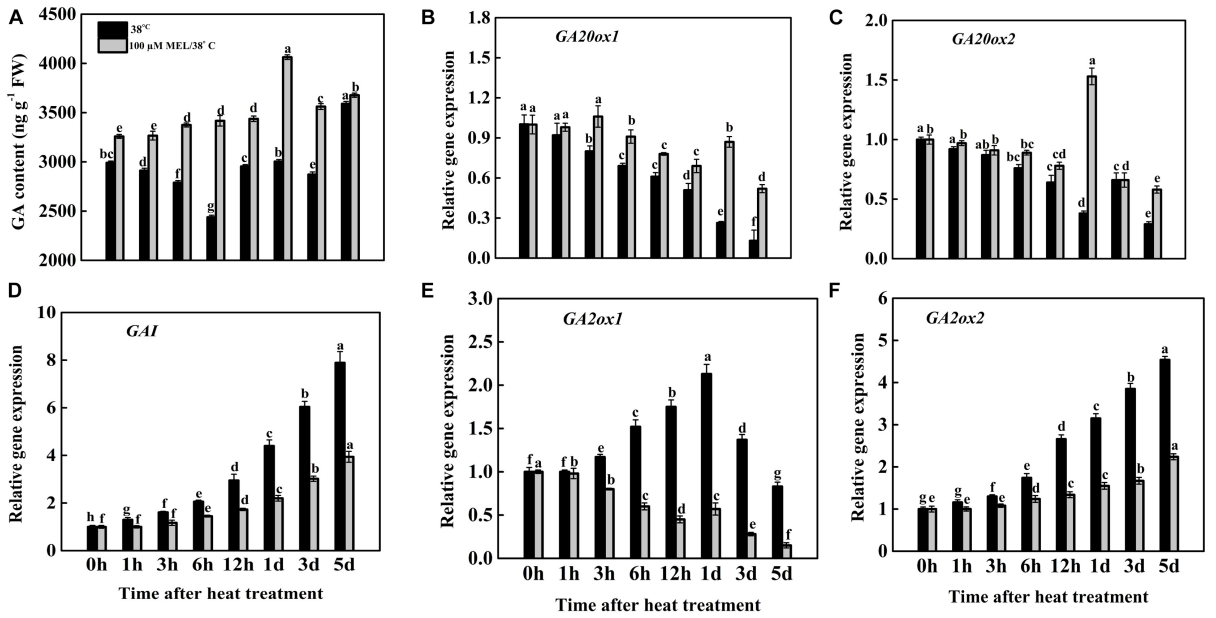

FIGURE 6 | Effects of exogenous melatonin treatment on the **(A)** endogenous GA content and **(B,C)** transcript abundance of GA biosynthesis genes (*GA20ox1* and *GA20ox2*), **(D)** GA signaling genes (*GAI*), **(E,F)** GA of catabolic genes (*GA2ox1* and *GA2ox2*) during heat stress with or without of melatonin treatment. Different letters denote the significant variations between the treatments and the average values were measured by Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$. Data represented as the mean ± standard error of triplicate biological replicates.

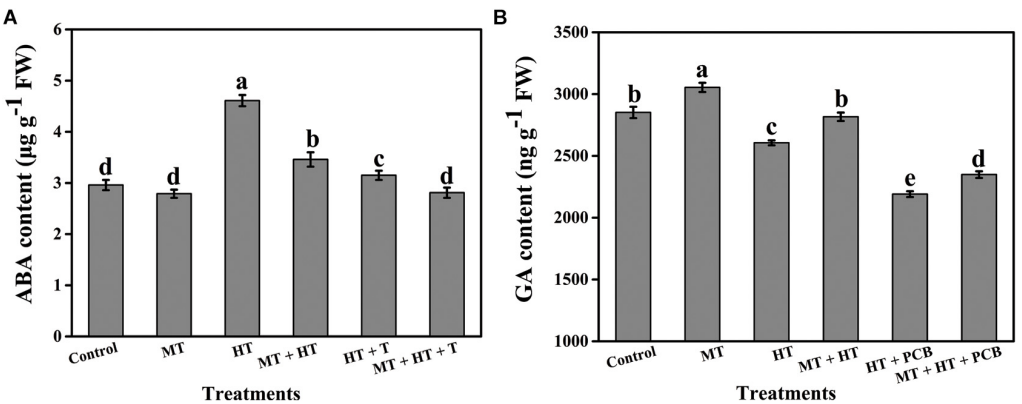

FIGURE 7 | Effects of 1 mM sodium tungstate (ST, an ABA biosynthesis inhibitor) and 1 mM paclobutrazol (PCB, a GA biosynthesis inhibitor) on the **(A)** endogenous ABA and **(B)** endogenous GA content during heat stress with or without of melatonin treatment. Different letters denote the significant variations between the treatments and the average values were measured by Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$. Data represented as the mean ± standard error of triplicate biological replicates.

Pearson's Correlation Coefficient Relationship Among the Key Genes of Melatonin, GA and ABA Pathways

To gain more understanding among the relationship of the three key molecules MT, GA and ABA biosynthesis, we performed Pearson's coefficient test (**Supplementary Tables S2, S3**). The correlation has differentially shown among these three biosynthesis pathways at different treatment conditions. Melatonin biosynthesis gene was significantly positive correlation with the GA biosynthesis gene, whereas, it strongly negative relation with ABA signaling pathway related gene. Similarly, GA biosynthesis was significantly opposite to ABA biosynthesis; by contrast, the ABA signaling pathway related gene was positively correlated with the GA signaling gene. In summary, we concluded that melatonin has a positive relation with GA to mitigate heat-induced leaf senescence and vice versa with ABA at the various treatment combinations.

DISCUSSION

A set of phytohormones stimulates stress-induced or natural leaf senescence, whether abscisic acid, ethylene,

jasmonic acid promoting senescence, and auxin, cytokinin (CK), and gibberellins prolonging the senescence process (Fan Z.-Q. et al., 2018; Tan et al., 2018). Melatonin acts as an anti-senescence factor, suppresses chlorophyll catabolism and other senescence-associated promotion of gene expression (Shi et al., 2015a; Zhang et al., 2016a; Arnao and Hernández-Ruiz, 2019). In the current experiment, we found that melatonin application significantly suppressed chlorophyll degradation-associated gene (*SGR1*, *SGR2*, *NYC*, *NOL*, *PPH*, *PAO*, and *RCCR*) and senescence marker gene (*SAG12*) expression (**Figure 3**). The total chlorophyll content, Fv/Fm ratio, and photosynthetic attributes (P*n* and G*s*) declined in only heat-stressed tomato seedlings (**Figures 2A–F**). Conversely, melatonin pretreatment effectively elevated these processes, leading to a substantial decline in pigment loss and maintaining photosystem integrity, implying that melatonin played a vital role in mitigating heat-induced leaf senescence in tomato (**Figure 2**). In agreement with our results, slowed leaf senescence has been documented in melatonin treated Chinese flowering cabbage (Tan et al., 2020), rice (Liang et al., 2015), bentgrass (Ma et al., 2018a), and kiwifruit (Liang et al., 2018). Plants face oxidative stress due to over ROS production, which directly participates in senescence acceleration (Choudhary et al., 2020). A constant elevation of REL, MDA, and H_2O_2 indicated a decline in cell membrane integrity through excess ROS production, and melatonin addition significantly attenuated this oxidative damage (**Figures 2G–I**). RBOHs are widely studied enzymatic stocks of ROS generation and play critical roles in altering ROS production (Wang et al., 2019). The transcript levels of RBOH (*RbohB*, *RbohC*, and *RbohD*) increased continuously with increasing treatment period, while exogenous spraying of melatonin differentially repressed *RbohB*, *RbohC*, and *RbohD* expression, resulting in lowered accumulation of ROS (**Figures 2J–L**). These results suggest that the lowered generation of ROS in melatonin pretreated seedlings is indirectly related to inhibition of RBOH gene expression. Our results are also in line with recent findings and indicate that the protective effect of melatonin on stress-induced ROS accumulation is related to *RBOH* gene regulation as well as other metabolite functions (Jahan et al., 2019a; Tan et al., 2020).

Melatonin pretreatment or overexpression or transient expression of melatonin biosynthesis genes might enhance the *in vivo* melatonin level and increase plant stress tolerance (Ahammed et al., 2019). In the current experiment, exogenous addition of melatonin led to elevated melatonin content, and the transcript abundances of *TDC*, *T5S*, *ASMT* and *SNAT* were significantly downregulated in tissues subjected to only heat stress (**Figure 4**). Correspondingly, the decline in mRNA levels of these biosynthetic genes was suppressed in melatonin-treated tissues exposed to high temperature. A couple of former studies indicated that melatonin content, and melatonin biosynthesis genes expression were significantly upregulated upon melatonin treatment (Zhang et al., 2017; Ma et al., 2018a; Tan et al., 2020).

Melatonin could potentially interact with plant hormones or signaling molecules, employing beneficial roles in stress management. Several recent studies have indicated that melatonin is symbiotic or contrary to other phytohormones throughout physiological processes in stress responses (Arnao and Hernández-Ruiz, 2014, 2015, 2019; Reiter et al., 2015; Kanwar et al., 2018). The elevated levels of ABA promote leaf senescence (Yang et al., 2002). Melatonin treatment significantly suppressed ABA accumulation as well as ABA biosynthesis genes expression and upregulated the expression of ABA catabolic genes under salt and water stress (Zhang H. J. et al., 2014; Li et al., 2015). In this study, heat-stressed induced higher ABA levels and elevated expression of ABA biosynthesis (*NCED1*, *NCED2,* and *AAO3*) and signaling transcription factor (*ABI3* and *ABI5*) genes, while ABA catabolic genes (*CYP707A1* and *CYP707A2*) were suppressed under the same treatment (**Figure 5**). However, the opposite trends were observed in the melatonin pretreated plants. Our findings were consistent with previous work and found that melatonin application relieved high temperature-induced leaf senescence by repressing ABA induction, lowering the expression of ABA synthesis and signaling genes (Zhang et al., 2016a) and upregulating catabolic gene expression (Li et al., 2015). In addition, ABA biosynthesis and signaling transcription factors are elevated during stress as well as in natural senescence environments (Finkelstein and Rock, 2002). In line with these findings, it can be hypothesized that the addition of melatonin decreases ABA production and enhances melatonin contents by concurrently inhibiting ABA biosynthetic gene expression and increasing melatonin biosynthesis gene expression, thus ultimately inhibiting heat-induced leaf senescence damage.

Bioactive GA is an essential element that plays an active role in delaying leaf senescence in plants subjected to stressful environments (Fan J. et al., 2018; Xiao et al., 2019). In the present investigation, the content of endogenous GA accumulation along with GA biosynthetic encoding enzyme gene (*GA20ox1* and *GA20ox2*) expression was significantly elevated in melatonin-treated plants under heat stress conditions, and the opposite trend was notified in only heat-stressed seedlings (**Figure 6**). Furthermore, melatonin application significantly repressed the transcript level of the DELLA protein-encoding gene *GAI* (used for GA deactivation or as a GA suppressor) as well as GA catabolic regulating gene (*GA2ox1* and *GA2ox2*) expression (**Figure 6D**). Our findings are also supported by previous works, which noted that the application of GA delays natural or stress-induced leaf senescence (Fan J. et al., 2018; Xiao et al., 2019). The supplementation with melatonin increases active GAs under salinity stress in cucumber seedlings by amplifying GA biosynthetic genes (Zhang N. et al., 2014) and delays plant senescence by preventing ROS production and optimizing antioxidant enzyme activities (Wang et al., 2017). Collectively, the cumulative effects of elevated melatonin content, melatonin biosynthesis and GA signaling gene transcription levels could result in increased heat tolerance and delayed leaf senescence in tomato.

Generally, the plant hormones GA and ABA interact antagonistically at different plant growth stages as well as under diverse stress conditions, including high temperature (Ahammed et al., 2020b). The cross-talk between the GA and ABA signaling pathways and the inconsistent combination between these two plant growth regulators also directly activate corresponding stress responses (Liu and Hou, 2018; Ahammed et al., 2020b). High temperature enhances the higher accumulation of ABA and

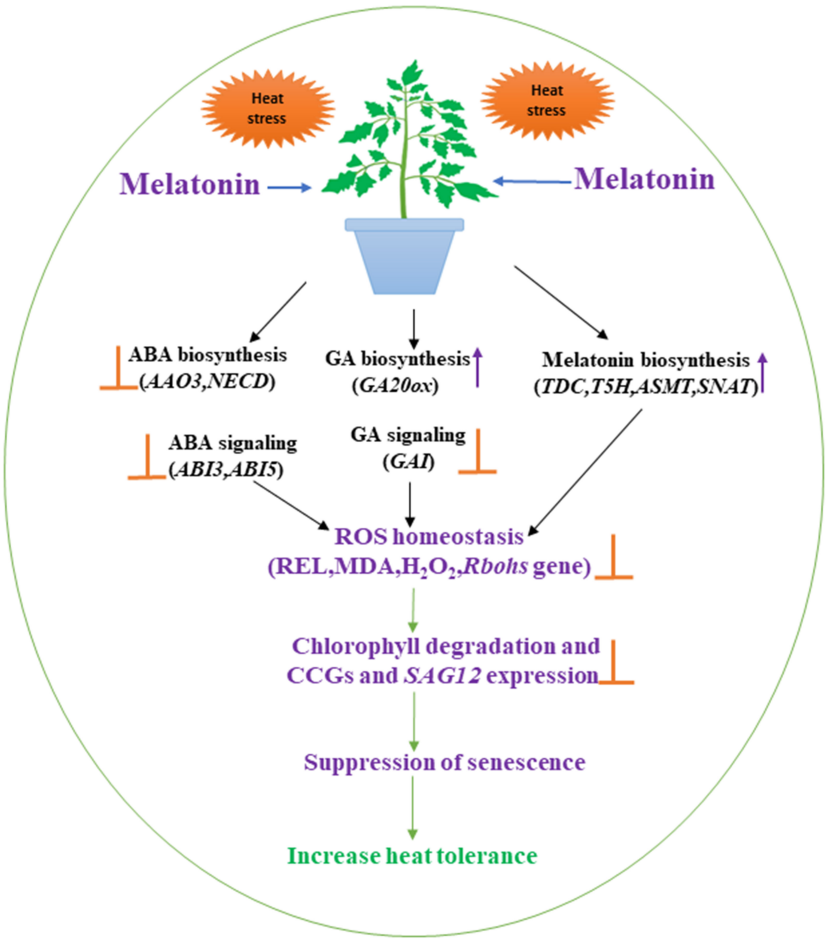

FIGURE 8 | A probable mechanism of melatonin-mediated heat-induced leaf senescence resulting in coordination with ABA and GA biosynthesis and signaling pathway. The arrow denotes increases and bar denotes decreases.

suppresses GA content in Arabidopsis while elevating ABA levels triggers upregulation of ABA biosynthesis enzyme genes, but lower GA accumulation occurs due to downregulation of GA biosynthesis enzyme genes under the same stress conditions (Toh et al., 2008). DELLAs govern a group of downstream genes at the transcript level by emulating TFs implicated in ABA signaling. For example, the GA signaling suppressors GAI and RGA in Arabidopsis act upon temperature stress by correlating to ABA signaling transcription factors, including *ABI3* and *ABI5*, and explicitly initiating small ubiquitin-related modifier (SOM), which revamps ABA and GA biosynthesis in Arabidopsis (Lim et al., 2013). We observed that melatonin application significantly influenced both GA and ABA biosynthesis and signaling pathways in the current investigation. In conclusion, the above results indicated that there might be a strong cross-connection among these three signaling molecules and that melatonin treatment repressed heat-induced leaf senescence in tomato either directly or indirectly by redesigning GA and ABA metabolism or modulating chlorophyll catabolic pathways.

Finally, as depicted in **Figure 8**, the present study revealed that melatonin application prolongs heat-induced leaf senescence of tomato seedlings through the upregulation of *in vivo* melatonin and GA content, whereas, inhibition of ABA formation and reduction of chlorophyll degradation. The positive contributions of melatonin and GA in enhancing heat tolerance were indicated by plant physiological attributes and suppression of ROS overproduction. These findings reveal the cross-talk among the three molecules and related directly or partially to their biosynthesis pathways. Thus allowing mitigation of the heat tolerance of tomato plants and facilitating an understanding of their interactions. Further studies are required via transgenic or VIGS approaches to insight more understanding about their interactions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SG contributed to conceptualization, design of the experiment, methodology, and fund acquisition. MJ performed the experiment and contributed to data curation and original draft preparation. YW revised the manuscript and contributed to software. SS contributed to supervision and editing. MH and DH prepared the figure. AE-Y revised the manuscript. NA contributed to data analysis. MA contributed to the collection of literature. JS supervised and reviewed the original manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.650955/full#supplementary-material

Supplementary Table 1 | List of primer pairs used in this experiment.

Supplementary Table 2 | Pearson's correlation coefficient relationship among the key genes of melatonin, GA and ABA pathways (For only heat stress).

Supplementary Table 3 | Pearson's correlation coefficient relationship among the key genes of melatonin, GA and ABA pathways (For Melatonin and heat stress).

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REVIEW
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A Systematic Review of Melatonin in Plants: An Example of Evolution of Literature

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Melatonin (N-acetyl-5-methoxy-tryptamine) is a mammalian neurohormone, antioxidant and signaling molecule that was first discovered in plants in 1995. The first studies investigated plant melatonin from a human perspective quantifying melatonin in foods and medicinal plants and questioning whether its presence could explain the activity of some plants as medicines. Starting with these first handful of studies in the late 1990s, plant melatonin research has blossomed into a vibrant and active area of investigation and melatonin has been found to play critical roles in mediating plant responses and development at every stage of the plant life cycle from pollen and embryo development through seed germination, vegetative growth and stress response. Here we have utilized a systematic approach in accordance with the preferred reporting items for systematic reviews and meta-analyses (PRISMA) protocols to reduce bias in our assessment of the literature and provide an overview of the current state of melatonin research in plants, covering 1995–2021. This review provides an overview of the biosynthesis and metabolism of melatonin as well as identifying key themes including: abiotic stress responses, root development, light responses, interkingdom communication, phytohormone and plant signaling. Additionally, potential biases in the literature are investigated and a birefringence in the literature between researchers from plant and medical based which has helped to shape the current state of melatonin research. Several exciting new opportunities for future areas of melatonin research are also identified including investigation of non-crop and non-medicinal species as well as characterization of melatonin signaling networks in plants.

Keywords: melatonin, indoleamine, plant signaling, morphogenesis, development, stress

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INTRODUCTION

Melatonin (N-acetyl-5-methoxy-tryptamine) is a mammalian neurohormone, antioxidant and signaling molecule that was first discovered in plants in 1995 (Dubbels et al., 1995; Hattori et al., 1995). The first studies investigated plant melatonin from a human perspective quantifying melatonin in foods (Dubbels et al., 1995; Hattori et al., 1995) and medicinal plants (Manchester et al., 2000), and questioning whether its presence could explain the activity of some plants as medicines (Murch et al., 1997; Chen et al., 2003). Starting with these first handful of studies in the late 1990s, plant melatonin research has blossomed into a vibrant and active area of investigation (**Figure 1**) with the first 2 papers cited > 530 times each. These 2 papers provide a set starting

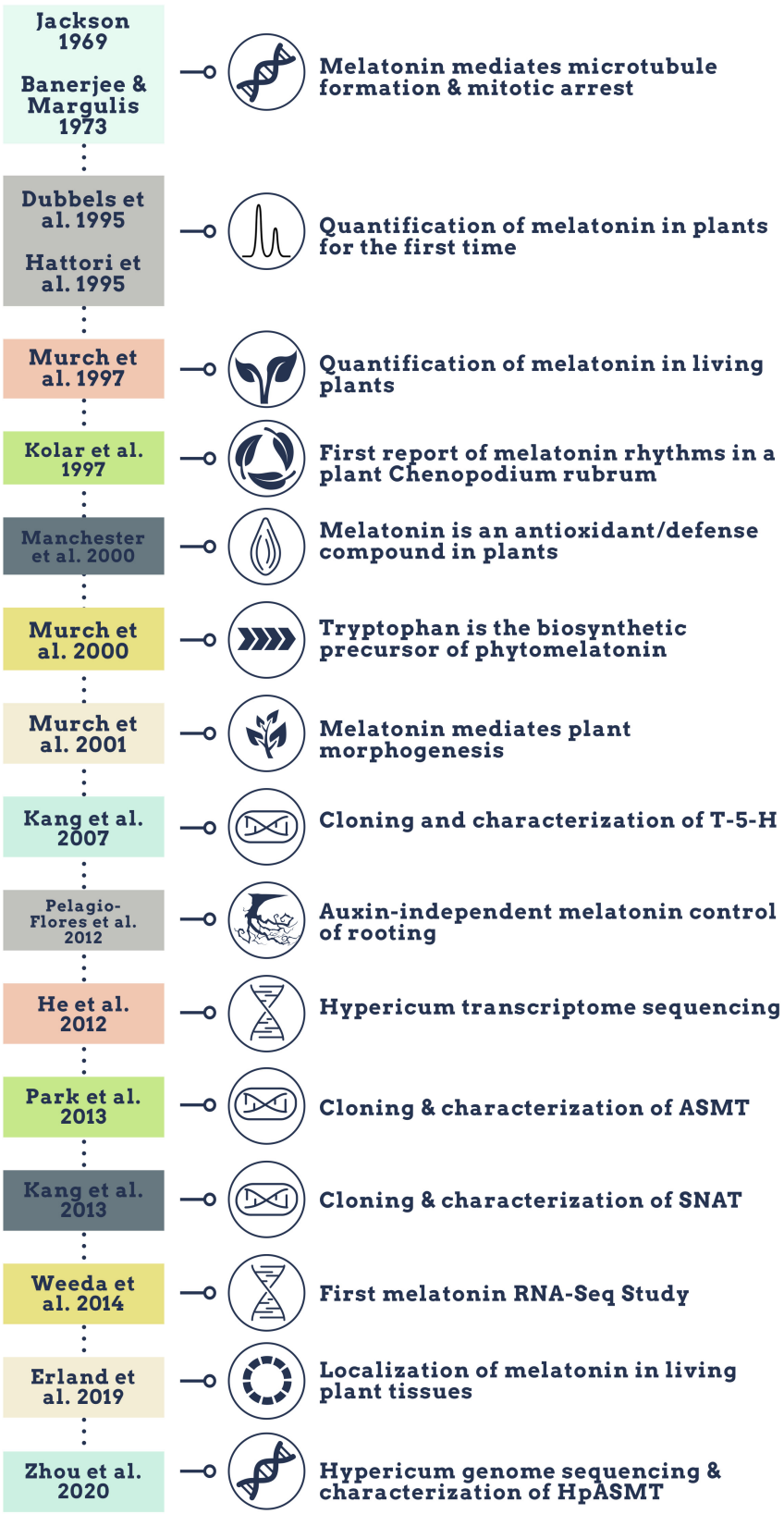

FIGURE 1 | Timeline of qualitative major melatonin research developments.

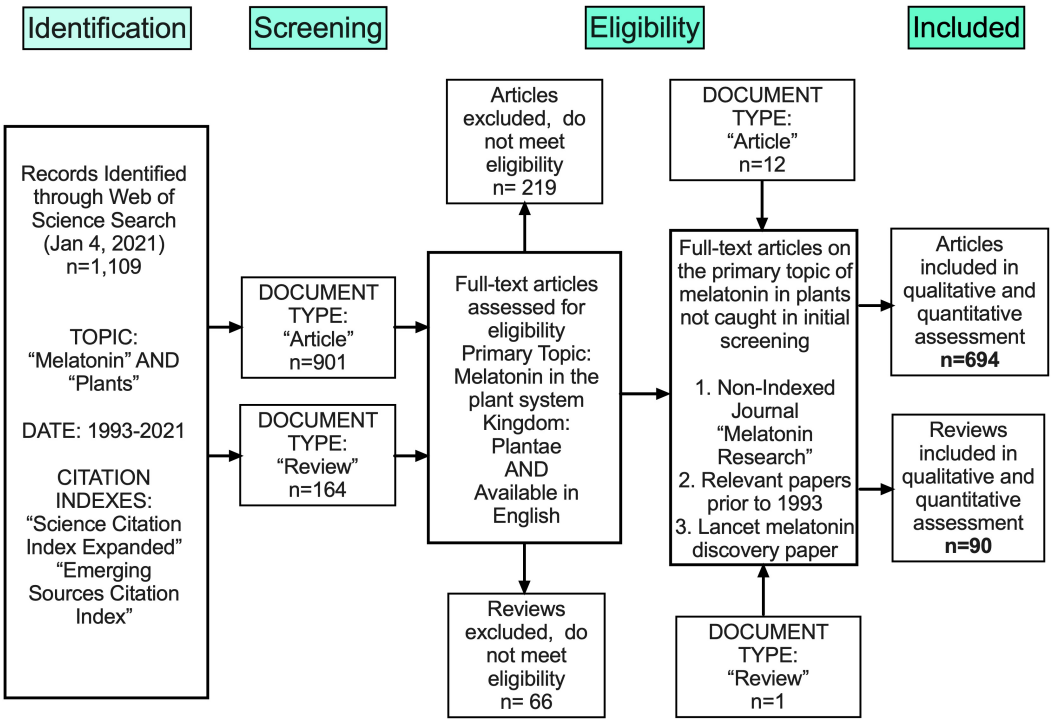

FIGURE 2 | Flow chart outlining methods of unbiased collection of literature following the PRISMA protocol.

point for an evaluation of how the scientific literature evolved in melatonin research (**Figure 1**). The initial goal of this systematic review was to understand the important roles of melatonin in plant physiology, growth, and metabolism. Application of network analysis approaches identified that divergent research groups have led investigations in divergent directions that influenced the relative weight of research outcomes. We have used a systematic approach to this review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocols to reduce bias in our assessment of the literature (**Figure 2**) and a full bibliography is included in the **Supplementary Materials** (**Supplementary Tables 1**, **2**). Our goal was to assess how the literature in the field has developed over the last 25 years. Some of the potential biases in our data include: (a) only manuscripts published in English were included, (b) our search was limited to primarily literature from peer-reviewed and indexed publications and (c) reports and data from governments, regulatory agencies, public or private corporations or proprietary processes were not included. Our assessment of the literature covers publications that appeared between 1995 – 2021. We found 692 manuscripts containing original research data and results describing distribution, metabolism, and mechanisms of melatonin in plants. A further 93 manuscripts are reviews of aspects of the literature from a viewpoint. Interestingly, the last 5 years have been particularly active in plant melatonin research with an exponential growth in publications (R^2 = 0.9939, **Figure 3**). Over time, the number of review papers on the topic of plant melatonin has kept pace with primary original research (R^2 = 0.914, **Figure 3**). In total, 785 scientific publications were assessed in this systematic review. The major developments are shown in a timeline of keystone papers (**Figure 1**).

BIREFRINGENCE IN THE LITERATURE

Our research finds that over time, the plant melatonin community diverged into dichotomous fields and clusters of authors (**Figure 4**). In general, researchers with backgrounds in medicine and human health where melatonin is the master regulator of circadian rhythms look to mammalian systems for inspiration and initially focused on understanding the implications of dietary sources of melatonin on humans (Dubbels et al., 1995; Hattori et al., 1995; Reiter et al., 2005). This has been a productive area of research and has made significant advancements in understanding the health outcomes of humans and animals consuming plants containing high amounts of melatonin as foods and/or medicines. The role of melatonin as a neurohormone in humans led to significant initial interest in melatonin as a bioactive or in synergy with other bioactives in medicinal plants, herbal products and dietary supplements including sleep aids [e.g., valerian (*Valeriana officinalis* L.), passionflower (*Passiflora incarnata* L.), chamomile (*Matricaria chamomilla* L.), ashwagandha (*Withania somnifera* (L.) Dunal)], psychoactive plants (e.g., *Datura metel* L., *Cannabis sativa* L., *Papaver somniferum L.*) and treatment of neurological conditions, such as feverfew (*Tanacetum parthenium* L.) in the treatment of migraines

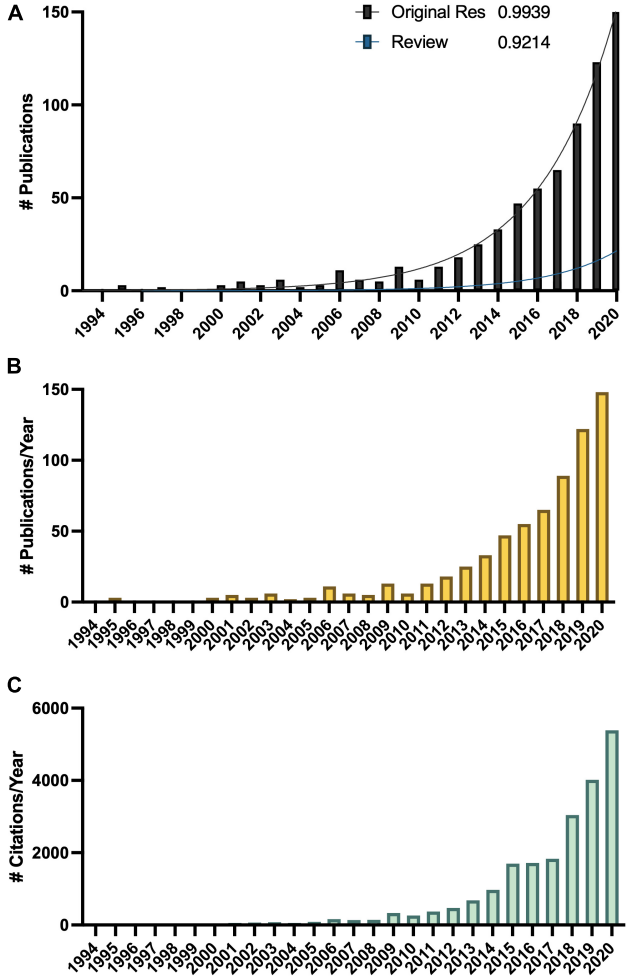

FIGURE 3 | Growth in the melatonin literature **(A)** total number of publications, with R^2 indicating best fit to an exponential curve, **(B)** total number published per year, and **(C)** total number of citations of articles captured in this analysis per year according to Web of Science.

(Vogler et al., 1998; Murch et al., 2009; Allegrone et al., 2019). Plant-based melatonin as a vegan supplement is growing in popularity, particularly in light of surging demands for melatonin for the treatment of coronavirus disease-19 (COVID19; Arnao and Hernández-Ruiz, 2018b; Arnao and Hernández-Ruiz, 2019b; Pérez-Llamas et al., 2020). A second stream of investigation has been led by researchers with expertise in the plant sciences who were inspired by the structural similarity between melatonin and the plant growth regulator indole-3-acetic acid (IAA; auxin). Research along these lines has led to significant advancements in understanding the roles of melatonin in root growth, seed germination and control of plant morphology (Pelagio-Flores et al., 2012; Zhang et al., 2014; Erland et al., 2018). Another avenue of investigation seems to have evolved with an emphasis on the ecological implications of plant melatonin and the potential roles of melatonin in adaptation, evolution and mitigating environmental stresses (Tan et al., 2014; Arnao and Hernández-Ruiz, 2019c). In this review, we have attempted to bring together divergent approaches and viewpoints to understand the synergy between approaches and the potential for discovery in the intersections between disciplines (**Figure 4**).

BIOSYNTHESIS OF PLANT MELATONIN VIA MULTIPLE DIVERSE AND REDUNDANT PATHWAYS

Evidence suggesting that melatonin in plants is produced from the aromatic amino acid tryptophan was first reported 20 years ago (**Figure 1**) where radiolabeling studies using ^{14}C-tryptophan demonstrated rapid conversion to melatonin in under an hour in *in vitro* grown plantlets (Murch et al., 2000). Over the last 20 years, molecular approaches have elucidated a full pathway for biosynthesis with several alternate mechanisms (**Figure 5**). In the primary pathway tryptophan is first converted to tryptamine through a decarboxylation reaction catalyzed by tryptophan decarboxylase (TDC; Zhou et al., 2020). Interestingly, this mechanism may not be conserved through evolution in all plant species since TDC1 is absent from some ecotypes of *Arabidopsis* and indeed different ecotypes of *Arabidopsis* have been found to respond differentially to melatonin exposure (Zia et al., 2019). Tryptamine is then hydroxylated to serotonin (5HT; 5-hydroxytryptamine) by tryptamine-5-hydroxylase (Kang et al., 2007). While TDC is highly regulated in most plant species, the conversion of tryptamine to 5HT appears to occur rapidly and with little feedback or regulation, aside from competition for tryptamine, which also serves as a precursor for many secondary metabolic pathways (Kang et al., 2007; Erland et al., 2018). In mammals, 5-hydroxytryptophan (5-HTP) is an intermediate between tryptamine and 5HT, but this part of the mechanism is not as well understood in plants. Quantification of 5-HTP in plants suggests it may be involved in 5HT biosynthesis or catabolism but an enzyme catalyzing these reactions has not been identified at the time of writing this review (Reynolds et al., 1983). Biosynthesis of melatonin from 5HT occurs through two main intermediates; (1) N-acetylserotonin (NAS), in a reaction catalyzed by serotonin-N-acetyltransferase (SNAT; Kang et al., 2013) following the same pathway as in animals, or (2) 5-methoxytryptamine (5-MT), which is catalyzed by a caffeic acid-O-methyltransferase (COMT; Lee et al., 2014b; **Figure 5**). SNAT can use tryptamine as a substrate for NAS production, skipping the need for production of 5HT, and can also catalyze the acetylation of 5-MT to form melatonin (Byeon et al., 2013; **Figure 5**). Recently, a novel deacetylase enzyme, NAS deacetylase (ASDAC), has been characterized that catalyzes the reverse reactions converting NAS to 5HT, or melatonin to 5-MT allowing for the possibility of interconversion between 5HT and melatonin (Lee et al., 2018). The final step in the primary pathway is methylation of NAS to melatonin catalyzed by the enzyme NAS methyl transferase (ASMT; Park et al., 2013a), or COMT (Byeon et al., 2014; **Figure 5**). Melatonin biosynthesis has been shown to occur in both the chloroplast (Zheng et al., 2017) and the

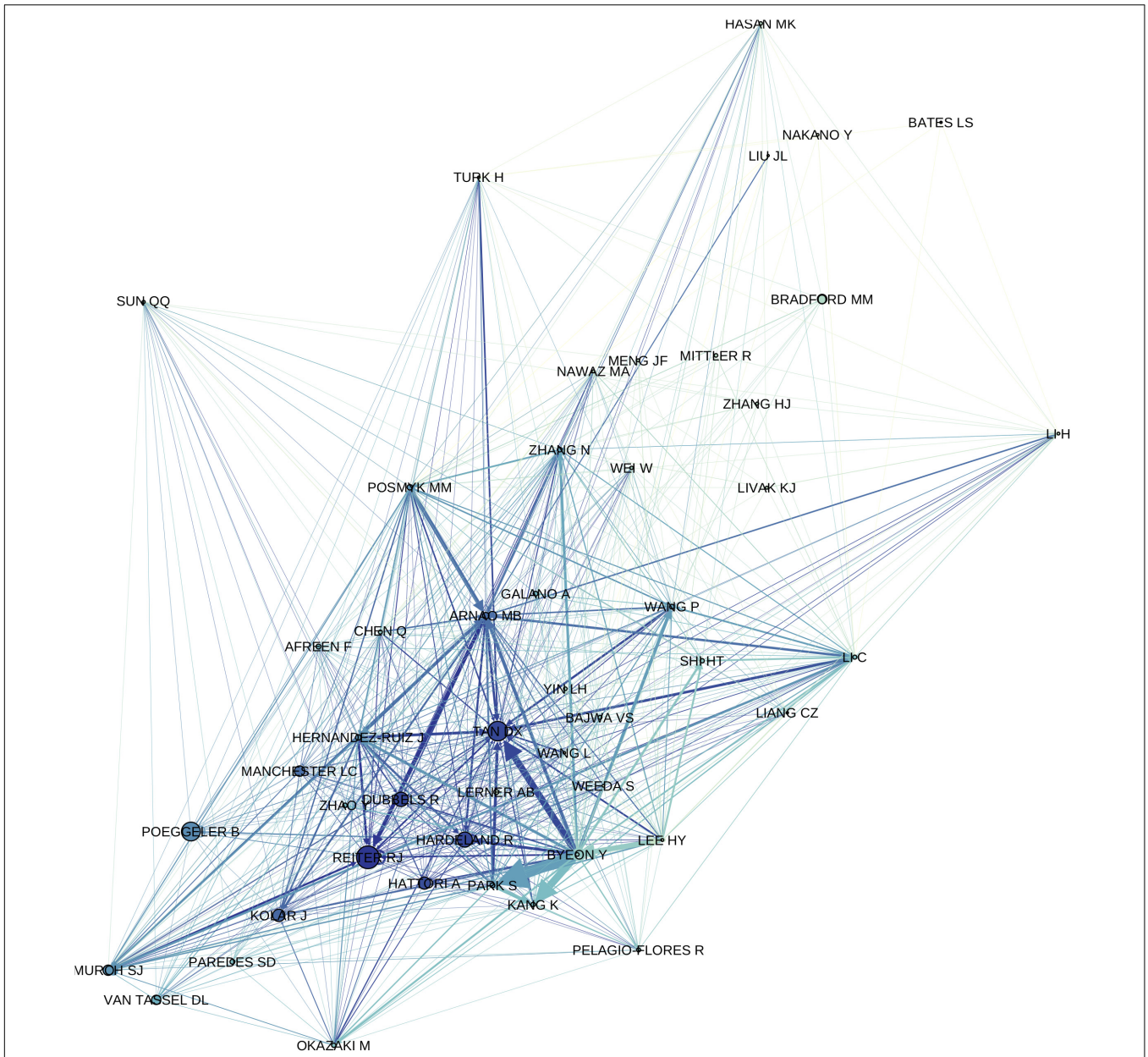

FIGURE 4 | Author correlation network showing relationship between first and last authors of original research articles included in the study and first authors of citing articles, filtered to an in-degree cutoff of >100. Increasing edge thickness indicates a stronger relationship (higher edge weight), edges are colored to match target node, increasing node size indicates higher page rank (network importance), nodes are colored based on in-degree, i.e., number of authors citing with authors with a greater number of citations being darker.

mitochondria (Wang et al., 2017) with some possible cytosolic involvement. To date, melatonin transport proteins have not been characterized in plants and this is an area in which further research efforts are warranted. The significant diversity that exists in the melatonin biosynthetic pathway leaves open many areas of future research. More investigations are needed to understand the significance of alternate pathways, evolutionary conservation, divergence or convergence and diversity between families and/or species. The multiple biosynthetic mechanisms create significant complications for studies to create enzyme specific mutants or to manipulate specific genes to determine function as dormant or redundant pathways are activated to recover functions.

MELATONIN IS A PRECURSOR FOR BIOACTIVE METABOLITES

Melatonin is metabolized to produce a number of important bioactive molecules in plants that can generally be grouped

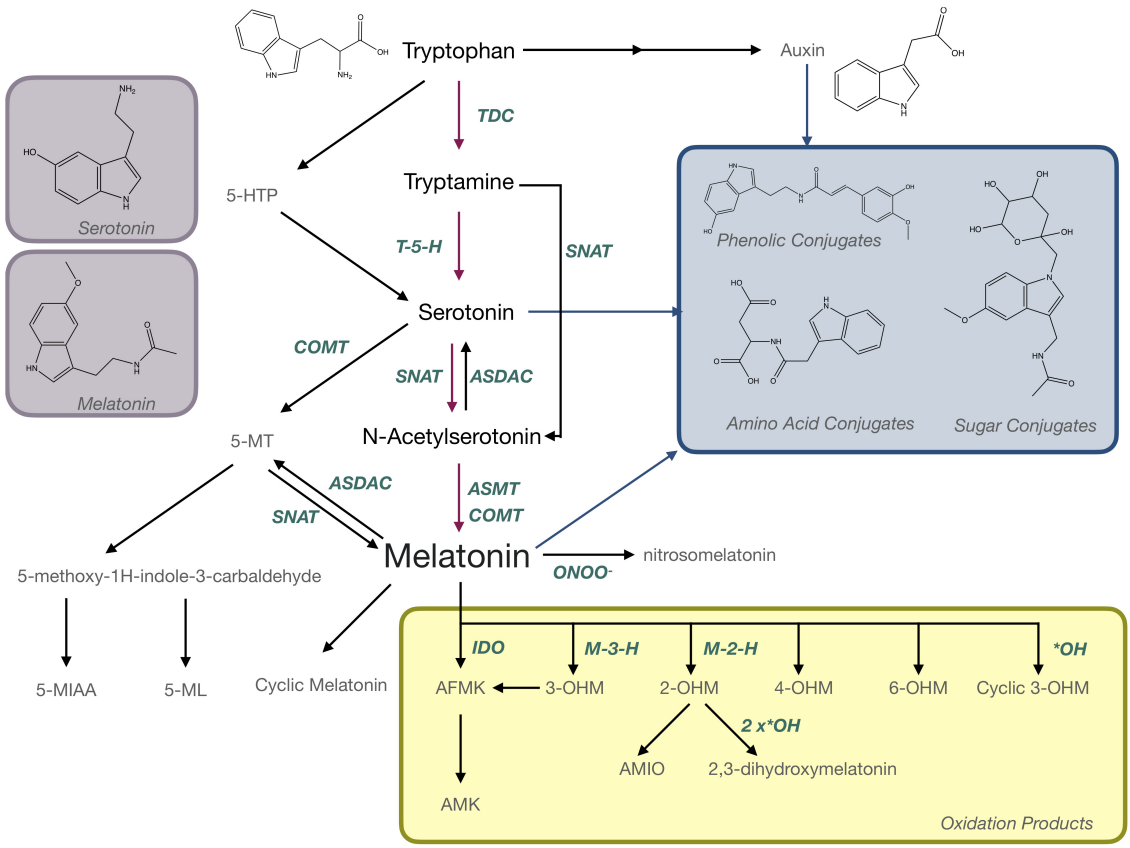

FIGURE 5 | Biosynthesis and metabolism of melatonin in plants. AFMK, N-acetyl-N-formyl-5-methoxykynuramine; AMIO, 3-acetamidoethyl-5-methoxyindolin-2-one; AMK, N-acetyl-5-methoxykynuramine; ASDAC, N-acetylserotonin deacetylase; ASMT, N-acetylserotonin-O-methyltransferase; COMT, caffeic acid-O-methyltransferase; 5-HTP, 5-hydroxytryptophan; IDO, indoleamine 2,3-dioxygenase; M-3-H, melatonin 3-hydroxylase; M-2-H, melatonin 2-hydroxylase; 5-MIAA, 5-methoxyindole-3-acetaldehyde; 5-ML, 5-methyoxytryptophol; 5-MT; OHM, hydroxymelatonin; SNAT, serotonin N-acetyltransferase; TDC, tryptophan decarboxylase.

as; (a) products of oxidation reactions, (b) products of catabolism and (c) conjugates and derivatives (**Figure 5**). Nitrosomelatonin, N-acetyl-N-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) are products of oxidation reactions as are several of the 5-MT derivatives (**Figure 5**). AFMK and AMK were the first melatonin metabolites described in plants (Tan et al., 2007a) and function as antioxidants with the capacity to quench reactive oxygen (ROS) and reactive nitrogen species (RNS; Tan et al., 2007b; Schaefer and Hardeland, 2009). It has been proposed that AFMK may be produced by both enzymatic and non-enzymatic reactions, particularly conversion by indole-2,3-dioxygenase (IDO; Okazaki et al., 2010). This hypothesis is still somewhat controversial as other authors argue that tryptophan is generally the substrate for IDO and can react with RNS or ROS (Hardeland, 2014). The levels of AFMK have also been found to vary in coordination with melatonin (Tan et al., 2007a) making it unlikely that melatonin is the primary precursor. Evidence of the function of 5-MT metabolites, 5-methyoxytryptophol (5-ML) and 5-methoxyindole-3-acetaldehyde (5-MIAA) have been investigated to an even lesser extent in plants, though their role as catabolism products of melatonin in dinoflagellates is fairly well described (Hardeland, 2014). Nitrosomelatonin as well as melatonin oxidation products, play important roles as antioxidants in plant, while conjugates have been hypothesized to play roles as sequestration or storage forms. This is the case for phenolic-conjugates of the melatonin precursor, 5HT, which play roles in defense (Ishihara et al., 2008), though the presence and function of melatonin-phenolic compounds is as yet not experimentally confirmed (Erland et al., 2020b). Though the presence of melatonin metabolites is well accepted, their presence and functions in plants have generally not been widely investigated. "Hydroxymelatonin" and "isomer" are mentioned in 14 titles/abstracts each, with the most mentioned, 2-hydroxymelatonin (2-OHM) still only being mentioned in 12 abstracts/titles. Several articles have proposed that some metabolites, particularly 2-OHM and 3-hydroxymelatonin (3-OHM; Byeon et al., 2015b) which are produced by melatonin 2-hydroxylase (M2H; Byeon and Back, 2015) and melatonin 3-hydroxylase (M3H; Lee et al., 2016), respectively, may actually be the predominant forms of melatonin in plants. For example, 2-OHM is reported to confer tolerance to abiotic stresses, such as cold (Lee and Back, 2016b; Back, 2020), drought (Lee and Back, 2016b; Back, 2020), salt (Choi and Back, 2019b),

and heavy metal stress (Byeon et al., 2015a; Shah et al., 2019), through both direct antioxidant function and upregulation of antioxidant enzymes (Shah et al., 2019, 2020). 2-OHM may also activate mitogen-activated protein kinase (MAPK) signaling cascades (Lee and Back, 2016a) and modulate gene expression including stress related transcription factors and transport proteins (Lee and Back, 2016b). With so many potential roles and interactions, further investigation of the functions of these metabolites and isomers is warranted. These future investigations should include not just quantification of these compounds but also aim to understand the roles of these metabolites in plants metabolism.

MELATONIN IS AN ANTIOXIDANT

The story of melatonin as an antioxidant is often repeated and comparatively well understood. The antioxidant potential of melatonin is postulated as a mechanism for stress responses, as well as protection of photosynthetic apparatus and reproductive development/germ tissues. At least 51 of the publications we assessed mention melatonin and ROS specifically. Readers who are interested in further detail are referred to a proliferation of reviews on this topic published in the last 2 years (n = 11 published in 2020, 2021; **Supplementary Table 2**). Interest in this area of research is driven by the hypothesis that the strong antioxidant potential of plant melatonin conferred an evolutionary advantage to the first living organisms on Earth during the great oxygen epoch (Tan et al., 2014; Tan and Reiter, 2020). Mechanistically, melatonin quenches up to 10 units of ROS per molecule and requires no recycling mechanism (Rodriguez-Naranjo et al., 2012). Isomers and metabolites of melatonin including AFMK, AMK and 3-OHM have all been shown to be capable of scavenging free radicals through hydrogen atom transfer (HAT), single electron transfer (SET) and radical adduct formation (RAF) mechanisms and to form a unique free radical scavenging cascade which does not require enzymatic activity (Reina and Martínez, 2018). Melatonin can react with both ROS and RNS to form oxidized or nitrogenated metabolites. For example, reaction of melatonin with the hydroxyl radical yields 2,3-dihydroxymelatonin and cyclic 3-OHM (Back, 2020), while reaction with oxoperoxonitrate ($ONOO^-$) yields 1-nitrosomelatonin (Mukherjee, 2019). The capacity to neutralize ROS also plays an important role in melatonin's control of ROS and reactive nitrogen species (RNS) signaling networks within plants, in particular hydrogen peroxide (H_2O_2) and nitric oxide (NO) signaling networks which allow for rapid signaling in response to stimulus (Lee and Back, 2017).

PRECISE, ACCURATE AND SENSITIVE QUANTIFICATION OF MELATONIN REVEALS ISOMERS

Methodologies for detecting and quantifying endogenous melatonin have seen a significant shift since 1995 (Dubbels et al., 1995; Hattori et al., 1995; **Figure 6**). The first studies quantifying melatonin in plants used antibody-based techniques, namely radioimmunoassay (RIA; Dubbels et al., 1995; Hattori et al., 1995), however, mass spectrometry (MS)-based techniques including quadrupole, time of flight and tandem systems now make up the majority of methods (**Figure 6**). Development of new technologies are reflected in the trends of preferred analytical method for example a shift from RIA to enzyme-linked immunosorbent assays (ELISA; **Figure 6**). Overwhelmingly, the preferred approach for quantification of melatonin has become liquid chromatography (LC)-MS based approaches (**Figure 6**). In addition to the higher sensitivity and specificity of LC-MS based approaches, this increase is also likely due to the increased ease of use of these platforms, a greater number of published protocols and decreasing cost of entry. LC-MS approaches generally also have improved ease of use and higher throughput as compared to electrochemical detection methods which often require long equilibration times and or gas-chromatography (GC) based methods which require derivatization prior to analysis. Another advantage to LC, and particularly LC-MS based approaches, is the capacity for simultaneous quantification of multiple analytes. For example, detection of multiple indoleamine compounds, metabolites or conjugates or quantification of structurally or functionally related signaling molecules or plant growth regulators with a single injection. One topic which has not been widely addressed in methods for quantification of melatonin is the existence of several isomers of melatonin (Tan et al., 2012; Kocadagli et al., 2014). As a result of improved methods, several melatonin isomers (Rodriguez-Naranjo et al., 2011) have been discovered and a nomenclature proposed (Tan et al., 2012), though some controversy exists as to whether these isomers are truly melatonin isomers or instead tryptophan ethylesters (Gardana et al., 2014; Iriti and Vigentini, 2015). As research into the relevance and functions of melatonin isomers and metabolites increases, methods for the accurate, precise and sensitive quantification of this class of compounds will be instrumental in informing these studies.

OMICS

Omics technologies for studies of melatonin metabolism have exploded in the last decade. Transcriptional effects of melatonin in plants is a "hot topic" of investigation with > 150 publications mentioning transcriptomics and > 40 which used genomics or microarray approaches (**Figure 9C**), in addition to more targeted polymerase chain reaction (PCR)-based strategies. Diverse genes including numerous transcription factors have been described as modified by melatonin and play important roles in melatonin-mediated plant stress responses (Weeda et al., 2014). It is therefore well established that melatonin can serve as a master regulator of gene expression in plant tissues to induce protective responses in plants (Arnao and Hernández-Ruiz, 2019a). Melatonin has been found to both induce translocation of transcription factors from the cytoplasm to the nucleus, and to increase levels of transcription factors and downstream transcripts. Depending on the pathways targeted, the genes up

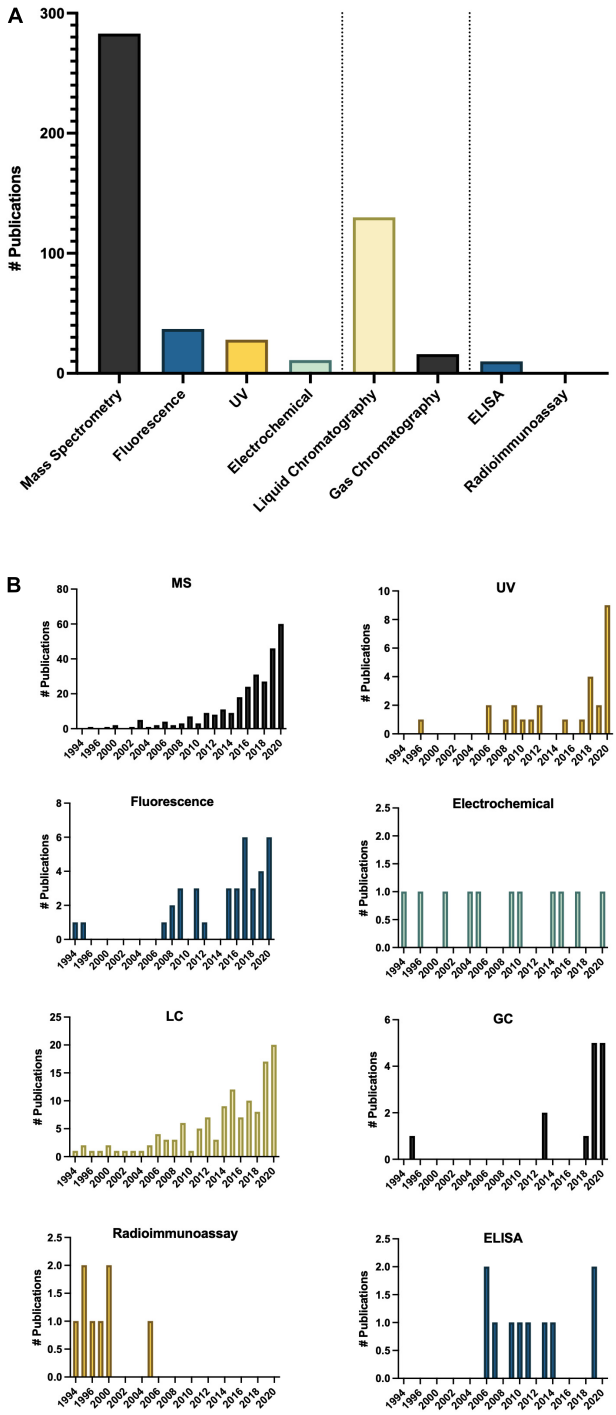

FIGURE 6 | Methods of analysis of melatonin in plants **(A)** total number of publications by technique over the time period queried and **(B)** Number of publications by technique by year. ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; UV, ultra violet detection.

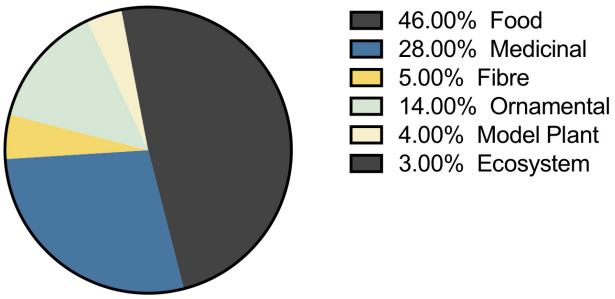

FIGURE 7 | Selection of plant species for melatonin studies is largely driven by economic value. Where species can fit two categories the primary use was included for plotting.

or down regulated can include antioxidant pathways, signaling pathways, primary metabolic pathways and secondary pathways, essentially allowing for fine tuning of almost every chemical system (Weeda et al., 2014; Shi et al., 2015a; Hu et al., 2016). While transcriptomics studies have built the platform upon which further research can be built, much is still needed to fully understand the downstream effects of melatonin. The biological relevance of these gene expression changes are less well described for melatonin. It is well accepted that though transcript levels may change this does not always lead to a phenotypic effect due to the necessity for translation and post translational modifications, transport to the necessary compartment and end function of the protein and catalysis of metabolites are all required. This is increasingly being reflected in the literature as proteomic and metabolomic studies start to rise.

The question of over-emphasis on gene expression data from both targeted gene expression and untargeted transcriptomics studies is particularly highlighted in the many studies which examine the effects of a treatment or condition, e.g., metal, salt, drought on melatonin gene expression levels without quantifying the level of melatonin itself. Our understanding of melatonin biosynthetic pathways continues to improve, and with it an appreciation for the complexity and diversity built into the pathway. While this redundancy allows for significant resilience in the melatonin system, and emphasizes the importance of melatonin in plants, it makes targeted gene expression studies potentially problematic as they provide information on only the target gene. The use of transgenics has been used extensively to improve our understanding of the role of melatonin in plants with more than 50 studies published utilizing this tool. With the advent of CRISPR and more routine and specific gene editing technologies the use of transgenic overproduction or knockout lines has significant inherent value to understanding the function of the pathways in which they participate and have been instrumental in characterizing the melatonin biosynthetic pathway. Transgenic studies which are coupled with quantification of melatonin (n = 49) and its metabolites have been especially powerful as confirmatory tools in identifying roles for melatonin metabolites as well. This serves as an important model for consideration in the design of melatonin studies integrating genomics tools and approaches with quantification of the phenotypic effect.

To date five articles have utilized proteomics approaches to understand the mechanisms of melatonin in seed germination,

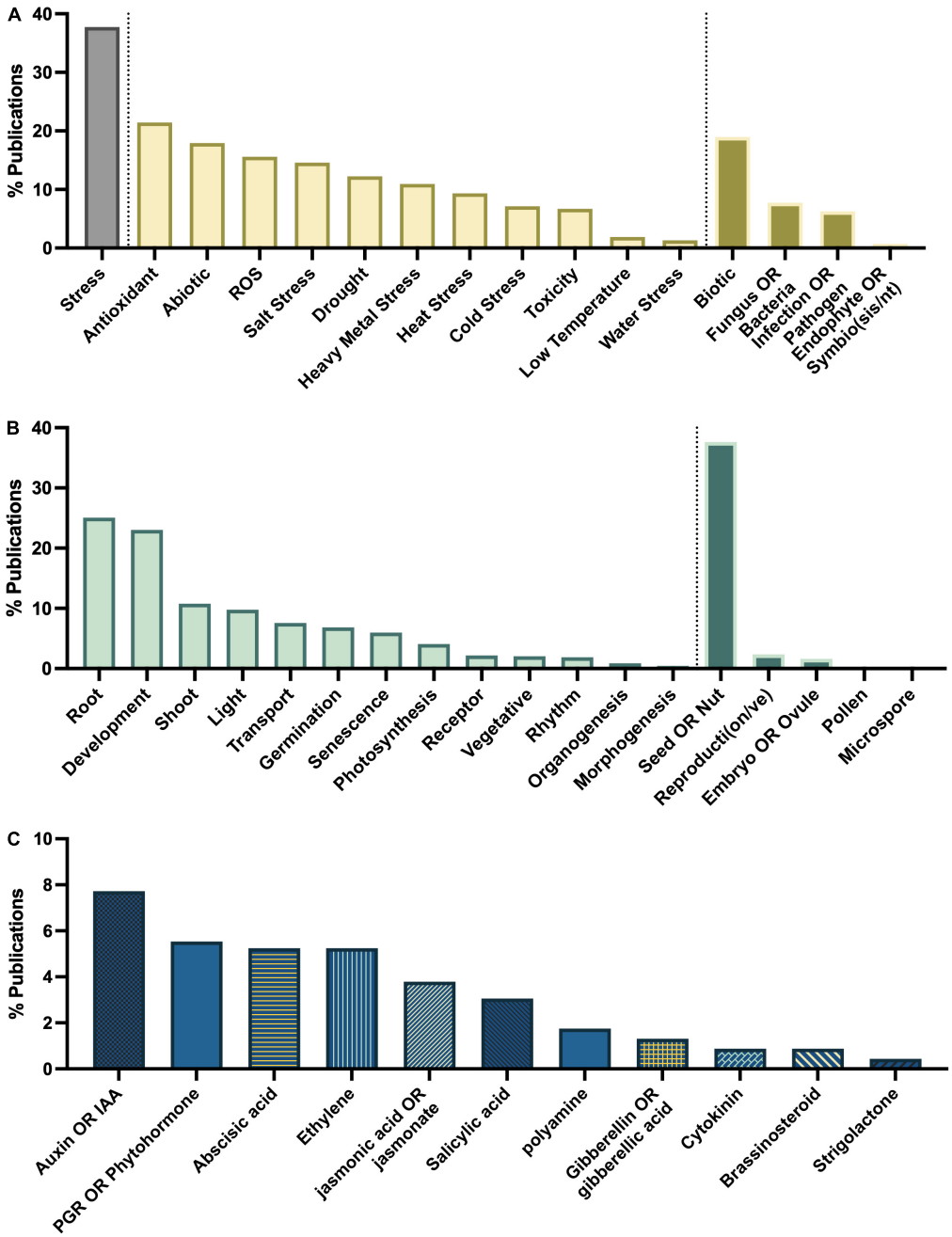

FIGURE 8 | Major themes in melatonin literature number of papers on topics associated with **(A)** stress, **(B)** growth and development, **(C)** interaction with phytohormones.

chilling stress, leaf senescence, oxidative stress and fruit ripening in maize (*Zea mays* L.), crab apple (*Malus hupehensis*), tomato (*Solanum lycopersicum* L.) and Bermudagrass (*Cynodon dactylon* (L.) Pers; Wang et al., 2014; Shi et al., 2015b; Kolodziejczyk et al., 2016a,b; Sun et al., 2016). Melatonin was found to enhance polyamine metabolism, ribosome pathway, carbohydrate metabolite, photosynthesis, redox and amino acid metabolism leading to tolerance as demonstrated by improved growth and reduced cell damage and ROS accumulation in response to hydrogen peroxide treatment in Bermudagrass (Shi et al., 2015b). Melatonin-mediated changes in the proteome have been reported in response to chilling stress in maize as well as in response to melatonin application in senescing crab apple leaves, particularly with respect to stress and antioxidant related proteins (Kolodziejczyk et al., 2016b). In crab apple, modified proteins were more commonly associated with the plastid

(Wang et al., 2014). Almost twice the number of metabolomics studies have been published in the area of plant melatonin research as compared to proteomics studies (n = 9) and have more diverse areas of interest as compared to proteomics studies. All but one have examined melatonin as a downstream metabolite mediated in the process or state studied, in contrast to proteomics and transcriptomics studies which generally focus on the impact of treatment. For example, morphogenesis in hazelnut (Erland et al., 2020b), fusarium wilt infection in watermelon (Kasote et al., 2020), low phosphorus resistance (Xu et al., 2020) and root induction in tomato (Soundararajan et al., 2017). This is a particularly exciting new area for melatonin research as it allows for the examination of the role of the melatonin naturally present in plants and has generated hypotheses of novel melatonin metabolism pathways.

SPECIES STUDIED REFLECTS HUMAN USES

The selection of species that have been studied in the melatonin literature has been highly influenced by economic and commercial value. Our queries of the literature identified 236 plant species representing 191 genera from 94 families for which endogenous melatonin has been quantified and its metabolism investigated (**Supplementary Appendix A1** and **Tables 1**, **2**). The greatest species diversity is represented in the Lamiaceae (n = 23), Leguminosae (n = 20), Poaceae (n = 18), Rosaceae (n = 17), and Solanaceae (n = 13; **Table 1**). Unsurprisingly, the majority of species that have been investigated are economically valuable with only 4% of studies performed on classic plant model species and 3% of studies representing plant communities or ecosystems (**Figure 7**). Overwhelmingly these species are of commercial importance with 46% of studies on food species and 28% of studies on medicinal plants (**Figure 7**). Assessment of the top 10 species investigated by number of papers finds that 7 are foods including: rice (*Oryza sativa* L.), corn (*Zea mays* L.), pear (*Pyrus communis* L.), grapes (*Vitis vinifera* L.), cucumber (*Cucumis sativa* L.), wheat (*Triticum aesativum* L.), and tomato (*Solanum lycopersicum* L.). The remaining three species include, unsurprisingly, the model species *Arabidopsis thaliana* (L. Heynh.) while the other two are medicinal species: St. John's wort (*Hypericum perforatum* L.), which has also been considered a melatonin model species, and tobacco (*Nicotiana tabacum* L.), a valuable agricultural crop. Examination by genus shows an even greater skew toward food and feed crops with only *Arabidopsis* (n = 70) and *Nicotiana* (n = 41) remaining in the top 10 species investigated. The most well studied genera include *Solanum* (n = 119, nightshades), *Oryza* (n = 87, rice), *Malus* (n = 53, apples), *Brassica* (n = 44, mustards), *Prunus* (n = 41, plums, cherries, peaches, apricot, almond), *Cucumis* (n = 32, cucumbers and melons), *Zea* (n = 32, corn, maize), and *Pyrus* (n = 32, pears; **Table 2**). It is worth noting that the top ranked genus *Solanum* includes a diversity of both medicinal and food species, which is perhaps responsible for its high levels of investigation (17% of all publications). The growth and production regions of the top species investigated are also generally highly correlated; the countries from which the greatest number of publications originate, for example China and South Korea are major producers of rice, while Southern Europe including Italy and Spain are major grape and wine producing regions (**Supplementary Figure 1**).

TABLE 1 | Summary of genera in which melatonin has been investigated.

Genus	Number of Papers Citing
Solanum	119
Oryza	87
Arabidopsis	70
Malus	53
Brassica	44
Prunus	41
Nicotiana	41
Cucumis	32
Zea	32
Pyrus	32
Vitis	29
Triticum	27
Hypericum	18
Citrullus	13
Glycine	12
Capsicum	12
Manihot	11
Avena	11
Scutellaria	10
Citrus	10
Morus	9
Raphanus	9
Dracocephalum	8
Medicago	8
Lupinus	8
Musa	7
Cynodon	7
Fragaria	6
Salvia	6
Festuca	6
Thymus	6
Camellia	6
Hordeum	6
Helianthus	6
Actinidia	6
Ocimum	5
Armoracia	5
Gossypium	5

A total of 191 genera have been investigated, only genera where five or more papers have cited the genus are included.

MAJOR RESEARCH THEMES

To determine the major themes in the literature, the dataset was queried for keywords by study type (**Figure 8A**), tissue type (**Figure 8B**), interaction with plant growth regulators (**Figure 8C**), environmental conditions of the study (**Figure 9B**),

TABLE 2 | Summary of families in which melatonin has been investigated.

Family	Number of Species
Lamiaceae	23
Leguminosae	20
Poaceae	18
Rosaceae	17
Solanaceae	13
Compositae	10
Brassicaceae	9
Apiaceae	5
Rubiaceae	4
Anacardiaceae	3
Myrtaceae	3
Moraceae	3
Rutaceae	3
Amaranthaceae	3
Amaryllidaceae	3
Zingiberaceae	3
Malvaceae	3
Boraginaceae	2
Actinidiaceae	2
Juglandaceae	2
Compositaceae	2
Polygonaceae	2
Araliaceae	2
Caprifoliaceae	2
Crassulaceae	2
Asparagaceae	2
Curcubitaceae	2
Betulaceae	2
Discoreaceae	2
Rhamnaceae	2
Ericaceae	2
Violaceae	2
Euphorbiaceae	2
Gentianaceae	2
Oleaceae	2

A total of 94 families have been investigated, only families where two or more species have been investigated are displayed.

type of melatonin exposure (**Figure 9A**), and type of study (**Figure 9C**). A network analysis by digital object identifier (DOI) identified relationships between the literature themes such that some aspects are over-represented, and some are undervalued in the current publication database (**Figure 10**). The majority of all melatonin research focuses on exogenous application (**Figure 9B**). Topic area had a significant effect on the importance of a paper in the network analysis. By comparing the % representation of the query areas examined across the literature between the complete set of literature and the total literature several trends emerged (**Figure 10B**). Papers which investigate stress responses and particularly mention melatonin as an antioxidant were found to be overrepresented by almost 20% in the high impact papers, while papers examining morphogenetic responses such as root or shoot growth were underrepresented by journal impact. Together these analyses show that it is not the papers which make the greatest advances in the field which end up having the greatest impact on the evolution of research. Rather, specific topic areas are over amplified and driving research theme relevance. One example of the impact of publication trends is relatively few citations of the early work by Jackson (1969) or Banerjee and Margulis (1973) in modern studies that use exogenous application of melatonin to induce physiological responses and better integration of these older studies with modern data may lead to exciting discoveries. Our analysis of the literature uncovered 6 major research themes in plant melatonin metabolism.

Major Research Theme – Abiotic Stress Responses

Queries by topic area found that the greatest areas of research interest have focused on stress responses with 37% of articles mentioning "stress" in their title or abstract, particularly with respect to melatonin's status as an antioxidant with 21% of articles mentioning "antioxidant," and 16% mentioning "ROS" (**Figure 8A**). Papers are evenly split between biotic and abiotic stress (19 and 18%, respectively; **Figure 8A**). The most mentioned abiotic stresses include salt (15%), drought (12% + additional 2% mentioning water stress), heavy metal (11%) and temperature stress (heat 9%, cold 7%, low temperature 2%). With more than 382 papers published on the topic of melatonin and stress this function of melatonin is one of the best described. Our meta-analysis identified 79 review papers, with several recent reviews which discuss the topic of melatonin-mediated plant stress responses (**Supplementary Table 2**) and we refer readers there for in-depth discussion on this topic. Melatonin has been found to have multidimensional mechanisms in mediating plant stress responses. These include induction of plant signaling cascades including MAPK (n = 6), calcium/calmodulin (n = 14), nitric oxide (n = 14), ROS associated signaling cascades (n = 3), as well as its role as a direct antioxidant (n = 86) and upregulation of other cellular antioxidant pathways.

The publications show that melatonin has widespread impacts on transcriptional networks inducing stress-associated transcription factors (n = 20) such as ZAT10/12 (n = 4), drought responsive element binding (DREBs; n = 9), CBF (n = 5), bZIP (n = 5), MYB (n = 7), NAC (n = 3) and defensive proteins, e.g., heat shock proteins (n = 7). Interactions with other phytohormones have also been examined (n = 14), particularly, abscisic acid (n = 19), auxin (n = 13), ethylene (n = 13), jasmonic acid (n = 9), polyamines (n = 8), salicylic acid (n = 6), cytokinins (n = 4), gibberellins (n = 3), and brassinosteroids (n = 2). As well, melatonin has been found in a significant number of studies to upregulate the ascorbate glutathione cycle, and to increase antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase, which have become common and non-specific screening assays in many melatonin papers with the specific enzymes mentioned in the title or abstract of 87, 72, and 75 articles, respectively and 48 mentioning "antioxidant enzyme" more generally. Likewise, quantification of ROS in plant tissues in response to melatonin treatment has also

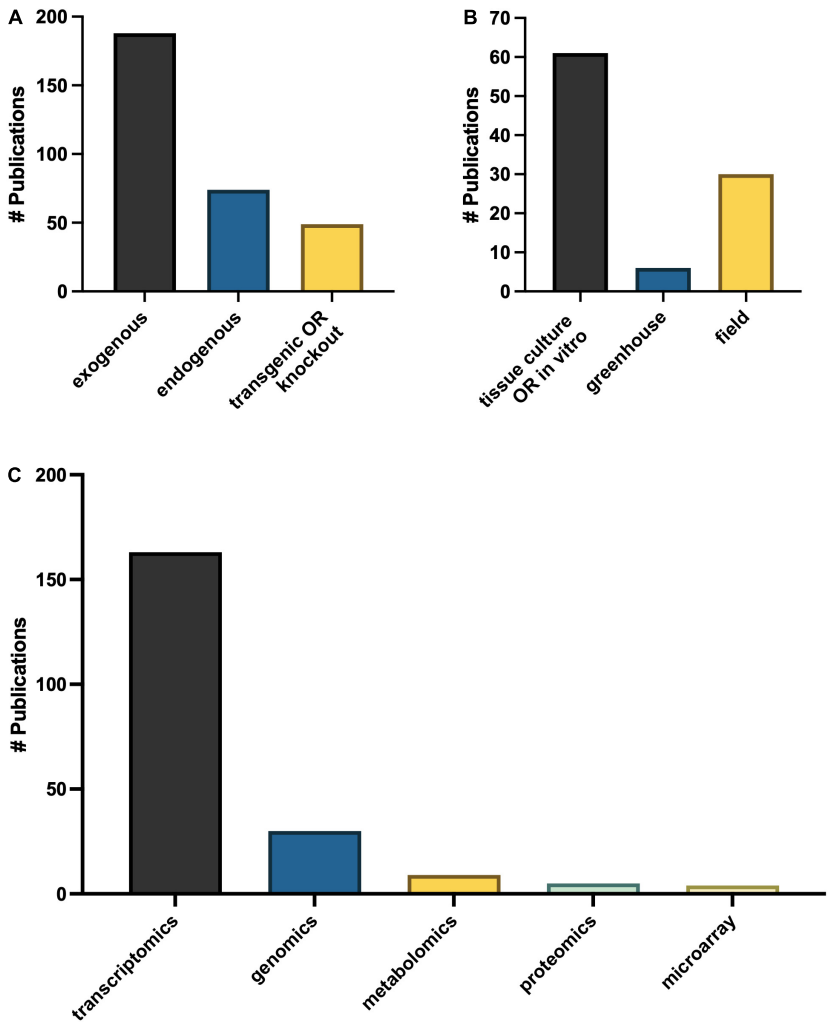

FIGURE 9 | Major themes in approaches to melatonin research in plants **(A)** growth methods, **(B)** melatonin treatment type and **(C)** OMICs technologies.

become routine, particularly with respect to H_2O_2 (n = 118), superoxide (n = 98), and malondialdehyde (indicator of lipid peroxidation, n = 85) levels which are broadly found to be reduced through melatonin treatment. Melatonin's antioxidant activity has also been shown to mediate ROS and RNS based signaling, through quenching of H_2O_2 rapid signaling, one of the means by which plants perceive stresses including temperature, salinity stress and wounding (Gong et al., 2017), as well as interacting with downstream of signaling NO and ROS signaling cascades (Chen et al., 2017; Panossian et al., 2018). Melatonin has also been found to improve function and efficiency of the photosynthetic apparatus in diverse species (Szafranska et al., 2016). This mechanism has been reported to occur primarily via quenching of excess ROS species, levels of which are increased when the efficiency of the apparatus starts to break down due to stress or age leading to an inability to absorb excess light energy (Szafranska et al., 2017). Maintenance of ion homeostasis (n = 49) is important particularly in heavy metal and salinity stress and melatonin has been found to improve performance in several species. Melatonin-induced NO signaling (Yan et al., 2020) as well as maintenance of energy levels necessary for H + -ATPase activity and downstream effects on sodium/potassium balance have in particular been found to be modified by melatonin (Yu et al., 2018). Melatonin moves through the apoplasm and via the vasculature in plant tissues (Erland et al., 2019). The transport has been observed to occur over long distances and to occur relatively rapidly (Li H. et al., 2017). Translocation of melatonin has also been found to occur in response to temperature stress (Erland et al., 2019), which results in a diffuse pattern of melatonin localization and transport to distal organs where it functions as an antioxidant and has been found to induce cold tolerance in *Citrullus lanatus* (L.; Li H. et al., 2017) and salt tolerance in *Dracocephalum kotschyi* Boiss (Vafadar et al., 2020).

A significant body of research on melatonin in plant stress responses was generated in studies with *A. thaliana* (n = 37) and crop species, with the greatest number of articles reported in *Solanum* species (n = 55) of which 17 of those in tomato, followed by rice (n = 30), corn (n = 25), *Malus* species

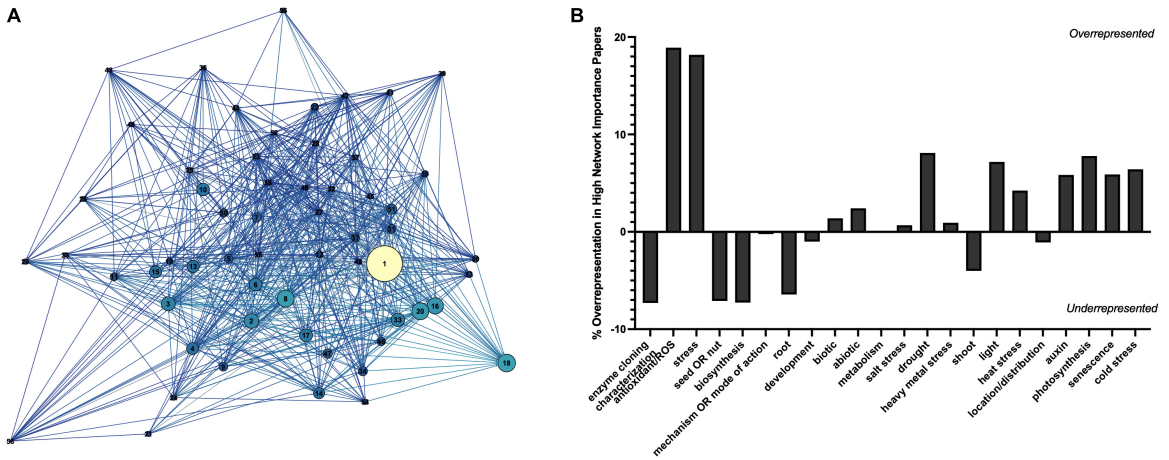

FIGURE 10 | Network analysis by digital object identifier (DOI) **(A)** and representation of studies in the plant melatonin literature **(B)**. Correlation network shows relationship between top cited DOIs filtered to an in-degree cutoff of >100. Increasing node size indicates higher page rank (network importance), nodes are colored based on in-degree, the greater the number of articles citing the lighter the color. Key can be found in **Supplementary Figure 3**.

(n = 23), wheat (n = 21), cucumber (n = 16), and *Brassica* species (n = 15). With a growing global population, decreasing amounts of land which are traditionally suitable for agricultural cultivation, and increasing climactic uncertainty with global climate change, melatonin represents a potentially invaluable means by which to maintain or enhance agricultural production under less favorable growing conditions. The emphasis on melatonin mediated stress responses in agricultural species to date has created an interesting niche which has yet to be explored, including the potential implications of melatonin application on soil microbiomes and persistence which will be an important consideration (Madigan et al., 2019). These same factors which are having dramatic impacts on agricultural species are also impacting wild and native species around the word. These species represent culturally significant species either for ceremony, medicine or traditional food sources, as well as ecologically important species. Application of the knowledge generated in commercially or scientifically valuable species to ecologically and culturally important species holds great potential for expanding our understanding of melatonin function in plants as well as having interdisciplinary applications. The knowledge generated through these types of studies may, for example, help in determining and implementing management and conservation priorities for traditional harvest, commercial wild harvest or conservation status of species.

Major Research Theme – Roots Are Important

A major theme in the literature is melatonin as a mediator of vegetative growth and development, most notably root system architecture (n = 172 articles for the term "root," **Figure 8B**). In fact, the first reports of melatonin in plants, focused on melatonin in the root system reporting that melatonin treatment could control mitotic spindle development in root tips, inhibiting cellular replication and providing the first mechanism by which melatonin inhibits primary root growth (Jackson, 1969; Banerjee and Margulis, 1973). Mediation of rooting has since been reported in 76 species with the greatest number of studies having been conducted in *Arabidopsis* (n = 23) which have enabled mechanistic investigation of the function of melatonin in control of root growth, branching and development. Rice (n = 17), apple (n = 11), tomato (n = 11), wheat (n = 11), corn (n = 10), canola (n = 10), and pear (n = 10) all also have more than 10 citing papers, though these mostly report the effect of melatonin in rooting, rather than investigate the mechanisms underlying this function. In general melatonin inhibits primary root elongation, while promoting secondary or lateral root formation favoring a short more branched root network. This likely confers an advantage to these plants when nutrients are available in the higher soil layers where a more branched root system allows better nutrient acquisition.

One of the first hypothesized mechanisms of melatonin action in plants was based on the structural similarity between melatonin and auxin. This is reflected in the literature overall as ∼8% of all the original research studies queried in this meta-analysis mention the terms "auxin" or "IAA" (**Figure 8C**) and of the papers which mention "root" twenty-one explicitly mention auxin or IAA. The actual function, as is often the case, has turned out to be much more nuanced. The first in-depth mechanistic investigation of melatonin mediation of root system architecture in 2012 reported that melatonin stimulated lateral and adventitious root formation through an auxin independent mechanism using transgenic and reporter lines (Pelagio-Flores et al., 2012). Complimentary studies in corn reported that melatonin does not meet the threshold for auxin activity in any of the three classical auxin assays: inhibition of 1-aminocyclopropane-1-carboxylate synthase (ACC) activity, coleoptile elongation and root growth, leading the authors to reject this hypothesis (Kim et al., 2016).

In contrast several studies have found that melatonin interacts with auxin and accomplishes its actions not through mimicking

auxin itself but by inducing and interacting with auxin signaling and transport mechanisms. This appears to be especially true with respect to primary root growth. Melatonin has been found to inhibit polar auxin transport through mediation of PINFORMED (PIN) auxin transport proteins, as well as auxin biosynthesis leading to reduced primary root meristem size, inhibition of primary root growth and subsequent lateral root promotion in *Arabidopsis* (Wang et al., 2016; Ren et al., 2019). In St. John's wort (*H. perforatum*), where melatonin was first reported to have an auxin-mediated effect on root organogenesis (Murch et al., 2001), melatonin has been demonstrated to have a distinct localization pattern in primary roots compared to auxin (Erland et al., 2019) and melatonin has also been found to be induced by auxin treatment (Erland and Saxena, 2019) suggesting a further level of complication in the story. In addition to auxin, melatonin interacts with other phytohormones which are important in root growth including ethylene (n = 11), jasmonic acid (n = 10), abscisic acid (n = 8), polyamines (n = 4), salicylic acid (n = 3), gibberellin (n = 2), brassinosteroids (n = 2), strigolactones (n = 1), cytokinins (n = 1). Melatonin has also been found to directly induce accumulation of H_2O_2 leading to ROS burst and calcium-dependent induction of lateral root formation (Chen et al., 2018). The importance of mediation of H_2O_2 levels in root growth and morphogenesis as a function of melatonin is highlighted with H_2O_2 mentioned in ∼22% of all papers on the topics of "root" and "melatonin." The importance of downstream signaling cascades has also been investigated, with calcium having been shown to play an essential role in mediating root growth (n = 7) including in *Mimosa pudica* L. (Ramakrishna et al., 2009). The use of transcriptomics approaches will likely continue to elucidate significant cross talk between auxin-dependent and auxin-independent mechanisms of melatonin action in plants which have been utilized extensively (n = 42) to study root physiology in response to melatonin treatment. Melatonin mediation of root growth can be considered to have a dual function, auxin-independent control of root branching, secondary, lateral and adventitious root growth, and auxin-dependent control of primary root growth.

Though not necessarily associated with the development of roots, captured in the "root" query is also investigation of the role of melatonin in tubers and other storage roots. Melatonin has been reported to maintain postharvest quality (n = 21) through its status as an antioxidant to maintain appropriate redox status of the tissues, induction of many of the same signaling cascades important in root development including calcium and MAPK signaling, as well as mediation of carbohydrate metabolism in particular (Ma et al., 2016; Hu et al., 2018). Cassava (n = 3) has been a particularly valuable plant systems in which these processes have been investigated.

Major Research Theme – Light Responses

Light is perhaps the most fundamental of all environmental cues in plants, beyond driving photosynthesis it serves as a physiological and developmental cue and at high levels induces stress. Plants have both daily and seasonal responses to light and melatonin has been found to serve as a signal of the seasons in plants. As may be expected, melatonin has been well reported to help stabilize photosynthetic pigments and the photosynthetic apparatus during seasonal senescence with 41 paper published on the topic of senescence, as well as delaying leaf abscission through interaction with the eponymous phytohormone abscisic acid (Wang et al., 2012). More intriguing is the seasonal variations of melatonin in vegetative tissues such as leaves over the season without dependence on maturity stage, supporting a role for melatonin in interpretation of light signals such as photoperiod (Korkmaz et al., 2017; Zhang H. et al., 2019). The literature shows that melatonin regulates the production of photosynthetic and defensive pigments, including chlorophylls (n = 120), anthocyanins (n = 23), carotenoids (n = 15), and betalain (n = 1) helping to maintain the photosynthetic apparatus under both light stress, and conditions such as temperature stress, which reduce efficiency of the photosynthetic apparatus. In animals, melatonin plays an important role in perception of light, or rather its absence, and is colloquially referred to as the chemical expression of darkness as it mediates circadian rhythms. As a result, interest in the capacity for melatonin to mediate circadian rhythms and light perception and responses in plants has been a significant area of research interest. Melatonin was first reported to have a daily rhythm in *Chenopodium rubrum* L. in 1997 (Kolar et al., 1997) and since has been found to show rhythmic (n = 13) and diurnal (n = 11) levels in: lupine (*Lupinus albus* L.; Hernandez-Ruiz and Arnao, 2008), water hyacinth (*Eichhornia crassipes* (Mart.) Solms; Tan et al., 2007a), sweet cherry (Zhao et al., 2013), eggplant (*S. melongena*; Korkmaz et al., 2017), rice (Wei et al., 2016), tomato (Arnao and Hernández-Ruiz, 2013), St. John's wort (Chung and Deng, 2020), and *Arabidopsis* (Li D. et al., 2020). Several melatonin metabolites have also been found to mimic melatonin expression patterns including 3-OHM (Choi and Back, 2019a) and AFMK (Tan et al., 2007a). Modulation of downstream pathways and functions associated with light dark cycles have been found to be affected by melatonin such as stomatal closure and sugar catabolism (Zhao et al., 2015). Wavelength of light is another important light signal which can be perceived by melatonin leading to downstream metabolic changes. Melatonin levels were found to have differential responses to red and blue light in *Scutellaria* species and in licorice (*Glycyrrhiza uralensis*; Afreen et al., 2006; Forsyth et al., 2020). Depending on concentration UV light can be both a signal and a stress inducer in plants, melatonin has likewise been found to have both functions, acting as an antioxidant and stress defense molecules as well as a signaling molecules. This is likely one of the reasons for which much more research has been undertaken in the UV range (n = 25) compared to visible light. Melatonin has previously been hypothesized to mediate plant responses through the constitutively overexpressed photomorphogenesis (COP)9 signalosome (Sanchez-Barcelo et al., 2016), an important mediator of ubiquitination and light-induced development or photomorphogenesis in plants. A recent in-depth study in *Arabidopsis* further supports this hypothesis showing that not only is the melatonin biosynthetic pathway upregulated and subsequently melatonin accumulated under UV-B exposure, but that melatonin mediated responses to UV-B exposure involves

interactions with COP1 and the transcription factors HYH and HY5 (Yao et al., 2021). Though the mechanism is not well understood, the authors hypothesize that UV-B receptor 8 (UVR8), the UV-B receptor, binds to COP1 activating HY5 and HYH and their downstream gene regulation networks (Yao et al., 2021). It is unclear where in this pathway melatonin is acting, as both *Arabidopsis cop1* and *hy5* mutants show decreased responses to UV-B exposure, however, this is particularly interesting since the chromophore in the UVR8 receptor is a series of repeating tryptophan residues (Lee, 2016).

Skotomorphogenesis is the redirection of plant movement in the absence of light. The role of melatonin in skotomorphogenesis is still in its early stages of investigation and has the potential to reveal new mechanisms. In rice melatonin has been found to play a role in this process in a brassinosteroid-dependent manner (Hwang and Back, 2018). Work in rice has found that SNAT, the enzyme which converts 5HT to NAS, melatonin treatment was found to induce brassinosteroid biosynthetic genes, including the gene for the rating limiting step in brassinosteroid biosynthesis DWARF4. Knock-out of SNAT lead to skotomorphogenetic effects (i.e., the opposite of photomorphogenesis) in dark conditions, including internode shortening and increased expression of light-inducible genes, which would normally be suppressed in the absence of light (Hwang and Back, 2018).

Major Theme – Interkingdom Communication

A unique and important feature of melatonin is its ubiquitous presence across all kingdoms of life, making melatonin an interesting candidate for mediation of interkingdom interactions. The ability for melatonin to mediate biotic stress defenses in plants has been extensively examined (n = 130), with melatonin being shown to improve plant resistance to fungal (n = 13), bacterial (n = 39), and viral infections (n = 9), through mechanisms including initial perception of threat, induction of signaling cascades and other phytohormone cascades, induction of innate immune response, and upregulation of defensive compounds. A growing area of research is the role melatonin may play in mediating symbiotic relationships between the plant root microbiome and plants (n = 4).

Melatonin acts as a signal to trigger downstream defense responses to pathogen challenge, particularly induction of defense-related genes and transcription factors (Lee et al., 2014a). Treatment with melatonin has been shown to be effective in increasing resistance of plants to viral, bacterial and fungal pathogens at low application levels (Moustafa-Farag et al., 2020) with the driving interest in this area being in the capacity for melatonin to improve resistance of agricultural crops with *Solanum* (n = 12), rice (n = 11), and tobacco (n = 7) being the most investigated species, followed by *Arabidopsis* (n = 6). Melatonin has been found to be able to induce defensive signaling pathways including ROS (n = 7) and NO signaling (n = 4), to upregulate phytohormones such salicylic acid (n = 8), jasmonic acid (n = 5), and ethylene (n = 3) and to increase production of defensive secondary metabolites such as lignans and polyphenols (n = 3). 2-OHM has also been reported to induce plant defenses similarly to melatonin (Lee and Back, 2019). Serotonin-phenolic conjugates are well described for their roles in the front-line mechanical defense against pathogen infection (Ishihara et al., 2008), particularly after wounding, and it has been recently hypothesized through untargeted metabolomics studies that melatonin may also form these conjugates (Erland et al., 2020a). Melatonin also upregulates transcription factors associated with plant defense and has been found to trigger plant innate immunity (Mandal et al., 2018), including pathogenesis-related (PR) proteins (n = 2) in response to pathogen challenge. Melatonin has also been found to induce overproduction of sugars and glycerol that are associated with plant resistance mechanisms in *Arabidopsis* (Qian et al., 2015). In some cases, pathogens have also been found to be capable of inhibition of host melatonin production, potentially enhancing melatonin susceptibility to infection, and indicating an important role for melatonin in pathogen response (Nehela and Killiny, 2018). This phenomenon has been investigated as an approach for enhancing efficiency of *Agrobacterium*-mediated transformation in recalcitrant species (Dan et al., 2015; Hou et al., 2019).

Not all interactions with prokaryotes are negative ones for plants and the function of melatonin in mediating symbioses is a new and exciting area of melatonin research. In recent years the importance of the plant microbiome has come to the forefront and has highlighted the important functions these symbioses play in improving plant health and performance, particularly under changing and stressful environmental conditions (Cordovez et al., 2019). Microbial symbionts are known to share amino acids and other nutrients with plant roots as well as to employ chemical signaling molecules, including phytohormones and NO to mediate these relationships (Whiteside et al., 2012; Cordovez et al., 2019). Melatonin is an important signaling and defense molecule in prokaryotic organisms as well as plants. Both tryptophan ethyl-ester and melatonin have been reported to be secreted by grapevine (*Vitis vinifera*, *Vitis labruscana* L.H. Bailey, and *Vitis amurensis* Rupr.) root bacterial symbionts, particularly the bacteria *Bacillus amyloliquefaciens* SB-9. In addition to secreting melatonin, *B. amyloliquefaciens* also induced melatonin biosynthesis in plant tissues through upregulation of transcripts for melatonin biosynthetic enzymes (Jiao et al., 2016). The authors also demonstrated that colonization of grapevine roots led to improved tolerance to drought and salinity stress, and reported a reduction in markers of antioxidant stress. This suggests that melatonin is an important signaling molecule mediating the symbiosis and this may be a mechanism by which bacterial symbionts improve plant stress tolerance (Jiao et al., 2016). Follow-up studies using the grape (*V. vinifera*) endosymbiont *Pseudomonas fluorescens* RG11, further demonstrated that conversion of tryptophan to melatonin using N15 labeled tryptophan in *P. fluorescens*, possibly via a tryptamine independent biosynthetic route, and demonstrated significant cross-talk between grape and bacterial melatonin biosynthetic pathways (Ma et al., 2017). Melatonin has also been found to be involved in arbuscular mycorrhizal (AM) symbioses in legumes (Zhang et al., 2020). In alfalfa, treatment with the AM *Rhizophagus irregularis* increased melatonin levels

in plants, particularly upon exposure to lead stress, which was associated with increased expression of ASMT, the final enzyme in the melatonin biosynthetic pathway (Zhang et al., 2020). Increased melatonin levels led to improved plant performance through enhanced antioxidant function and reduced uptake of lead (Zhang et al., 2020). Similarly, co-application of plant growth-promoting bacteria have also found to have synergistic effects with melatonin including in faba bean (*Vicia faba* L.) and spinach (*Spinacia oleracea* L.), and have been found to improve performance under abiotic stress (Asif et al., 2020; El-Ghany and Attia, 2020). Research into a greater number of species of both plants and symbionts will help to determine how widespread these interactions are, as well as the potential signaling pathways involved. This is likely to have a significant impact on our understanding of interkingdom melatonin signaling dynamics.

The possible trafficking of melatonin between symbionts or pathogens and melatonin dates back to the first reports of melatonin in plants, where the presence of melatonin in plants was distinguished from the possibility of microbial production and contamination through the use of *in vitro* culture (Murch et al., 2000). In this way the use of *in vitro* and sterile culture has been, and will continue to be, an essential strategy in understanding and differentiating the role of melatonin in plants vs. the role of melatonin as an interkingdom signal. While more than 60 papers specify having used *in vitro* culture systems, another 28 and 7, have used field and greenhouse studies, respectively (**Figure 9B**). As a better understanding of the role of endosymbionts play in plant melatonin levels and functions it will be interesting to see the potential impacts of this knowledge on the existing body of literature on melatonin. This is particularly true of root symbionts which are well known for their ability to improve plant performance under stressful or low nutrient conditions, modification of root architecture, as well as improving crop traits such as yield and mass accumulation, all of which have also been associated with melatonin.

Though still an upcoming area of research, melatonin has also been found to be a signal mediating responses to wounding and insect feeding, and to mediate responses between pathogens, insect damage and plant resistance ($n = 3$). American elm (*Ulmus americana* L.) populations have been decimated by infection with dutch elm disease (*Ophiostoma ulmi*), which is a fungal pathogen whose infection is greatly facilitated by elm beetle (*Scolytus multistriatus* Marsham) feeding (Saremba et al., 2017). Resistance to the disease has been found to be dependent on appropriate balances of jasmonic acid and salicylic acid levels in the tissues as insect feeding and pathogen challenge induce antagonistic phytohormone cascades in plants. Melatonin has been proposed as one of the initial signals which signals feeding damage in plants, and which may play a role in resistance through interaction with jasmonic acid and salicylic acid signaling (Sherif et al., 2016, 2017).

Major Research Theme – Protection of Germ Cells and Tissues

The first plant organs in which melatonin was quantified were reproductive tissues, and since these initial reports of melatonin in reproduction have continued to be by far one of the most active areas of melatonin research. More than 258 papers have investigated melatonin in seeds or nuts, and 78 in fruit, with more than half quantifying melatonin levels up to μg/g fresh weight. There is a clear role for melatonin in both the defense and direction of reproductive tissues in plants. Melatonin has also been found to have a function in pollen and microspore development ($n = 2$). Protection and defense of reproductive tissues is essential to maintain their integrity and ensure survival of populations and species. Melatonin plays an essential role in detoxifying both ROS and ammonium ions in seeds and embryos, and may also be important in controlling the progression of embryo development ($n = 11$). There appears to be a strong pattern of melatonin production with embryo development and fruit ripening, with a trade-off between melatonin and 5HT, with melatonin levels increasing during early embryo development and later stages seeing a shift toward higher 5HT content (Murch et al., 2009, 2010). In *Datura metel*, this pattern could be disrupted by cold exposure which induces a spike in melatonin levels (Murch et al., 2009). A transient spike in melatonin during seed development has also been reported in rice, which had a spike in melatonin levels associated with increased expression of melatonin biosynthetic genes in the panicle during flower development (Park et al., 2013b). In sweet cherry (*Prunus avium*) melatonin levels increased later in the season, which authors attributed to defense against high light stress and increased ROS load in the tissues (Zhao et al., 2013). Melatonin has also been found to accumulate in pollen in *Hypericum perforatum* (Murch and Saxena, 2002), and to prevent high temperature-induced pollen abortion in tomato (Qi et al., 2018).

Melatonin mediates flowering and floral timing ($n = 18$), and has been found to improve fruit and grain set, and thus yield ($n = 62$). These responses have been strongly tied to light, with melatonin potentially serving as an important signal of light quality to mediate these processes. The first plant in which this was reported is *Chenopodium rubrum* L., where exogenously applied melatonin reduced flowering when applied in the pre-during or first half of the dark phase (Kolar et al., 2003). In *Arabidopsis* the capacity for melatonin to mediate flowering has been linked to its capacity to stabilize DELLA proteins, negative regulators of gibberellins and flowering locus c, and lead to delay of flowering (Shi et al., 2016). Follow-up studies have shown that inhibition of endogenous melatonin biosynthesis by knockout of SNAT delays flowering (Lee et al., 2019), while strigolactone treatment has also been found to reduce melatonin levels leading to delay in flowering in *Arabidopsis* (Zhang Z. et al., 2019). In apple trees, melatonin levels have been found to respond to light levels in the field, particularly higher far-red and blue light levels, and that a drop in melatonin levels was associated with induction of flowers. Additionally, it was found that application of exogenous melatonin could lead to delay of flowering (Zhang H. et al., 2019). The capacity for melatonin to moderate reproductive growth has also been exploited for the improvement of postharvest quality of diverse fruit species. Melatonin has been reported to delay postharvest senescence and maintain quality of many fruits including tomato ($n = 3$), peach ($n = 3$), pear ($n = 3$), strawberry ($n = 3$), banana ($n = 2$),

mango (n = 2), bamboo (n = 1), citrus (n = 1), kiwi (n = 1), and grapes (n = 1). This has been associated with modifications to carbohydrate and starch metabolism (n = 8), inhibition of ethylene and ethylene associated signals (n = 6) as well as melatonin's antioxidant function (n = 5).

Postharvest melatonin levels have been reported to start to decrease in fruits and are possibly related to the shift in physiological function from embryo development to embryo maintenance and protection through germination. During seed germination melatonin levels have also been found to remain high and to subsequently drop as the plant matures (Korkmaz et al., 2017). Increased melatonin levels have been strongly associated with improved germination (n = 47) in more than 20 species, particularly under abiotic (n = 10) and biotic (n = 10) stress including salinity (n = 13), temperature (n > 10), drought (n = 6) and metal stress (n = 7). This has been associated with mechanisms including modulation of abscisic acid (n = 4), antioxidant function (n = 14) and induction of ROS signaling cascades (n = 4). Several in-depth transcriptomics studies have been undertaken (n = 8) detailing the transcriptomic cascades involved in these processes and have highlighted the roles of general pathways including energy metabolism, signal transduction, redox status and root development. Suppression of jasmonic acid biosynthesis (Hu et al., 2020) has also been found to be essential to improved germination of melon seedlings under copper stress, while melatonin was found to regulate sodium and chloride ion accumulation in rice seedlings experience salinity stress (Li X. et al., 2017). Several hydroxylated melatonin metabolites including 2-OHM (n = 2), 3-OHM (n = 2) and cyclic hydroxymelatonin (n = 2) are also active in improving seed germination.

Major Research Theme – Phytohormone and Plant Signaling

Plant signaling mechanisms are less well understood than other organisms and are difficult to study due to the complexity of cell to cell communication. Melatonin is highly conserved through evolution as a fundamental signaling molecule across all forms of life. Melatonin signaling dynamics are still an evolving and exciting area of research in plants. Numerous plant signaling cascades have been hypothesized to be mediated by melatonin, including MAPK (n = 12), COP signalosome (n = 2), ROS/H_2O_2/NADPH oxidase signaling (n = 37), nitric oxide signaling (n = 26), Ca^{2+}/CaM (n = 28), as well as interactions with phytohormones including auxin (n = 53), cytokinins (n = 6), salicylates (n = 21), jasmonates (n = 26), gibberellins (n = 9), strigolactones (n = 3), brassinosteroids (n = 6), abscisic acid (n = 36), polyamines (n = 12) and ethylene (n = 36). Melatonin mediates gene expression through these interconnected signaling cascades as well as mediation of microRNAs (n = 3) and transcription factors (n = 36). As with any growing field, much interest has been paid to the final effect and function of melatonin in plants; however, melatonin mediated signaling networks are the basis of melatonin function in plants and a better understanding of how plants use melatonin as a signaling molecule and how plants perceive both endogenous and applied melatonin will be fundamental in advancing the topic.

The search for plant melatonin receptors and melatonin-interacting proteins in plants is ongoing. In 2019 the first melatonin receptor CAND2-PMTR1 was described in *Arabidopsis* and reported to mediate stomatal closure in response to stress (Wei et al., 2018). The protein is a G-protein coupled receptor (GPR)50 type receptor, a group of orphan melatonin receptors and was identified based on its structural homology. The original article describes the characterization of the protein through the use of *Arabidopsis* mutant lines and binding kinetics between the protein AtCand2 and iodomelatonin (Wei et al., 2018). The authors report that loss of a functional receptor in the mutant lines leads to quenching of H_2O_2 production and calcium influx and propose that binding of the receptor with melatonin mediates stomatal closure (Wei et al., 2018). The protein has also been found to possess rhythmicity, which the authors associated with the previously described control of stomatal closure via ROS based signaling (Li D. et al., 2020). These results have, however, since been disputed, with efforts to repeat experiments on the receptor using transgenic, confocal microscopy (localization) and bioactivity studies being unsuccessful (Lee and Back, 2020). GPR50 proteins have previously been found to inhibit function of MT1 and MT2 receptor subtypes in animals through heterodimerization, therefore it is possible that while CAND2-PMTR1 maybe not be an authentic plant melatonin receptor, it may still be important in mediating melatonin signaling (Levoye et al., 2006). Several other melatonin interacting proteins have been reported in plants the first of which is Hyp-1, a PR 10 protein from *H. perforatum* which was shown in crystallography studies to form a complex with melatonin (Sliwiak et al., 2016). An analog has also been isolated in lupine, and was found to form a complex with both melatonin and the cytokinin zeatin (Sliwiak et al., 2018a,b). Overexpression of Hyp-1 in both tobacco (*N. tabacum*) and lettuce (*Lactuca sativa*) found that Hyp-1 localized to the nucleus, plasma membrane and cytoplasm of epidermal cells and was found to confer resistance to bacterial infection with *Agrobacterium tumefaciens* (Hou et al., 2019). Together these results suggest that Hyp-1 may be a mediator of melatonin-phytohormone cross talk to control responses to biotic challenge.

One common downstream target of initial signaling cascades is induction or repression of transcription factors. Thirty-six papers have been published which mention "transcription factor" in the title and abstract, and include numerous classes of stress-associated transcription factors including: MYB (n = 10), DREB (n = 10), bZIP (n = 5), ZAT (n = 4), WRKY (n = 11), bHLH (n = 3), NAC (n = 7), and HSFs (n = 14). Transcription factors associated with other phytohormone networks have also been reported to be induced by melatonin including the auxin response genes AUX/IAA and ethylene response factors (ERFs). Many of these transcription factors have been identified through transcriptomics studies where specific genes are generally not specified in the abstract, thus being excluded from our query. A subset have been characterized through RT-qPCR and fewer still having been validated using overexpression or knockout mutant lines (generally in *Arabidopsis*). These transcription

factors have been best characterized in plant stress responses with 58% of the queried studies mentioning "stress." The melatonin metabolite 2-OHM has also been found to be able to induce transcription factors including Myb4 and AP37 (Lee and Back, 2016b), further demonstrating important biological activities of melatonin metabolites in plants. Activation of transcription factors which then trigger downstream transcriptional activity is clearly an important function of melatonin, however, the upstream signaling mechanism which lead to induction of these transcription factors is still not known. Hopefully with the increasing interest in identification of melatonin interacting proteins this area will continue to grow.

In plants one of the first signaling cascades proposed to be induced and essential for melatonin action in plants is induction of calcium/calmodulin (Ca^{2+}/CaM) signaling cascades. Melatonin is well characterized for its interaction with calcium signaling cascades in animals and is the basis for one of the first investigations of melatonin function in plants. Banerjee and Margulis (1973) reported that melatonin had "colchicine-type disruption" in onion root tips, inhibiting mitotic spindle development (Banerjee and Margulis, 1973). Colchicine is a well characterized anti-mitotic drug which inhibits calcium influx into cells leading to microtubule disruption and the authors suggest a similar mechanism for melatonin (Banerjee and Margulis, 1973). Later studies *in vitro* reported that melatonin did in fact mediate cytoskeletal rearrangements through antagonism of calmodulin using an *in vitro* mammalian system (Huerto-Delgadillo et al., 1994). A later report investigated thidiazuron-induced morphogenesis in *Echinaceae purpurea* L. suggested interaction between melatonin and calcium, as treatment with calcium transport inhibitors lead to an increase in melatonin content (Jones et al., 2007). The first direct report of dependence of melatonin action in plants was reported in 2009, where melatonin induced shoot morphogenesis in *Mimosa pudica* L. (Ramakrishna et al., 2009). These effects were found to be inhibited by treatment with the calcium channel blocker verapamil, or addition of calcium chelators to the medium (Ramakrishna et al., 2009). Since then the importance of calcium signaling continues to be demonstrated with more than 28 papers included in our query that mention calcium. While the importance of calcium in mediating plant action potentials has been established for some time (Beilby, 1984), there have been significant recent developments in the tools and transgenic lines available for the investigation of calcium signaling in the past 10 years and the importance of rapid calcium action potentials in plants continues to grow. To date several reports have described melatonin- Ca^{2+}/CaM cross-talk in plant stress (n = 14) responses including salinity (n = 7), temperature (cold n = 3; heat n = 2), drought (n = 3) and heavy metals (n = 4). Given the dependence of melatonin on calcium signaling this is an area which should be a future focus.

Another signaling pathway which is highly coordinated with calcium signaling but a relatively recent development in the understanding of melatonin signaling pathways is its ability to mediate ROS signaling cascades. Melatonin has been found to induce rapid ROS signaling through interaction with the NADPH oxidase; respiratory burst oxidase homologue (RBOH), which catalyzes production of hydrogen peroxide via superoxide as an oxidation product of NADPH (Lee and Back, 2017). The importance of ROS and especially H_2O_2 in mediating plant responses is now well established in the literature functioning in morphogenesis, abiotic and biotic stress responses and associated physiological functions such as stomatal closure. The capacity of melatonin as an antioxidant, coupled with the growing body of literature on the important of ROS and especially H_2O_2 as a signaling molecule, led to interest in the interaction between the two pathways. This is an area of significant growing interest with 31 papers now published which mention "NADPH oxidase" or "RBOH" in diverse species including *Solanum* sp. (n = 7), particularly tomato (*S. lycopersicum*, n = 2), *Arabidopsis* (n = 4), corn (n = 3), rice (n = 3), wheat (n = 3), cucumber (n = 2), tobacco (n = 2), and pear (n = 2). ROS signals trigger diverse downstream pathways in plants, including many of the signaling cascades found to interact with melatonin including stress-associated transcription factors, MAPK, calcium signaling, and phytohormones in response to biotic and abiotic stresses (Miller et al., 2009; Gong et al., 2017). The H_2O_2 signals generated by RBOH have been found to occur rapidly and at the systemic scale traveling at rates of 8.4 cm/min (Miller et al., 2009). RBOH signaling exists in signaling loops which balance maintenance of sufficient levels of ROS to continue the self-propagating signal with detoxification by antioxidant systems in so called ROS signaling loops (Wrzaczek et al., 2013). Melatonin acts as an upstream signal triggering RBOH dependent H_2O_2 production in response to abiotic stresses including temperature, salinity and drought stress, leading to upregulation of genes associated with downstream stress-associated signaling cascades including MAPK, calcium dependent protein kinases (CDPK), heat shock proteins (HSP) and stress-associated transcription factors (Gong et al., 2017). Melatonin induction of H_2O_2 accumulation has also been described in the calcium-dependent control of lateral root formation in *Arabidopsis* and alfalfa (*Medicago sativa* L.; Chen et al., 2018) and seed germination in *Arabidopsis* and melon (*Cucumis melo* L.), where melatonin inhibits abscisic acid function through calcium-dependent activation of RBOH leading to H_2O_2 accumulation (Li H. et al., 2020). The importance of ROS and RBOH-mediated melatonin signaling has since also been established in response to biotic stress, with melatonin induction of RBOH and downstream MAPK/MKK signaling cascades in response to pathogen challenge (Lee and Back, 2017).

Mitogen-activated protein kinase serine/threonine kinases are one of the oldest signaling pathways known and are used by eukaryotic and prokaryotic organisms for the perception of environmental stimuli and control plant growth and development. They function downstream of receptor-like protein kinases (RLKs) leading to phosphorylation and induction of MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase) and finally MAPK/MPK which triggers transcription factors responsible for ultimate gene expression or secondary signal transduction pathway activation (Xu and Zhang, 2015). Melatonin-mediated responses to both biotic (n = 5) and abiotic (n = 5) cues, have been found

to be dependent on MAPK signaling cascades (n = 11). Induction of MAPK by melatonin treatment was first reported in *Arabidopsis* and tobacco where they were found to have differential expression patterns by species. In *Arabidopsis* MPK3 and MPK6 were induced by melatonin, NAS or 2-OHM treatment. Knockout experiments identified MKK4, 5 and 7 to be the upstream MAPK kinases (MKK) mediating this response which lead to upregulation of genes associated with pathogen response and innate immunity including PRs, though induction of Hyp-1 interestingly has not been investigated (Lee and Back, 2016a). Both H_2O_2 and NO signaling have also been found to be activated by MAPKKK3 in *Arabidopsis* defense responses downstream of RBOH (Lee and Back, 2017), which may be triggered by melatonin-receptor binding, for example with PMTR1 (Lee and Back, 2020; Li D. et al., 2020), or potentially via interaction with the PR protein Hyp-1 though this hypothesis has yet to be investigated.

Involvement of NO signaling cascades has been reported for melatonin-induced root development (n = 9) and resistance to biotic (n = 7) and abiotic (n = 7) stress including bacterial (n = 3) and viral infection (n = 1), metal toxicity (n = 9), salt stress (n = 5), nutrient deficiency (n = 3), heat (n = 1), high light (n = 1), and maintenance of postharvest quality (n = 1). NO signaling in plants mediates diverse plant physiological processes, however, the structure of its generation and signaling cascades are still an area of active investigation with a common target being post-translational modification of proteins in plants (Hancock and Veal, 2020). Not only has melatonin has been found to induce NO signaling, but the physiological effects of melatonin have also been found to be dependent on induction of this signal. The downstream targets of melatonin-induced NO signals are not, however, well described. Melatonin and NO signaling interactions have been found to be associated with other signaling cascades including ROS and phytohormone signaling cascades, including ethylene (Liu et al., 2019), auxin (Wen et al., 2016), and polyamines (Ding et al., 2018), are clear. Coordination between NO and H_2O_2 has also been found to be important in biosynthesis of melatonin in responses to stress stimuli (n = 7). For example, in rice both NO and H_2O_2 are required for activity of melatonin biosynthetic enzymes, and apparently also for trafficking of the intermediates between cellular compartments in response to cadmium exposure (Lee et al., 2017).

Melatonin is now well accepted as a master regulator and mediator of many other phytohormone networks. It plays a central role to balance, fine-tune and direct more classical phytohormone signaling cascades with the particular action and response varying depending on the function (Erland et al., 2015; Arnao and Hernández-Ruiz, 2018a, 2020). Melatonin may drive the balance between well-known phytohormone pairs including auxins and cytokinins in morphogenesis (Murch et al., 2001) and jasmonic acid and salicylic acid in responses to pathogen and insect attack (Sherif et al., 2016). Melatonin interaction with auxin in particular, and in interaction with ethylene (n = 11), polyamines (n = 4), jasmonic (n = 9) and abscisic acid (n = 8), as well as interactions with Ca^{2+}/CaM (n = 11), H_2O_2 (n = 37), and NO signaling (n = 9) have been found to be essential to melatonin-mediated effects on root growth. Interactions between zeatin and melatonin, potentially at the Hyp-1 protein may be important in pathogen defense (Sliwiak et al., 2016), while interaction with gibberellin (n = 2) and abscisic acid (n = 4) mediates seed germination.

An interesting novel signaling mechanism for melatonin is its interaction with lipid signaling. Melatonin has been found to mediate fatty acid composition in various species, which has been primarily investigated with respect to membrane integrity in response to abiotic stress (n = 15). Melatonin has recently been reported to be a regulator of endoplasmic reticulum (ER) stress (Ozgur et al., 2016) and to mediate phospholipid levels (Yu et al., 2018). Phospholipids are synthesized and trafficked between the chloroplast and ER where they can induce signaling cascades which have previously been described to be induced by treatment with the melatonin precursor 5HT, mimicking red light exposure (Chandok and Sopory, 1994). Melatonin has been reported to increase both phosphatidic acid (PA) and phosphatidylinositol (PI) levels in *Ipomea batatas* (L.; Yu et al., 2018), and several reports have found that melatonin levels are increased in response to red light exposure (Forsyth et al., 2020) suggesting that perhaps a similar mechanism could occur for melatonin. This will certainly be an exciting novel area of plant melatonin research.

DISCUSSION

Bias in the Literature

A significant, but to date, unacknowledged bias exists within plant melatonin literature. To investigate our anecdotal observations of bias we undertook a network analysis of all of the original research papers meeting our search terms, and which possess a DOI to identify the papers which are the most influential in the field of melatonin literature as a function of their citation network (**Figure 10A**). An examination of the papers with highest network impact (as defined by degree > 100, i.e., min 100 papers cited or citing; **Figure 10**) was then undertaken. Our results show poor agreement between our qualitative keystone papers and the papers with highest network importance, suggesting that the papers with the greatest influence on the literature are not those which have made keystone discoveries in the field. As the network was created using DOI as the identifier for papers, papers which do not have a DOI were excluded from the analysis and this is responsible for the absence of several of these keystone papers including Hattori et al. (1995), which was one of two papers first quantifying melatonin in foods, Murch et al. (1997), the first report of melatonin quantification in vegetative plant materials and Jackson (1969), the first report of melatonin activity in the plant system. To further investigate what factors are driving high network importance we examined the journal title and topics in which these papers were published. The keystone papers show a generally well distributed list of publication titles, while the high network importance papers are heavily skewed to publication in

the Journal of Pineal Research (JPR) with 76% of High Network Importance but only 33% of the Keystone Papers, while an even lower proportions of all papers, only 20%, are published in JPR (**Supplementary Figure 2**). Interestingly, journals such as the Journal of Experimental Botany (JXB) which represent the next greatest proportion of high impact papers 9% is not included in the keystone papers and represents only a fraction of all papers. Examination of the author network reveals several author collaborator research hubs.

CONCLUSION

Plant melatonin research is a rapidly expanding field of research and will continue to be an active area which will have applications for ecosystem function, agriculture and human health. We have identified several areas of bias within the literature, and highlighted key gaps. In particular, future studies should investigate non-medicinal and non-crop species as well as emphasizing not just the function of melatonin, but also the mechanisms of melatonin action, as well as characterization of melatonin signaling cascades, melatonin receptors and melatonin interacting proteins represent new opportunities in plant melatonin research.

AUTHOR CONTRIBUTIONS

LE and SM participated in design, analysis, presentation, and writing of manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.683047/full#supplementary-material

Supplementary Appendix | Species in which melatonin has been investigated, listed in order by family. The top 10 most investigated species are shaded. A total of 236 species and 94 families have been investigated.

Supplementary Data 1 | Complete bibliography with original research articles included in this study and query terms.

Supplementary Data 2 | Complete bibliography of review articles included in this study and query terms.

Supplementary Data 3 | Complete list of species queries in original research articles included in this study.

Supplementary Figure 1 | Country of corresponding author for original research articles, where country is specified filtering for countries with > 5 publications. Numbers in legend indicate number of publications.

Supplementary Figure 2 | Top journals publishing plant melatonin research by **(a)** highest network importance, **(b)** Keystone papers included in **Figure 1**, and **(c)** all original research articles.

Supplementary Figure 3 | Top papers included in DOI network (in degree cut-off of 100) with numbers in table corresponding to node numbering also displayed in **Figure 10**.

Supplementary File 1 | Summary of methods used for literature review.

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Roles of Endogenous Melatonin in Resistance to *Botrytis cinerea* Infection in an *Arabidopsis* Model

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Melatonin is an important bioactive molecule in plants. Two synthetases, N-acetylserotonin methyltransferase (ASMT) and serotonin N-acetyltransferase (SNAT) are involved in the final two steps of melatonin synthesis. Melatonin participates in responses to a variety of biotic and abiotic stresses in plants, but few studies have addressed the roles of endogenous melatonin in pathogen resistance. We investigated the role of endogenous melatonin in resistance to *Botrytis cinerea* infection in an *Arabidopsis thaliana* model system. Plant lines that overexpressed *ASMT* or *SNAT* through genetic manipulation showed upregulated expression of resistance genes *PR1* and *PR5*, transcription factor gene *WRKY33*, and jasmonic acid (JA) defense pathway marker gene *PDF1.2*, and downregulated transcription factor gene *MYC2* in JA signaling pathway. Higher melatonin content also enhanced the activity of antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD), increased JA content, reduced plant disease symptoms, and reduced lesion size in leaves. These findings indicate that endogenous melatonin enhances plant resistance to *B. cinerea* infection. In contrast, *ASMT* and *SNAT* gene silencing lines showed opposite results and were more susceptible to *B. cinerea*. Thus, it can be demonstrated that melatonin functions as an effective regulator of plant stress resistance at the genetic level. A schematic model is presented for its role in resistance to *B. cinerea* infection. Our findings also helped to elucidate the associated signal transduction pathways and interactions between melatonin and other plant hormones.

Keywords: melatonin, *Botrytis cinerea*, *Arabidopsis thaliana*, N-acetylserotonin methyltransferase, serotonin N-acetyltransferase

INTRODUCTION

Melatonin is a signaling molecule that ubiquitously exists in animal (Lerner et al., 1958) and plant (Dubbels et al., 1995) cells, and its synthesis pathways in plants have been discovered (Back et al., 2016). Serotonin N-acetyltransferase (SNAT; Kang et al., 2013) and N-acetylserotonin methyltransferase (ASMT; Park et al., 2013), as the main enzymes in the synthesis pathway, directly determine the endogenous melatonin level. The function of melatonin in plants has been widely reported (Fan et al., 2018; Sun et al., 2020). It can respond to various

biotic (Mandal et al., 2018; Zhao et al., 2021) and abiotic (Chen et al., 2017; Yao et al., 2020) stresses to resist the influence of environmental changes during plant growth and development. The immune response of melatonin in plants requires the participation of many signaling molecules, such as reactive oxygen species (ROS; Pardo-Hernández et al., 2020) and Nitric Oxide (NO; Zhu et al., 2019), to transmit both intracellular and intercellular signals. In the field of biotic stress, melatonin is involved in plant resistance to numerous fungus (Moustafa-Farag and Almoneafy, 2019). The growth of certain plant fungi, including *Alternaria* spp. and *Fusarium* spp., is inhibited by treatment with melatonin. Increased melatonin levels in plants enhance resistance to *Sphaerotheca fuliginea* and oomycetes, and sensitivity to *Phytophthora infestans* (Zhang et al., 2017; Mandal et al., 2018). Melatonin pretreatment increases the resistance of apple (*Malus prunifolia*) to Marssonina apple blotch (*Diplocarpon mali*), by promoting expression of chitinase genes, regulating hydrogen peroxide (H_2O_2) and pathogenesis-related proteins (PR proteins; Yin et al., 2013). In banana (*Musa acuminata*), melatonin treatment induces the production of defense-related plant hormones [IAA, salicylic acid (SA), JA, ethylene] by regulating the expression of *MaHSP90*, thereby enhancing resistance to Fusarium wilt (Wei et al., 2017).

Botrytis cinerea often termed "gray mold," is a necrotrophic pathogenic microorganism (fungus) responsible for major economic losses (related to its wide hosting range) considered second only to those of penicillin fungal pathogens (Dean et al., 2012). It kills host cells by secreting the sesquiterpene metabolite botrydial, and by producing ROS that induces oxidative outbreaks (Feng and Shan, 2014). *Botrytis cinerea* also degrades pectin in plant cell walls by synthesizing polygalacturonase, keratin enzyme, and cell wall degradation enzyme, thereby promoting invasion and damage to the plant (Liu et al., 2017). Local immune responses of plants to *B. cinerea* infection result in integration and expression of PR proteins, steady-state regulation of plant hormones, ROS production, and accumulation of secondary metabolites such as JA (Pieterse et al., 2009).

Plant hormone signal transduction is an important component of the local immune response, and JA in particular plays a crucial role in defense against *B. cinerea* (AbuQamar et al., 2017). In *Arabidopsis*, a JA-knockout mutant (*coi1*) defective in the perception of JA signal shows higher susceptibility to *B. cinerea* (Pingping et al., 2017). Transcriptional coactivator mediator subunit 25 (Med25) promotes plant resistance to *B. cinerea* by inducing the expression of the JA-dependent defensive gene (An and Mou, 2013). The invasion of *B. cinerea* to plants activates the expression of a marker gene of the JA pathway (plant defensin 1.2; *PDF1.2*; Frey et al., 2018). Conversely, *MYC2* plays a negative regulatory role in JA-mediated immunity and it interacts with JAZ [jasmonate zinc-finger inflorescence meristem (ZIM) domain] to promote JA decomposition, thereby reducing *MYC2* expression following *B. cinerea* infection (Liu et al., 2019). Transcriptome analyses indicate the alteration of thousands of transcripts in the body of an amoeba host following *B. cinerea* invasion, whereby mitogen-activated protein kinases (MAPKs) undergo phosphorylation changes during transcription of downstream genes, in which key transcription factors are involved in the regulation of plant defense responses (Mulema and Denby, 2012). WRKY proteins are major components of transcription factors that play essential roles in both PAMP triggered immunity (PTI) and Effector triggered immunity (ETI) during plant resistance to *B. cinerea* infection (Jones and Dangl, 2006). WRKY proteins regulate defensive responses to biotrophic and necrotized pathogens (Chen et al., 2018). WRKY33 also participates in the regulation of its expression through a clear feed-forward mechanism in combination with its promoter in *B. cinerea*-infected plants (Mao et al., 2011).

We investigated the role of endogenous melatonin in resistance to *B. cinerea* infection in an *Arabidopsis thaliana* model. Using genetic manipulation techniques, the expression of *SNAT* and *ASMT* was altered. After *B. cinerea* infection, we found that *SNAT* and *ASMT* overexpressed lines exhibit obvious disease resistance characteristics compared with Columbia (Col-0). On the contrary, disease-resistant ability in *SNAT* and *ASMT* mutant lines was weakened. The results clarified that melatonin can improve plant disease resistance to the stress of *B. cinerea* by regulating the expression of related genes and the content of phytohormone JA. These pieces of evidence further prove the role of melatonin in the field of biotic stress, especially for enhancing plant resistance to fungal invasion.

MATERIALS AND METHODS

Plant Material and Pathogen Inoculation Procedure

Arabidopsis thaliana ecotype Col-0 was used in this study. Plants were grown for 4 weeks at 22°C under long-day conditions (16 h of light/8 h of dark) and light intensity 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

The *B. cinerea* (B05.10) used in this study was cultivated on potato dextrose agar (PDA) medium (Zheng et al., 2015) in the dark for 5 days at a 28°C incubator to induce conidia production. Spores were collected and then suspended in 2% glucose solution to dilute to 1×10^5 conidia/ml. Leaves (3–4 per plant) were added dropwise with 10 µl spore suspension, and kept in the dark for 24 h and then transfer to 16 h light/8 h dark condition and light intensity 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Obtain Transgenic Plants

Using the technique of artificial miRNA (amiRNA; Schwab et al., 2006), we successfully constructed miR172-*snat* and miR172-*asmt* vectors. Then they were separately transferred into Col-0 to silence melatonin synthesis genes *via* the *Agrobacterium*-mediated transformation method. Seeds were selected by adding 50 mg/ml kan and 30 mg/ml cef in 1/2 MS medium and T3 generation transgenic lines (*asmt-1*, *asmt-2*, *snat-1*, and *snat-2*) were used in the following experiments. Genes overexpression lines (*ASMT-OE-1*, *ASMT-OE-2*, *SNAT-OE-1*, and *SNAT-OE-2*) were constructed in our laboratory by inserting *SNAT* and *ASMT* gene complementary DNA (cDNA) into the binary vector pRI101-*AN* (TaKaRa, Tokyo, Japan).

Quantitative Real-Time RT-PCR Analysis

After inoculation with *B. cinerea* strains for 48 h, total RNA was isolated from leaves using RNeasy Plant Mini Kit (TaKaRa, Tokyo, Japan), then reverse-transcribed to cDNA with the PrimeScript™II1st strand cDNA Synthesis kit (TaKaRa, Tokyo, Japan). The expressions of relevant genes were evaluated by quantitative real-time RT-PCR (qRT-PCR; model CFX96; Bio-Rad).

The *Actin8* (AT1G49240) genes were used as internal controls. Each experiment was repeated at least three times.

Determination of Activities of Antioxidant Enzymes Superoxide Dismutase and Peroxidase

After *B. cinerea* treatment for 24, 48, or 72 h, plant leaves (0.1 g) were ground separately in liquid nitrogen, incubated with 1.5 ml of phosphate buffer (PB; pH 7.8), and centrifuged (4,000 rpm, 15 min). The supernatant was taken as the solution to be tested.

Superoxide dismutase (SOD) and peroxidase (POD) activity was determined following Zang et al. (2015).

Quantification of Melatonin and JA Content

Plant tissues (0.1 g) treated by *B. cinerea* for 24, 48, or 72 h were grounded in liquid nitrogen and added 900 μl 0.01 M PBS (pH 7.4), then ultrasonicated for 30 min and centrifuged (12,000 rpm, 10 min)at 4°C, The supernatants were ready to measure the content of melatonin and JA.

Melatonin content was quantified using the Melatonin Enzyme-Linked Immunosorbent Assay Kit (Jianglai, Shanghai, China), with absorbance read at 450 nm. JA content was determined using the Plant JA ELISA KIT (Jianglai, Shanghai, China) containing anti-JA polyclonal antibodies, with absorbance read at 490 nm. All measurements were performed in triplicate with samples collected from three biological replicates.

Trypan Blue Staining

Leaves treated by *B. cinerea* after 3 days were stained with 0.4% (w/v) Trypan Blue solution for 2 h at 37°C, and chlorophyll was eliminated with 95% ethanol. The samples were observed under a dissecting microscope (Nikon C-LEDS; Nikon, Tokyo, Japan).

Determination of Malondialdehyde Content

Following *B. cinerea* treatment for 24, 48, or 72 h, plant tissues (0.1 g) were ground in liquid nitrogen and added with 900 μl of PB. After centrifugation (4,000 rpm, 10 min), 50 μl of the supernatant was used as the reaction solution and assayed with an malondialdehyde (MDA) kit (Jiancheng, Nanjing, China). The absorbance of the supernatant was detected at the wavelengths of 532 nm.

Leaf Damage Area Calculation

Leaves of plants treated with *B. cinerea* for 4 or 6 days were removed and photographed. Leaf damage area data were taken for the 6-day-treated plants and statistically analyzed using Image J 1.52 s software program (NIH Image, Bethesda, MD, United States).

Statistical Analysis

Analyses were performed using the Origin software program V. 8.0, with values expressed as mean ± SE. Differences between means were evaluated by one-way ANOVA and considered significant for $p < 0.05$.

RESULTS

Endogenous Melatonin Alters Stress Response in *B. cinerea*-Infected Plants

To evaluate the role of endogenous melatonin in response to *B. cinerea* invasion and regulation of plant defensive signaling pathways, we analyzed the expression of resistance genes *PR1*, *PR5*, *PDF1.2*, *MYC2*, and *WRKY33* (**Figure 1**). Spore suspension droplets were taken from leaves of *B. cinerea*-infected plants at 48 h. *PR1* expression was significantly higher in the overexpression lines (most notably *ASMT-OE-1*) than in Col-0. In contrast, its expression was lower in the gene silencing lines (most notably *asmt-1*; **Figure 1A**). Expression of *PR5*, another important gene in defensive responses, was similarly increased in overexpression lines following *B. cinerea* infection (**Figure 1B**). Expression of *PDF1.2*, an important gene in the JA-dependent pathway, was increased 37-, 4.8-, 7-, and 2.8-fold (respectively) in *SNAT-OE-2*, *ASMT-OE-1*, *ASMT-OE-2*, and *SNAT-OE-1* relative to Col-0. In contrast, its expression was lower in *asmt-1* and *snat-2* (respectively ~33 and 37% of Col-0 value), and not notably different in *asmt-2* or *snat-1* (**Figure 1C**). Expression of *MYC2*, a negative regulatory gene in the JA-dependent signaling pathway, showed an opposite trend. It was lower in overexpression lines (*SNAT-OE-2* value ~17% of Col-0 value) and increased in gene silencing lines (**Figure 1D**). Expression of *WRKY33*, a transcriptional regulator gene, varied less in the modified lines than that of *PR1* or *PR5*. The transcription level of *WRKY33* was increased in the *SNAT*-overexpression lines, particularly *SNAT-OE-2*, whose value was 2.86 times that of Col-0. *WRKY33* expression was lower in gene silencing lines, most notably *snat-1* (value ~50% that of Col-0; **Figure 1E**).

These findings indicate that the expression of *ASMT* and *SNAT* genes plays an important role in regulating the expression of *PR1*, *PR5*, *PDF1.2*, *MYC2*, and *WRKY33*. Downregulation of *ASMT* and *SNAT* inhibits expression of *B. cinerea* infection resistance genes, thereby increasing susceptibility of plants to fungal pathogens. Upregulation of *ASMT* and *SNAT* in overexpression lines enhances resistance to *B. cinerea*. In summary, plant defensive responses to *B. cinerea* infection are strongly affected by alterations in levels of the two melatonin synthesis enzymes or of endogenous melatonin.

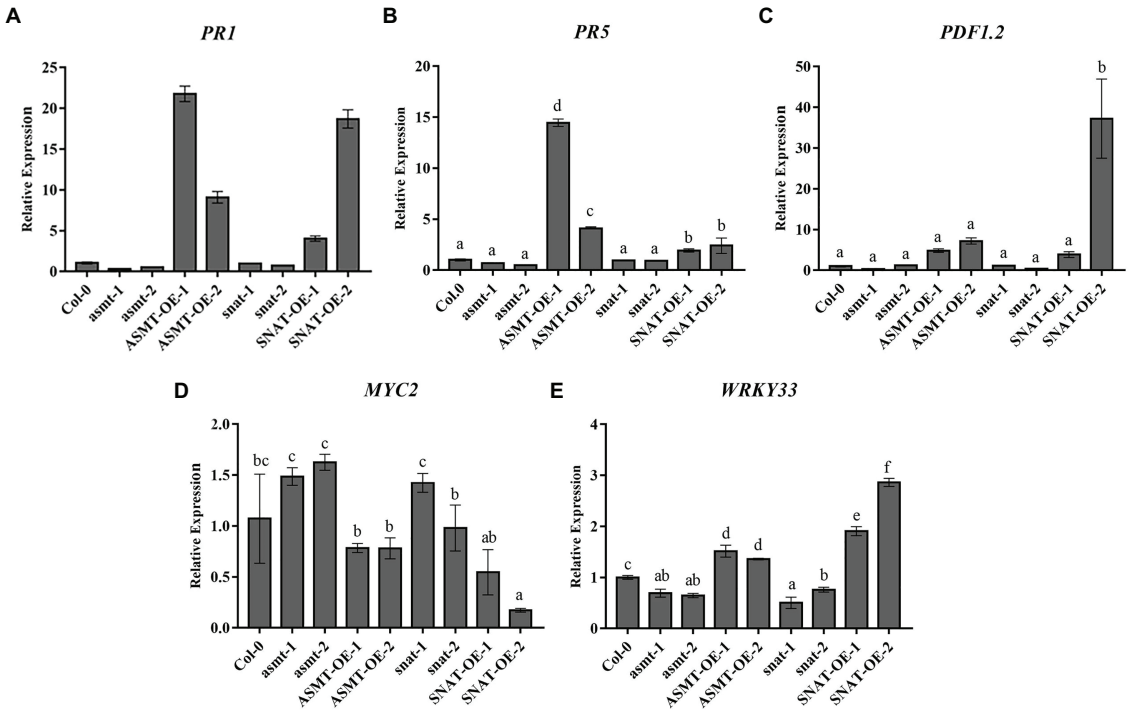

FIGURE 1 | Expression of defends signaling-related gene following *Botrytis cinerea* treatment. The transcript levels of *PR1* **(A)**, *PR5* **(B)**, *PDF1.2* **(C)**, *MYC2* **(D)**, and **(E)** *WRKY33* were determined by RealTime Quantitative Polymerase Chain Reaction (RT-qPCR). RNA extracted from rosette leaves of 4-week old wild-type (Col-0), N-acetylserotonin methyltransferase (*ASMT*)-overexpressed (*ASMT-OE-1*, *ASMT-OE-2*) plants, serotonin N-acetyltransferase (*SNAT*)-overexpressed (*SNAT-OE-1*, *SNAT-OE-2*) plants, *snat* silencing lines (*snat-1*, *snat-2*), and *asmt* silencing lines (*asmt-1*, *asmt-2*) at 48 h after the *B. cinerea* infection. *PR1* (*AT2G14610*), pathogenesis-related protein 1; *PR5* (*AT1G75040*), pathogenesis-related protein 5; *PDF1.2* (*AT5G44420*), plant defensin 1.2; *MYC2* (*AT1G32640*); and *WRKY33* (*AT2G38470.1*). The data (mean ± SD) were calculated using three replicate assays, with the SEs indicated by the vertical bars. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

Effects of Melatonin Levels on Resistance to *B. cinerea* Infection

To further investigate the roles of *ASMT* and *SNAT* genes in pathogen resistance, we measured endogenous melatonin levels in the overexpression and gene silencing lines. Melatonin levels were higher after 48 h *B. cinerea* treatment relative to 0 or 24 h treatment, most notably for *ASMT-OE-1* (**Figure 2**). The increase was significant for overexpression lines, but not for gene silencing lines or Col-0. Melatonin levels were lower in all lines at 72 h, most notably for *asmt-1*. These findings indicate that endogenous melatonin content is affected by up- or downregulation of *ASMT* and *SNAT* genes, suggesting a positive regulatory effect of endogenous melatonin in resistance to *B. cinerea* infection.

Effects of Endogenous Melatonin on Pathogenic Processes in *B. cinerea*-Infected Plants

Superoxide dismutase is resistant to oxidative damage, and plays a key role in the removal of free radicals in plants (López-Cruz et al., 2016). SOD activity in our plant lines varied depending on *B. cinerea* treatment time (**Figure 3A**). At 24 and 48 h, SOD activities of overexpression lines were higher than that of Col-0, whereas those of gene silencing lines were significantly lower. SOD activities of the various lines were generally lower at 72 h than at 48 h, but the overall trend was consistent. POD activities in the lines did not differ significantly at 0 h, similarly to SOD activities (**Figure 3B**). These findings indicate that *ASMT* and *SNAT* cope with peroxide damage by alterations of endogenous melatonin level, thereby enhancing plant resistance to fungal invasion.

Spores (conidia) of *B. cinerea* attach to the plant surface and germinate, and the degree of leaf damage is proportional to infection time (Williamson et al., 2007; Wang et al., 2013). *Botrytis cinerea* treatment did not initially cause notable damage to plants, and MDA content at 0 h did not differ significantly among the lines, similarly to SOD activity. At longer treatment times, *B. cinerea* spores reproduced parasitically on leaves, leading to a greater degree of lipid peroxidation and more damage to the plants. MDA content was the highest at 72 h in all strains (**Figure 3C**). These findings indicate that upregulation of *ASMT* and *SNAT* enhances endogenous melatonin level and reduces cell membrane damage, thus increasing resistance to *B. cinerea* and relieving biotic stress, whereas downregulation of *ASMT* and *SNAT* has the opposite effect.

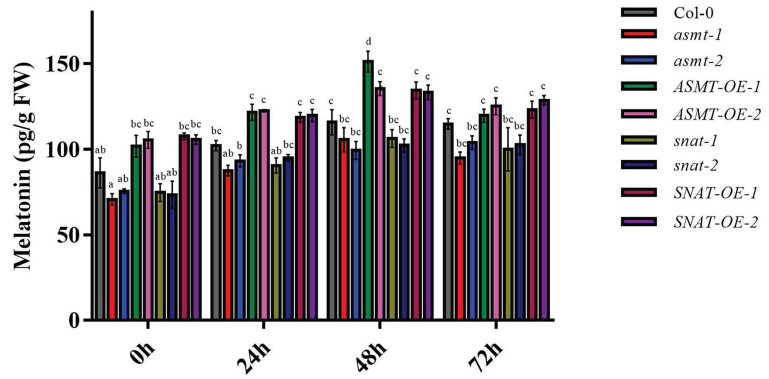

FIGURE 2 | Melatonin content following *B. cinerea* treatment. The content of melatonin in 4-week old wild-type (Col-0), *ASMT*-overexpressed (*ASMT-OE-1*, *ASMT-OE-2*) plants, *SNAT*-overexpressed (*SNAT-OE-1*, *SNAT-OE-2*) plants, *snat* silencing lines (*snat-1*, *snat-2*), and *asmt* silencing lines (*asmt-1*, *asmt-2*) were measured at different time points (0, 24, 48, and 72 h) after inoculation with *B. cinerea*. The data (mean ± SD) were calculated using three replicate assays, with the SEs indicated by the vertical bars. Different lowercase letters indicate statistically significant differences ($p < 0.05$). FW, fresh weight.

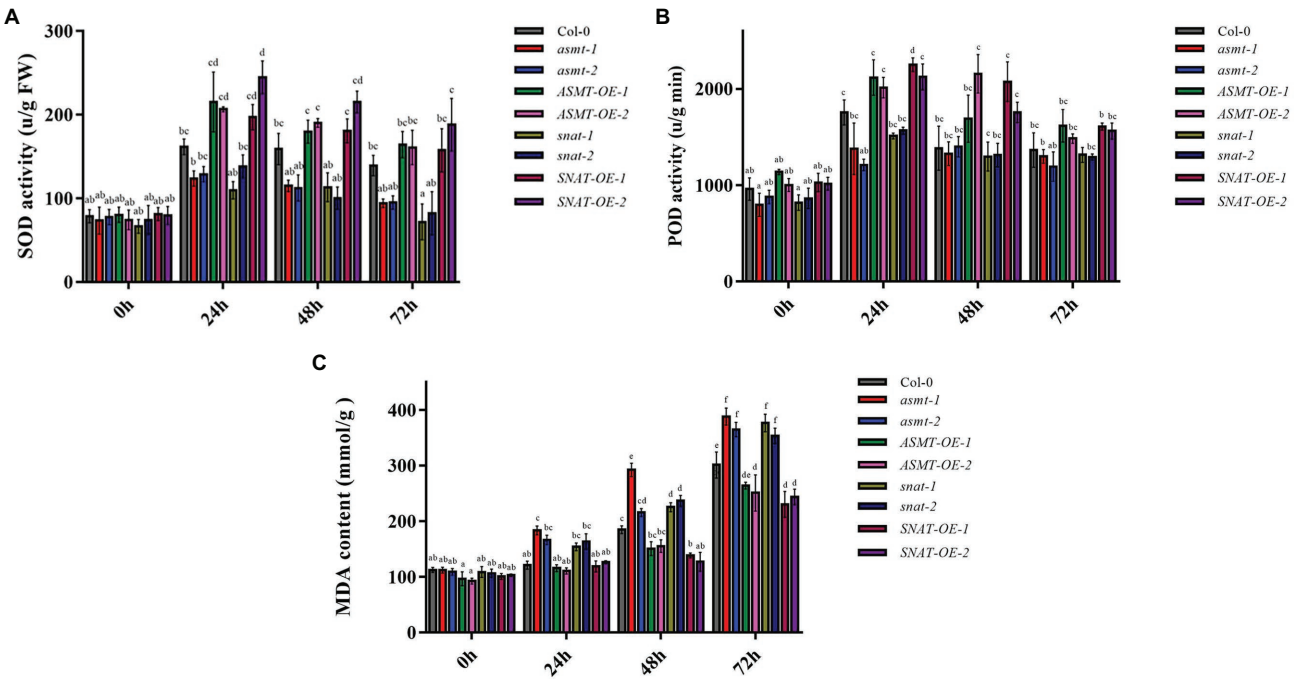

FIGURE 3 | The activities of antioxidant enzymes **(A)** superoxide dismutase (SOD) activity and **(B)** peroxidase (POD) activity and **(C)** the content of malondialdehyde (MDA) following *B. cinerea* treatment. Significant differences among 4-week old wild-type (Col-0), *ASMT*-overexpressed (*ASMT-OE-1*, *ASMT-OE-2*) plants, *SNAT*-overexpressed (*SNAT-OE-1*, *SNAT-OE-2*) plants, *snat* silencing lines (*snat-1*, *snat-2*), and *asmt* silencing lines (*asmt-1*, *asmt-2*) at different time points (0, 24, 48, and 72 h) after the *B. cinerea* infection. The data (mean ± SD) were calculated using three replicate assays, with the SEs indicated by the vertical bars. Different lowercase letters indicate statistically significant differences ($p < 0.05$). FW, fresh weight.

Effects of Leaf Damage in *B. cinerea*-Infected Plants

Botrytis cinerea infection of leaves results in damage to cell membranes, such that the dye can enter cells. The degree to which cells are dyed blue reflects the extent of the disease (Ramírez et al., 2011). In Col-0, blue spots were scattered on various parts of the leaves. Blue spots on *asmt-1*, *snat-1*, *asmt-2*, and *snat-2* leaves were deeply colored, indicating presence of more dead cells and greater susceptibility to *B. cinerea*. The overexpression lines showed only a few scattered dark blue spots, indicating a lesser degree of leaf cell death than in Col-0, and greater resistance to *B. cinerea* invasion (**Figure 4A**).

Reactions to *B. cinerea* infection varied among Col-0, gene silencing lines, and overexpression lines. At 4 days after inoculation (dai), gene silenced lines than in Col-0 showed greater susceptibility to *B. cinerea* more severe disease symptoms

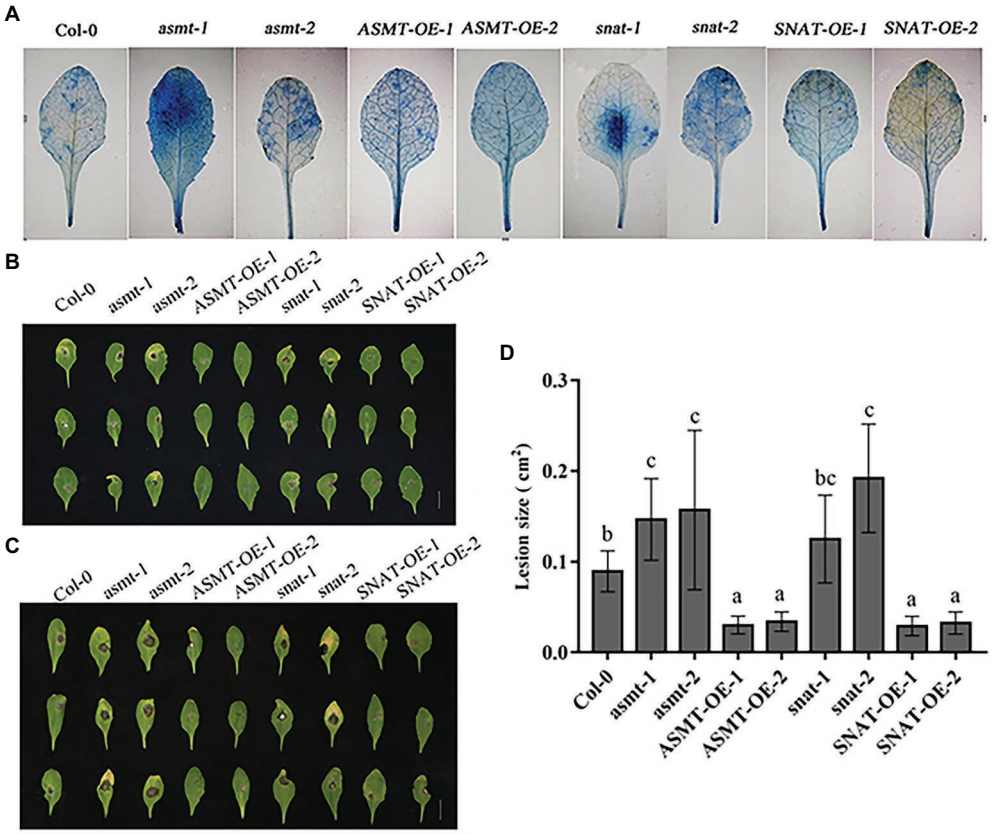

FIGURE 4 | Effects of endogenous melatonin on disease resistance to *B. cinerea*. **(A)** Trypan blue staining of leaves from different lines following *B. cinerea* treatment at 48 h. **(B,C)** Photographs of rosette leaves cut from 4-week-old Wild-type (Col-0), *ASMT*-overexpressed (*ASMT-OE-1*, *ASMT-OE-2*) plants, *SNAT*-overexpressed (*SNAT-OE-1*, *SNAT-OE-2*) plants, *snat* silencing lines (*snat-1*, *snat-2*), and *asmt* silencing lines (*asmt-1*, *asmt-2*) plants after infection with *B. cinerea* spores. **(B)** Leaves phenotype on 4 days. **(C)** Leaves phenotype on 6 days. **(D)** Lesion size. Lesion size of leaves phenotype on 6 days was measured using Image J software. The data (mean ± SD) were calculated using three replicate assays, with the SEs indicated by the vertical bars. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

and more fungal growth. In contrast, overexpression lines showed lesser and more slowly developing disease symptoms, small disease area, and limited spore growth (**Figure 4B**). Leaf necrosis was more severe on 6 dai (**Figure 4C**) than on 4 dai (**Figure 4B**) for gene silencing lines, but overexpression lines showed only a minor difference between these days. These findings indicate that *ASMT* and *SNAT* gene knockdown reduces plant resistance to *B. cinerea* infection.

A graph of lesion size in the various lines (**Figure 4D**) also illustrates the relationship between *B. cinerea* and *ASMT*/*SNAT*. Leaf damage area for the four-gene silencing lines (most notably *snat-2*) was significantly larger than for Col-0. Upregulation of *ASMT* and *SNAT* promoted host defensive response.

Effect of Endogenous Melatonin on JA Content

Jasmonic acid signaling plays a key role in the *B. cinerea* infection process. We examined the effects of altered endogenous melatonin levels on JA content. At 0 h, JA content did not differ notably among Col-0, gene silencing lines, and overexpression lines. JA content was significantly higher at 24 h than at 0 h for overexpression lines (particularly *SNAT-OE-1*), but such change was less notable for gene silencing lines (**Figure 5**). Maximal values were observed at 48 h for Col-0 and overexpression lines. JA content at 48 h was significantly higher for overexpression lines (particularly *SNAT-OE-2*) than for Col-0. Values at 72 h were lower than those at 48 h for Col-0 and all four overexpression lines. These findings indicate that endogenous melatonin level affects JA content for signal transduction pathways involved in pathogen resistance.

CONCLUSION AND FUTURE PERSPECTIVE

Botrytis cinerea is a necrotrophic fungus with a broad host plant spectrum (Dean et al., 2012), Plants are infected mainly by *B. cinerea* spores, which are released from previously infected plants when disturbed (Williamson et al., 2007). Some researchers have uncovered the role of melatonin in plant-*B. cinerea*

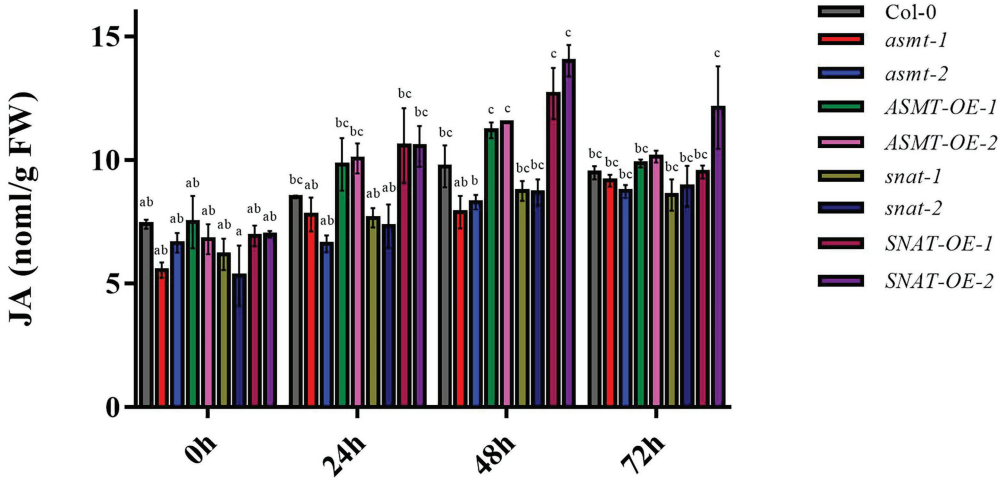

FIGURE 5 | Jasmonic acid (JA) content following *B. cinerea* treatment. The content of JA in 4-week-old wild-type (Col-0), *ASMT*-overexpressed (*ASMT-OE-1*, *ASMT-OE-2*) plants, *SNAT*-overexpressed (*SNAT-OE-1*, *SNAT-OE-2*) plants, *snat* silencing lines (*snat-1*, *snat-2*), and *asmt* silencing lines (*asmt-1*, *asmt-2*) plants at different time points (0, 24, 48, and 72 h) after inoculation with *B. cinerea*. The data (mean ± SD) were calculated using three replicate assays, with the SEs indicated by the vertical bars. Different lowercase letters indicate statistically significant differences ($p < 0.05$). FW, fresh weight.

interaction, for instance, tomato (Liu et al., 2019), *Fragaria ananassa* (Aghdam and Fard, 2017). We are going to study the resistance of melatonin to *B. cinerea* or related fungal species in *A. thaliana* and other model plants in future work. Enhancing effects of exogenous melatonin on plant resistance to fungi have been observed in many studies (Amaral and Cipolla-Neto, 2018). Much less is known regarding the role of melatonin (particularly endogenous melatonin) in plant resistance to *B. cinerea*. We examined the effects of altered endogenous melatonin levels on such resistance.

Plant defense mechanisms against pathogens involve complex signaling networks, including the expression of related genes (Senthil-Kumar and Mysore, 2013). *PR1* gene often plays a key role in plant resistance against necrotrophic pathogens (Frey et al., 2018). In this study, *PR1* expression in *ASMT* and *SNAT* overexpression lines was significantly increased relative to Col-0 but was reduced in gene silencing lines (**Figure 1A**). These findings are consistent with previous reports that the increase of *PR1* expression promotes plant defense against *B. cinerea* (Frey et al., 2018). *PR1* is considered to be associated with SA; however, it shows increased expression mainly in defensive responses against biotrophic fungi and not those against necrotrophic *B. cinerea*. Functional roles of SA are complex, and functions of SA signals in plant resistance to *B. cinerea* remain unclear (AbuQamar et al., 2017). *PR5*, a gene co-expressed with *PR1*, is also involved in signal transduction in plants (Siewers et al., 2005). We observed upregulation of *PR5* in overexpression lines, similarly to *PR1*, and downregulation in *asmt-1* and *asmt-2*. However, *PR5* expression in *snat-1* and *snat-2* did not differ notably from that in Col-0, possibly because *PR5* is not a single-label gene for resistance to *B. cinerea* (**Figure 1B**).

Transcription factor WRKY33 is an essential component in the regulation of plant defensive responses to fungal infection. *WRKY33* expression is induced in *B. cinerea*-infected plants. In our gene expression analysis, *WRKY33* was upregulated in overexpression lines but downregulated in gene silencing lines (**Figure 1E**), consistently with the increased susceptibility to *B. cinerea* observed for *WRKY33* mutants (*wrky33-1*, *wrky33-2*). In previous studies, *WRKY33* has been suggested to participate in JA-dependent pathways and to play a negative regulatory role in JA-mediated defensive responses. However, we did not observe an association between *WRKY33* increase and reduced JA content (**Figure 5**), indicating that this gene is not involved in the JA-dependent signaling pathway in this case. Recent reports show that *WRKY33* induction in *B. cinerea*-infected plants does not require a JA signal molecule (CORONATINE INSENSITIVE1; COI1; Wang et al., 2015), which illustrates that WRKY33 is activated *via* a JA-independent pathway. On the other hand, WRKY33 is phosphorylated by MPK3/MPK6 to induce synthesis of the phytoalexin camalexin in *B. cinerea* infected plants (Mao et al., 2011), suggesting that *WRKY33* expression in our *ASMT* and *SNAT* overexpression and gene silencing *Arabidopsis* lines may be related to MPK3/MPK6. We speculate melatonin can act directly on MAPKs and it further phosphorylates *WRKY33*.

Changes in growth and physiological processes of *B. cinerea*-infected plants are related to a variety of metabolic processes, including enzyme degradation and soluble sugar accumulation in cell walls, pH changes, and reduced production of antifungal compounds and secondary metabolites. Most of these changes are regulated by hormone signals, such as ethylene, Abscisic Acid, Jasmonic Acid and Salicylic Acid (Ingle et al., 2015). Studies based on transcriptome analysis suggest that the involvement of melatonin in plant-fungus interactions alters the expression of JA-related genes, and that melatonin can interact with JA to modulate plant defensive responses (Mandal et al., 2018). However, the role of melatonin in the JA pathway

remains unclear. We observed high JA content (**Figure 5**) in our overexpression lines, but low JA content in gene silencing lines. Thus, increased melatonin level promoted JA accumulation, and consequently resistance to fungal infection. Increased JA content triggers signaling pathways downstream of JA. JAZ protein has an inhibitory effect on JA signaling pathway, and increased JA content leads to JAZ protein degradation, thereby reducing interaction between JAZ and MYC2 (Ruan et al., 2019), consistently with the reduced *MYC2* transcription levels in our overexpression lines (**Figure 1D**). MYC2 is a regulatory factor that plays an essential role in the JA-dependent signaling pathway. It negatively regulates downstream signal genes (*ORA59*/*ERF1*) that activate plant defensive responses and trigger downstream expression of *PDF1.2*, which plays a positive regulatory role in JA-dependent disease resistance (Frey et al., 2018). Consistently, *PDF1.2* expression was increased strongly in overexpression line *SNAT-OE-2*, and also in *ASMT-OE-1*, *ASMT-OE-2*, and *SNAT-OE-1*, but was reduced in gene silencing lines *asmt-1* and *snat-2* (**Figure 1C**). These findings further confirm the involvement of melatonin in JA signaling pathway activation and in the enhancement of plant resistance to *B. cinerea* infection. ROS are among the earliest signaling molecules in the interaction between plants and pathogens. Plants have evolved a variety of enzymes, and non-enzyme antioxidant defense systems, that promote the removal of ROS and prevent oxidative damage to plant tissues. SOD and POD are well-studied antioxidant enzymes, and have been shown to help regulate oxidative reaction balance in resistance to *B. cinerea*. In our study, SOD and POD activities did not vary notably among the various lines at 0 h but increased as *B. cinerea* treatment time increased (**Figures 3A,B**). Similar results were obtained in studies of two apple cultivars (Bui et al., 2019). In our study, MDA content in all lines reached maximal value at 72 h (**Figure 3C**), possibly because the reduction of SOD and POD activities did not allow prompt relief of oxidative stress resulting from ROS accumulation. MDA levels in Col-0 and our gene silencing lines and overexpression lines were similar at later treatment times, as well as at 0 h.

Increased ROS levels in response to external stimuli in plants led to a rise in melatonin levels and consequent activation of antioxidant enzyme activity (Liu et al., 2019). We observed changes in endogenous melatonin levels (**Figure 2**) resulting from up- or downregulation of *ASMT* and *SNAT* genes. Melatonin acts synergistically with antioxidant enzymes to remove ROS, promote photosynthesis, delay metabolite biosynthesis, enhance antioxidant capacity of plants, reduce oxidative stress in cells, tissues, or whole organisms, and protect plants from harsh environments (Manchester et al., 2015). Thus, increased endogenous melatonin promotes SOD and POD activity, facilitating prompt removal of excess ROS, and thereby enhancing plant defense against *B. cinerea*. Similarly, melatonin reduces damage to citrus fruits by penicillin fungal pathogens by removing ROS.

Various molecules secreted by *B. cinerea* induce the death of host cells (Siewers et al., 2005). Trypan blue staining revealed fewer dead cells in overexpression lines, and more dead cells

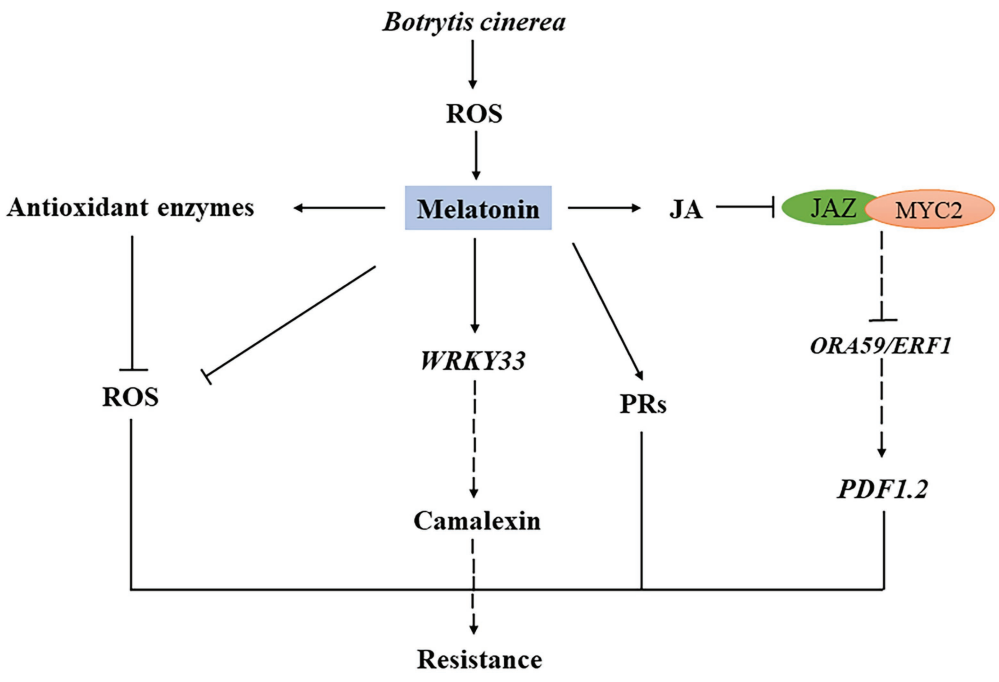

FIGURE 6 | Schematic model of the role of melatonin in plant resistance to *B. cinerea* infection. Under *B. cinerea* Infection, endogenous melatonin content increases and decreased transcript levels of *JAZ1* and *MYC2*, which are two negative genes in the JA signaling pathway. Increasing Increased melatonin content also reduces ROS level by stimulating antioxidant system enzyme genes. The increase of melatonin content leads to the upregulation of *WRKY33* and defense genes (*PRs*, *PDF1.2*) expression.

in gene silencing lines, relative to Col-0 (**Figure 4A**). A possible explanation is that necrotrophic stenosis induces ROS accumulation, triggering programmed cell death (PCD) in host cells and providing nutrients to fungi, thereby promoting their growth and reproduction, and the appearance of disease symptoms (Torres, 2010). Infection of leaves by *B. cinerea* results in obvious necrotic symptoms (Liu et al., 2017). *Botrytis cinerea* infection of apple plants similarly caused significant disease symptoms (Bui et al., 2019). Phenotypic observations of leaves in the present study revealed disease symptoms on 4 dai, and the appearance of large necrotic lesions by 6 dai (**Figures 4B,C**). Overexpression lines were less susceptible to *B. cinerea* relative to Col-0, whereas gene silencing lines were more susceptible and developed more obvious lesions (**Figure 4D**). Our findings suggest that *ASMT* and *SNAT* overexpression enhances plant resistance to *B. cinerea* by increasing melatonin level, consistently with previous reports that plant resistance to other fungi is enhanced by endogenous melatonin.

The growth of fungi was inhibited in a PDA medium containing melatonin, suggesting that melatonin enhances plant resistance (Arnao and Hernández-Ruiz, 2015). At various times (24, 48, and 72 h) following *B. cinerea* inoculation, the induced substrate for melatonin synthesis increases, and *SNAT* and *ASMT* overexpression leads to further melatonin synthesis (**Figure 2**), suggesting that melatonin is involved in resistance to *B. cinerea*. Plant infection with the pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) similarly caused the increase of melatonin levels (Shi et al., 2015). Melatonin levels were high in our overexpression lines but lower in gene silencing lines. Melatonin content was maximal in *ASMT-OE-1* at 48 h, in *ASMT-OE-2* at 24 h, and in *SNAT-OE-2* at 72 h. This finding suggests that melatonin is being synthesized *via* multiple biosynthetic pathways.

In conclusion, we observed that increased endogenous melatonin level enhanced plant resistance to *B. cinerea*, consistently with previous reports. Melatonin is involved in the basic defensive responses of plants to *B. cinerea* and plays an essential role in plant immunity. Our findings provide new insights into molecular mechanisms of plant defensive signaling initiated by melatonin during interaction with pathogens, particularly fungal pathogens (**Figure 6**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

The authors declare that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Effects of Melatonin on Morphological Characteristics, Mineral Nutrition, Nitrogen Metabolism, and Energy Status in Alfalfa Under High-Nitrate Stress

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Melatonin is an indoleamine small molecular substance that has been shown to play an important role in the growth, development, and stress response of plants. The effects of melatonin on the morphological characteristics, mineral nutrition, nitrogen metabolism, and energy status in alfalfa (*Medicago sativa* L.) under high-nitrate stress were studied. The alfalfa plants were treated with water (CK), 200 mmol L^{-1} nitrates (HN), or 200 mmol L^{-1} nitrates + 0.1 mmol L^{-1} melatonin (HN+MT), and then were sampled for measurements on days 0 and 10, respectively. The results showed that the HN treatment resulted in a decrease in the morphological characteristics (such as shoot height, leaf length, leaf width, leaf area, and biomass), phosphorus, soluble protein (SP), nitrogen-related enzymes activities and gene relative expression, adenosine triphosphate (ATP), and energy charge (EC). It also caused an increase in nitrogen, sodium, potassium, calcium, nitrate-nitrogen (NO_3^--N), ammonium-nitrogen (NH_4^+-N), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). However, these parameters were conversely changed in the HN+MT treatment. Besides, these parameters were closely related to each other, and were divided into two principal components. It reveals that melatonin plays an important role in modulating the morphology, mineral nutrition, nitrogen metabolism and energy status, thereby alleviating the adverse effects of high-nitrate stress and improving the growth of alfalfa.

Keywords: melatonin, mineral nutrition, nitrogen metabolism, energy status, high-nitrate stress, alfalfa

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INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is a tryptophan-derived low molecular weight indole amine that is widespread in bacteria, algae, animals, and higher plants (Back et al., 2016; Zhang J. et al., 2019). Melatonin is also a universal signaling molecule in mammals and plant species (Zhang Q. et al., 2019; Zhao et al., 2021). As an important free radical scavenger and antioxidant, melatonin is involved in various physiological processes, and it modulates plant tolerance or resistance to abiotic stresses, including salinity, drought, heat, cold, water, heavy metal, nutritional deficiency, osmotic and ionic stress, ultraviolet radiation and chilling injury (Li et al., 2012; Tan et al., 2012; Shi and Chan, 2014; Shi et al., 2015; Antoniou et al., 2017; Gong et al., 2017; Zhang R. et al., 2017;

Zhao et al., 2017; Kobylińska et al., 2018; Zhang Q. et al., 2019; Bose and Howlader, 2020). Hence, melatonin plays an essential role in improving plant growth, development and protecting plants from adverse conditions.

Alfalfa (*Medicago sativa* L.) is a widely cultivated perennial legume species in the world (Wolabu et al., 2020). As the most productive and highest quality forage crop, alfalfa can be used for grazing, silage, hay, and even human fresh food and health products (Tang et al., 2013; Jaime et al., 2019; Lin et al., 2020). Nitrogen is a component of amino acids, nucleic acids, proteins, nucleoproteins, chlorophyll, enzymes, hormones and secondary metabolites, as well as the basis of key genetic materials (Wen et al., 2020). Hence, the efficiency and availability of nitrogen have decisive influences on the growth, development, and metabolisms of plants (Hachiya and Sakakibara, 2017). For most plants, nitrate-nitrogen (NO_3^--N) and ammonium-nitrogen (NH_4^+-N) are major sources of inorganic nitrogen (Zhu et al., 2020). Although alfalfa can fix nitrogen through nodules, the amount of nitrogen fixation by nodules is 50 to 60% of its lifetime nitrogen demand. That is, root nodule nitrogen fixation cannot meet the normal growth needs of alfalfa. Furthermore, the soil of most alfalfa fields is low in NO_3^--N and organic matter, and these areas suffer from salinization, drought, and low temperatures. Therefore, rational application of nitrogen fertilizer is a guarantee for good growth, high quality and high yield of alfalfa, especially in areas with poor soils and harsh climates (Zeng et al., 2006; Liu et al., 2015; Yu and Ma, 2015; Hao et al., 2017).

NO_3^--N is the most prevalent in soil for the growth and morphological construction of most plants (Oldroyd and Leyser, 2020; Wen et al., 2020). However, in the alfalfa-growing areas with a dry climate and alkaline soil, soil nitrification is serious but denitrification is weak, and excess NO_3^--N is easy to accumulate in the soil (Ju et al., 2011). Besides, the excessive and unreasonable application of nitrogen fertilizer also affects the growth and yield of alfalfa in recent years (Hao et al., 2015, 2017). It has demonstrated that excessive NO_3^--N reduces photosynthesis and enzyme activities, and increases ionic toxicity, osmotic stress and reactive oxygen species (ROS), further affecting crop quality and yields (Yang et al., 2010; Zhang R. et al., 2017). Nitrate absorption and transformation are mainly regulated by the nitrogen metabolism pathway in alfalfa, which is mainly modulated by nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) (Zhang R. et al., 2017; Wen et al., 2020). In addition, plant growth and development are ultimately driven by light energy captured through photosynthesis, and the energy available state plays an important role in the metabolism of plants (De Col et al., 2017). Therefore, nitrogen mechanism and energy status are of great significance to the growth and yield of alfalfa under high NO_3^--N.

The roles of melatonin in regulating alfalfa adaptation to high-nitrate stress are seldom studied. In this study, the objective was to evaluate the effects of melatonin on morphological characteristics, mineral nutrition, nitrogen metabolism and energy status in alfalfa under high-nitrate stress. Thus, a comprehensive understanding of the role of exogenous melatonin for improving alfalfa nitrate stress tolerance was provided.

MATERIALS AND METHODS

Plant Growth and Treatments

The seeds of "Sanditi" (*Medicago sativa* L.) were sown in the pots using a 3:1:1 mixture of peat, vermiculite, and pearlite, respectively. Then the pots were placed in a growth chamber at 25°C with a 14-h daily photoperiod (20000 lx) and 10-h dark. At the three-leaf stage, the excess seedlings were removed so that there were nine seedlings in each pot. They were regularly watered. After 15 d, the pots were divided into three groups (six replications per group), namely normal control (CK), high nitrates (HN), and high nitrates + melatonin (HN+MT). The plants were treated with water, 200 mmol L^{-1} nitrates, and 200 mmol L^{-1} nitrates + 0.1 mmol L^{-1} melatonin, respectively. Nitrate and melatonin were used with the optimum concentration selected from the pre-experiment. And nitrate and melatonin were used by irrigation and foliar spraying, respectively. The nitrate was composed of calcium nitrate [$Ca(NO_3)_2$] and potassium nitrate (KNO_3) averagely. The treatments were applied every other day for a total of three times. Then, three replications of each group were sampled for measurements, which were labeled 0 d. The others continued to grow for 10 d and then were sampled. Shoot height, leaf length, leaf width, leaf area, shoot fresh weight (FW) and dry weight (DW) were measured both on days 0 and 10. The leaf samples were quickly frozen in liquid nitrogen and stored at −80°C for the other parameters.

Mineral Nutrition

The leaves were sampled and washed three times with distilled water. Then they were fixed at 105°C for 30 min, and dried for 48 h at 65°C. They were ground to powder to pass through a 1-mm sieve. Nitrogen and phosphorus were determined with a Continuous Flow Analyzer (Flowsys; Systea, Anagni, Italy) after wet digestion of H_2SO_4-H_2O_2. The powder (0.1 g) for sodium, potassium, and calcium was extracted in boiling water with deionized water for 2 h. Then, the concentrations were determined with a Flame Photometer (M410 blue notes, Sherwood, UK).

NO_3^--N and NH_4^+-N Concentrations

NO_3^--N and NH_4^+-N concentrations were measured using fresh leaf samples by the methods of Zhang R. et al. (2017). Fresh samples (0.5 g) were ground in 10 mL of water and then held in a boiling water bath for 30 min. The supernatant was diluted to 25 mL. Then, 0.1 mL solution was mixed with 0.4 mL of 5% salicylic-H_2SO_4 to react for 20 min. And 9.5 mL of 8% NaOH was added. After cooling to room temperature, absorbance was measured at 410 nm (UV-3000, Mapada, China) for NO_3^--N. As to NH_4^+-N concentrations, the fresh samples were ground in 10 mL of 10% acetic acid and the volume was made up to 100 mL with distilled water. Then, 2 mL of the solution, 3 mL of ninhydrin hydrate and 0.1 mL of 1% ascorbic acid were mixed and heated in boiling water for 15 min. After being cooled in

an ice bath, the mixture was measured at 580 nm (UV-3000, Mapada, China).

Soluble Protein (SP) and Proline (Pro)

SP concentrations were measured by the method of Li and Zhang (2016). The fresh sample (0.5 g) was ground with 5 mL distilled water and centrifugated at 3,000 g. The supernatant (1 mL) was added to 5 mL of 10% coomassie brilliant blue (containing 90% ethanol and 85% phosphoric acid). After 5 min, the absorbance was recorded at 595 nm (UV-3000, Mapada, China). SP concentration was calculated by the standard curve ($y = 5.8243x + 0.0236$, $R^2 = 0.9941$).

Pro was measured by the method of Li and Zhang (2016). The fresh samples (about 0.4 g) were extracted with 3% sulfosalicylic acid. Then, the extract (2 mL) was added to 2 mL of ice acetic acid and 2 mL of acidic ninhydrin in the boiling water for 30 min. It should be noted that acidic ninhydrin solution was obtained by dissolving 1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 mol L^{-1} phosphoric acid, stirring and heating (70°C). After 40-min toluene extraction, the upper toluene solution was absorbed to centrifuge for 5 min to determine the absorbance at 520 nm (UV-3000, Mapada, China).

Enzyme Activities

NR activity was measured by the method of Su et al. (2018). Leaf samples were added to a 10-mL tube with 5 mL of 0.1 mol L^{-1} phosphatic buffer solution (PBS) and 4 mL of 0.2 mol L^{-1} KNO_3. The sample was vacuumized for 10 min and kept warm at 30°C for 30 min. Then, 1 mL of 30% trichloroacetic acid (TCA) was added and shaken well. The extract (2 mL) was added to 4 mL of 1% sulfanilamide and 4 mL of 0.2% α-naphthylamine. The reaction continued for 30 min at 30°C. Absorbance was read at 520 nm (UV-3000, Mapada, China).

GS activity was assayed by the method of Oaks et al. (1980) with some modifications. The leaf samples were ground in 3 mL of 0.05 mol L^{-1} Tris-HCl (pH 8.0), then were centrifuged at 4°C. The reaction mixture contained a final volume of 1.6 mL with 0.1 mol L^{-1} Tris-HCl and 80 mol L^{-1} hydroxylamine hydrochloride (pH 7.4). Then, 0.7 mL of enzyme extract and 0.7 mL of 40 mmol L^{-1} adenosine triphosphate (ATP) were added to the reaction mixture. After incubation for 30 min at 30°C, 0.2 mol L^{-1} TCA, 0.37 mol L^{-1} $FeCl_3$, and 0.6 mol L^{-1} HCl were added to the mixture. After centrifugation at 5,000 g, the absorbance was measured at 540 nm (UV-3000, Mapada, China).

GOGAT activity was assayed by the method of Singh and Srivastava (1986) with some modifications. The method of enzyme extract was the same as that of GS. The reaction mixture contained 0.4 mL of 20 mmol L^{-1} L-glutamine, 0.05 mL of 0.1 mol L^{-1} α-oxoglutarate, 0.1 mL of 10 mmol L^{-1} KCl, 0.2 mL of 3 mmol L^{-1} NADH and 0.5 mL of the enzyme extract in a final volume of 3 mL, made up with 25 mmol L^{-1} Tris-HCl (pH7.6). The decrease in absorbance was recorded for 3 min at 340 nm (UV-3000, Mapada, China).

GDH activity was assayed by the method of Kanamori et al. (1972) with some modifications. The fresh samples were ground in 6 mL of 10 mmol L^{-1} Tris-HCl (pH 7.6, containing 1 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} EDTA, and 1 mmol L^{-1} β-mercaptoethanol). The enzyme extract was obtained after centrifugation at 4°C. The reaction mixture contained 0.3 mL of 0.1 mmol L^{-1} α-oxoglutarate, 0.3 mL of 1 mol L^{-1} NH_4Cl, 0.2 mL of 3 mmol L^{-1} NADH, and 1 mL of the enzyme extract in a final volume of 3 mL, made up with 0.2 mol L^{-1} Tris-HCl. The decrease in absorbance was recorded for 3 min at 340 nm (UV-3000, Mapada, China).

TABLE 1 | Sequences of the primers used for polymerase chain reaction.

Genes	Accession no.	Forward primer (5′-3′)
NR	3g073150-F	AATCGTCGCAAGGAGCAGAATATGG
	3g073150-R	ACAACGCTTGAGCACGGTTCTC
GS	8g062840-F	TTCGTCACCAGGTCCATATTGAAGC
	8g062840-R	ACACAGATTGAGCATCCACAGTTAGC
GOGAT	1g027020-F	GTGGTCAGTCGCTTGTGGTATGG
	1g027020-R	CTGTGCTTCTTGTTGAGGTCTTGTTG

Enzyme Genes

Total RNA was extracted from 100 mg leaf samples frozen in liquid nitrogen using an RNA Extraction Kit (TaKaRa Biomedicals, Japan). Quantitative RT-PCR was performed using an ABI StepOne Real-Time PCR System (USA). The primers were designed using DNAMAN software (**Table 1**). qRT-PCR was carried out in a 20-μL reaction mixture consisting of 10 μL of SYBR Premix Ex Taq II, 0.4 μL of ROX Dye, 0.8 μL of the forward PCR primer, 0.8 μL of the reverse PCR primer, 7 μL of ddH_2O and 1 μL of diluted template cDNA. The reaction conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ formula and standardized with the *β-actin* gene as an internal control.

Energy Status

ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP) and energy charge (EC) were measured by the method of Wang et al. (2017). The samples were ground in liquid nitrogen. Then, 6 mL of 0.6 mol L^{-1} perchloric acid was added to the samples. After a 10-min water bath, the homogenate was centrifuged at 8,000 g and 4°C for 15 min. The pH of the supernatant was neutralized to 6.8 using 1 mol L^{-1} KOH. The solution was diluted to 4 mL and passed through a 0.22-μm filter after another incubation step on ice and centrifugation. Then, 20 μL of the filter liquor was used to measure the concentrations of ATP, ADP, and AMP at 254 nm with an UltiMate 3000 HPLC (Thermo Fisher, Waltham, MA, USA) with a C18 column (5 μm; 250 × 4.6 mm; Phenomenex, USA). Mobile phase A (pH 7) consisted of 60 mmol L^{-1} K_2HPO_4 and 40 mmol L^{-1} KH_2PO_4, whereas mobile phase B was pure acetonitrile. The flow rate was 1 mL min^{-1}. ATP, ADP, and AMP concentrations were calculated via standard curves, while the EC was calculated with the formula: $EC = (ATP + 0.5 \times ADP)/(ATP + ADP + AMP)$.

Statistical Analysis

The experimental data were sorted and calculated using Excel 2019 (Microsoft Corp., Redmond, WA, USA), and were subjected to an analysis of variance (ANOVA) using SPSS 20 Statistics (SPSS Inc., Chicago, IL, USA). A significant difference was indicated at $p < 0.05$.

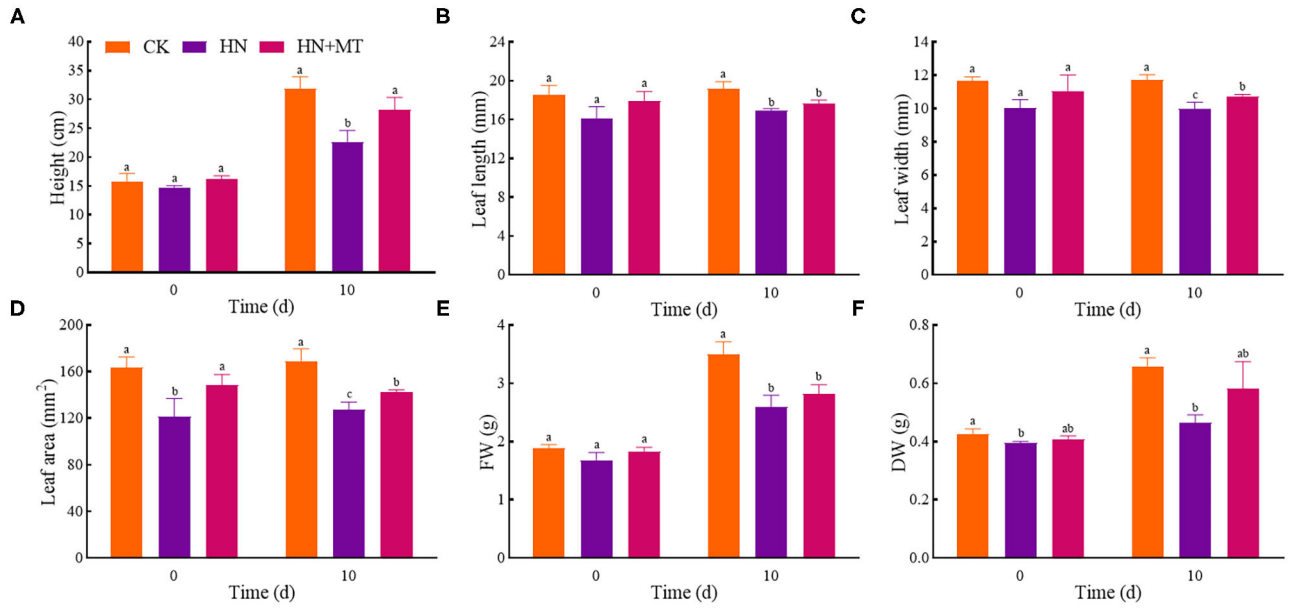

FIGURE 1 | Changes of shoot height **(A)**, leaf length **(B)**, leaf width **(C)**, leaf area **(D)**, FW **(E)**, and DW **(F)**. The groups on the same day followed with the same letter are not significantly different at $p < 0.05$. Bars indicate the SDs of the means.

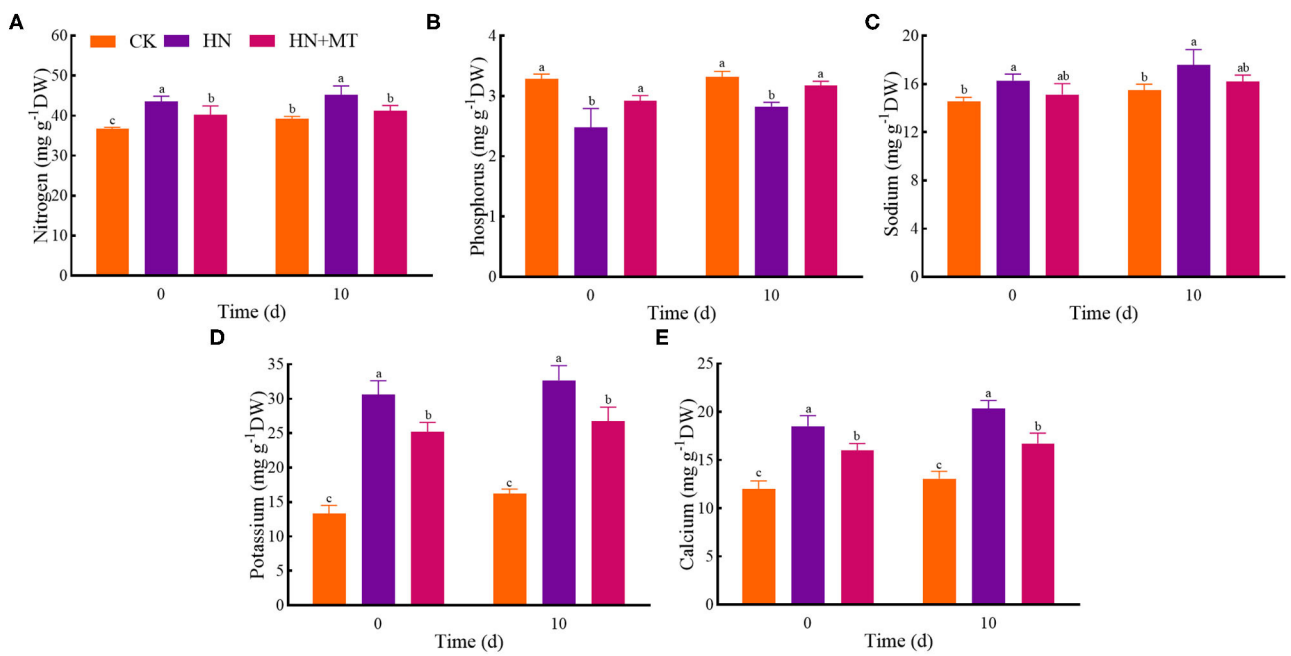

FIGURE 2 | Changes of nitrogen **(A)**, phosphorus **(B)**, sodium **(C)**, potassium **(D)**, and calcium **(E)**. The groups on the same day followed with the same letter are not significantly different at $p < 0.05$. Bars indicate the SDs of the means.

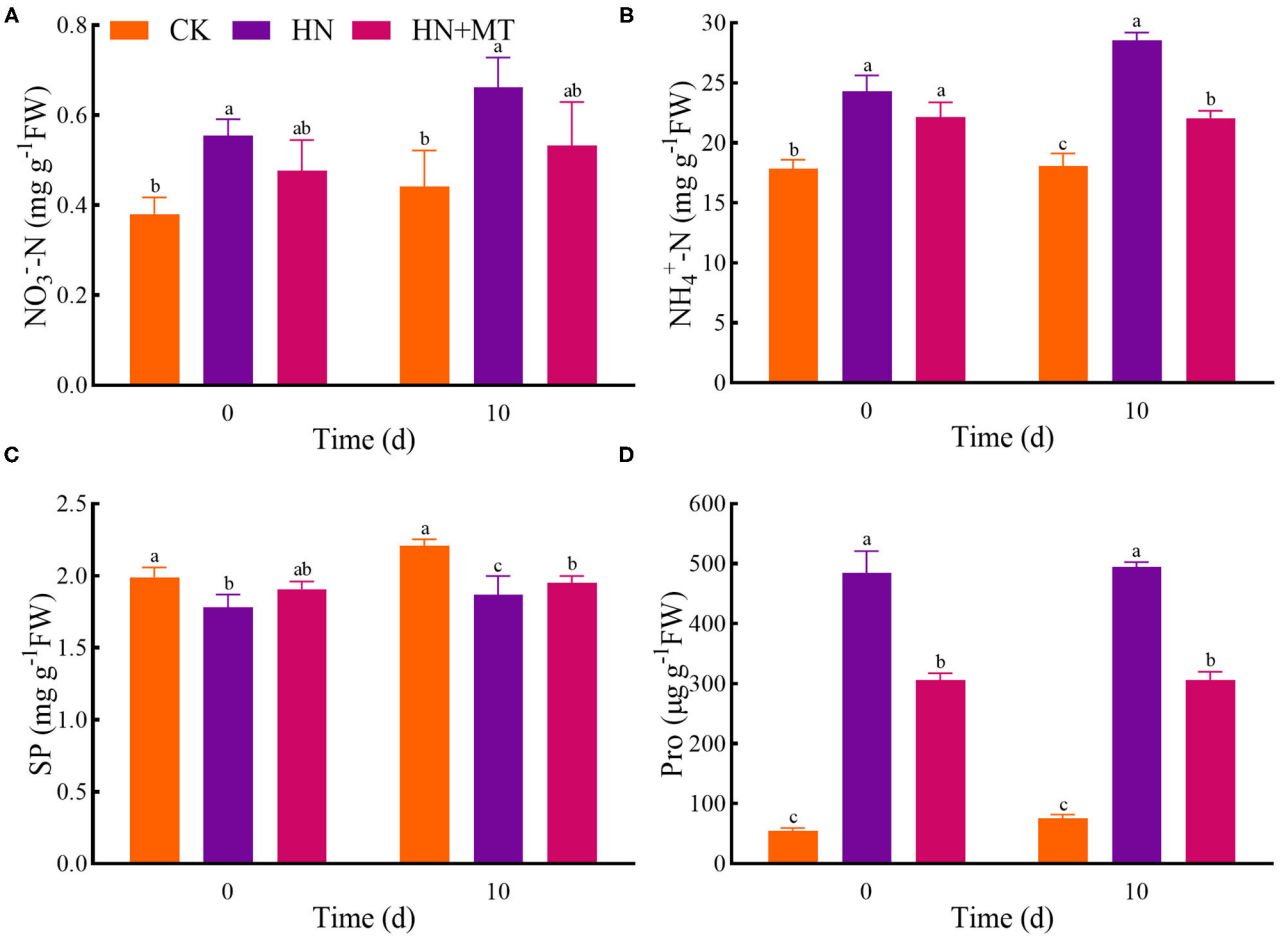

FIGURE 3 | Changes of NO_3^--N **(A)**, NH_4^+-N **(B)**, SP **(C)**, and Pro **(D)**. The groups on the same day followed with the same letter are not significantly different at $p < 0.05$. Bars indicate the SDs of the means.

RESULTS

Morphological Parameters

On day 0, the height, leaf length, leaf width, leaf area, FW and DW were the lowest, and there were significant differences in the leaf area and DW between the HN treatment and the CK (**Figure 1**). The HN+MT treatment increased these six parameters compared with the HN treatment, whereas they were still lower than the CK with no differences. On day 10, the HN treatment significantly decreased these six parameters compared with the CK. Whereas, the HN+MT treatment increased them, even dramatic differences were observed in the height, leaf width and leaf area.

Mineral Nutrition

In the HN treatment, nitrogen, sodium, potassium, and calcium had the highest values whereas phosphorus had the lowest value both on days 0 and 10 (**Figure 2**). Compared with the HN treatment, the HN+MT treatment almost dramatically decreased nitrogen, sodium, potassium, and calcium concentrations but increased phosphorus concentrations. It indicates that melatonin inhibits nitrogen, sodium, potassium and calcium but increases phosphorus in alfalfa under high-nitrate stress.

Nitrogen Types

Compared with the CK, the HN treatment significantly increased the concentrations of NO_3^--N, NH_4^+-N, and Pro, while it distinctly restricted the SP concentrations both on days 0 and 10 (**Figure 3**). The HN+MT treatment showed opposite changes in these four parameters.

Nitrogen Metabolism Enzymes

The HN treatment significantly inhibited the activities of NR, GS, GOGAT, and GDH, while the HN+MT treatment almost dramatically improved these activities (**Figure 4**). However, the activities in the HN+MT treatment still were significantly lower than those in the CK.

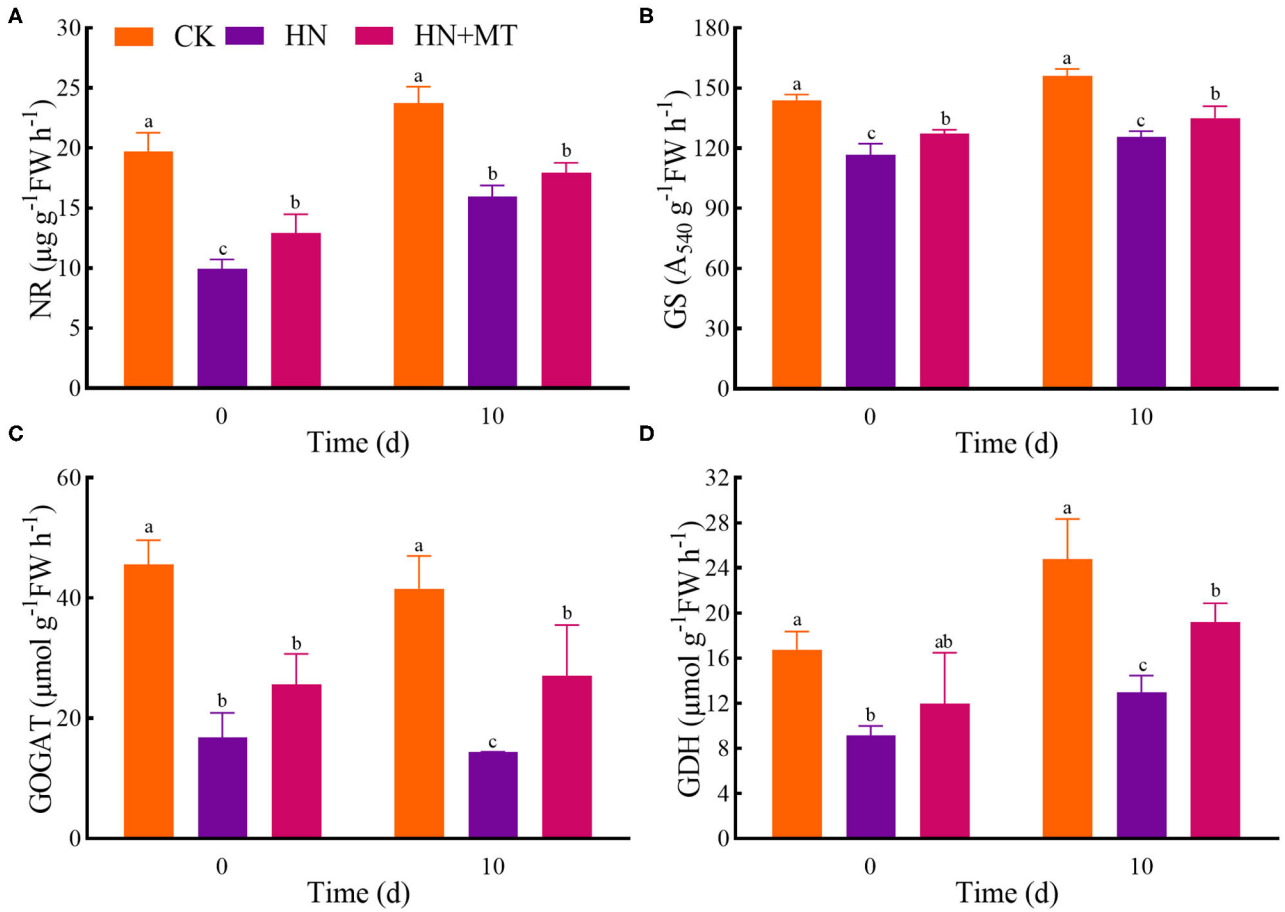

FIGURE 4 | Changes of NR **(A)**, GS **(B)**, GOGAT **(C)**, and GDH **(D)**. The groups on the same day followed with the same letter are not significantly different at $p < 0.05$. Bars indicate the SDs of the means.

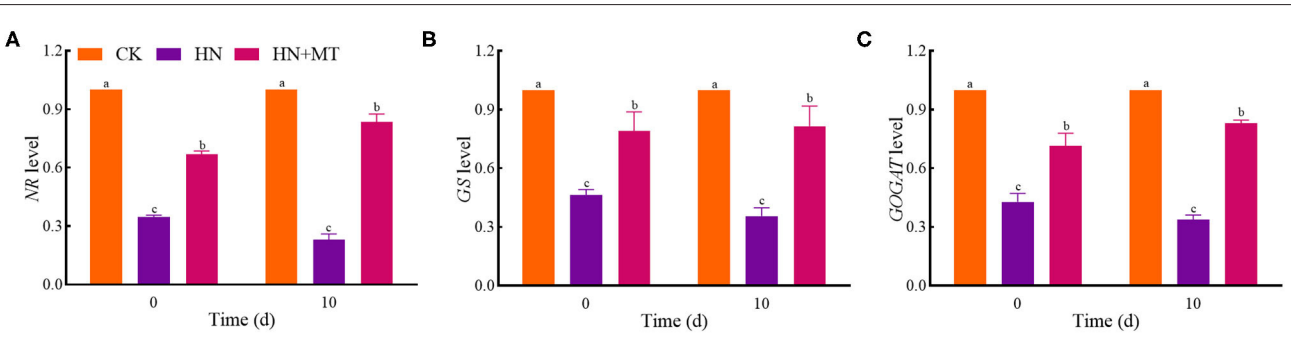

FIGURE 5 | Changes of *NR* **(A)**, *GS* **(B)**, and *GOGAT* **(C)** levels. The groups on the same day followed with the same letter are not significantly different at $p < 0.05$. Bars indicate the SDs of the means.

Gene Levels of Nitrogen Metabolism Enzymes

The expression of genes changed in a way similar to that of enzymes' activities both on days 0 and 10 (**Figure 5**). The relative expression of *NR*, *GS*, and *GOGAT* in the HN and HN+MT treatments was lower than those in the CK. The HN treatment significantly down-regulated the genes' expression, whereas the HN+MT treatment distinctly up-regulated the genes' expression.

Energy Status

In the HN treatment, ATP and EC had the lowest value, while ADP and AMP had the highest value both on days 0 and 10

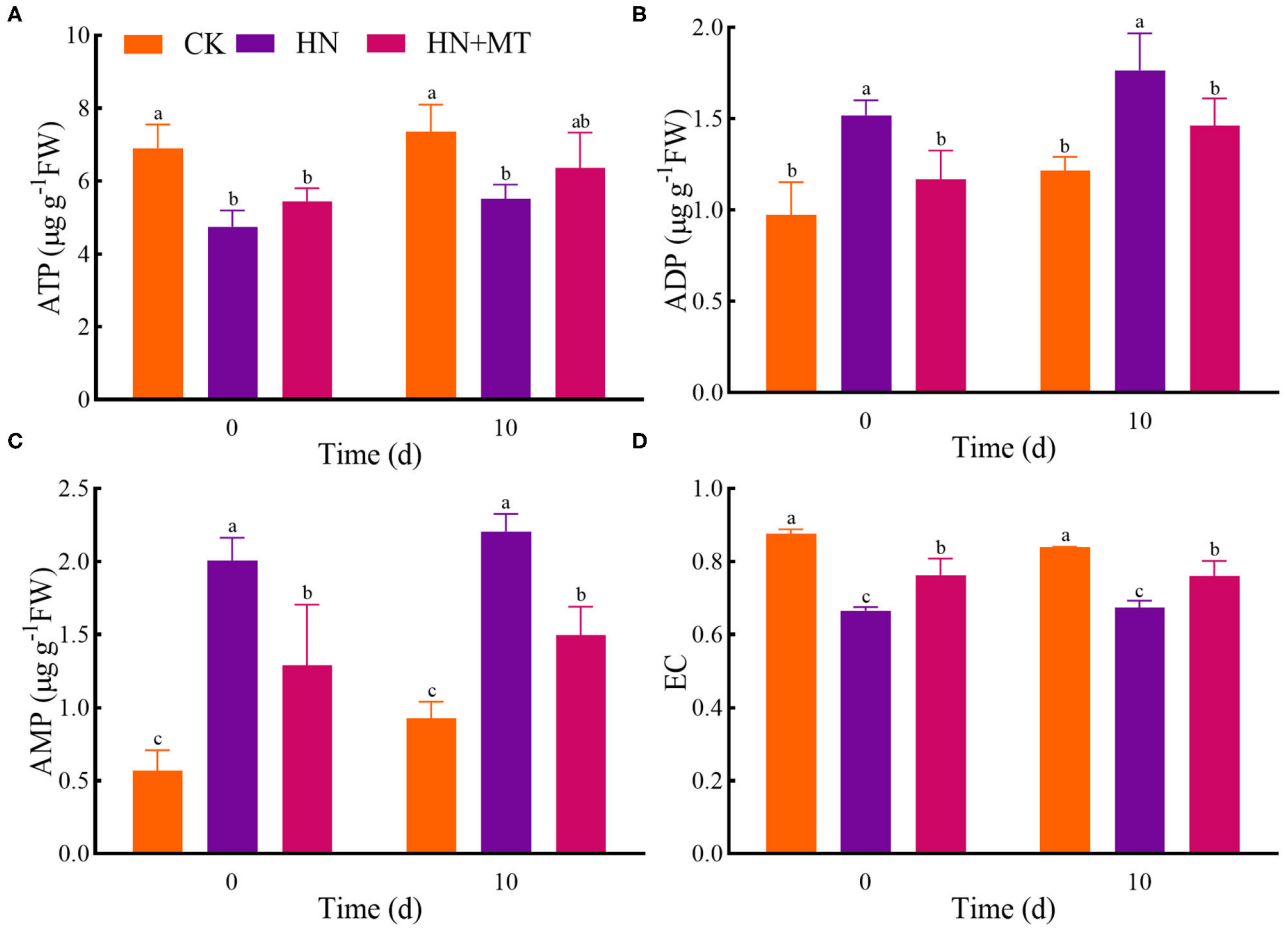

FIGURE 6 | Changes of ATP **(A)**, ADP **(B)**, AMP **(C)**, and EC **(D)**. The groups on the same day followed with the same letter are not significantly different at $p < 0.05$. Bars indicate the SDs of the means.

(**Figure 6**). Conversely, the HN+MT treatment increased ATP and EC whereas decreased ADP and AMP.

Correlation Analysis and Principal Component Analysis

Correlation analysis indicates that there was a positive relationship with each other in the height, leaf length, leaf width, leaf area, FW, DW, phosphorus, SP, NR, GS, GOGAT, GDH, ATP, and EC (**Figure 7A**). And these parameters were negatively correlated with nitrogen, sodium, potassium, calcium, NO_3^--N, NH_4^+-N, Pro, ADP and AMP, respectively. Nitrogen, sodium, potassium, calcium, NO_3^--N, NH_4^+-N, Pro, ADP, and AMP were positively correlated with each other. What's more, almost all of the relationships were significant, except for the relationships between FW and sodium, FW and ADP, DW and sodium, nitrogen and sodium.

Principal component analysis reveals that the 23 parameters were divided into PC 1 (88.6%) and PC 2 (4.1%). And PC 1 and PC 2 explained 92.7% of the differences in the 23 indicators. In addition, these 23 parameters were divided into two categories. Nitrogen, sodium, potassium, calcium, NO_3^--N, NH_4^+-N, Pro, ADP, and AMP were distributed in the second and third quadrants, and all of them showed an opposite relationship with the other parameters distributed in the first and fourth quadrants (**Figure 7B**).

DISCUSSION

As an evolutionarily highly conserved molecule, melatonin has a wide range of functions in plants. Studies have revealed that melatonin plays positive roles in improving the seedling growth and development of maize (Turk and Erdal, 2015), cucumber (Bałabusta et al., 2016; Zhang R. et al., 2017), soybean (Zhang J. et al., 2017), *Malus hupehensis* Rehd. (Gong et al., 2017), watermelon (Li et al., 2017), and saffower (Amjadi et al., 2021) under adverse conditions. In addition, a high-nitrate treatment inhibited shoot height, stem diameter, shoot/root dry weight, and total dry weight, whereas the pretreatment with 0.1 mmol L^{-1} melatonin dramatically increased these parameters under high-nitrate treatment (Zhang R. et al., 2017). In the present

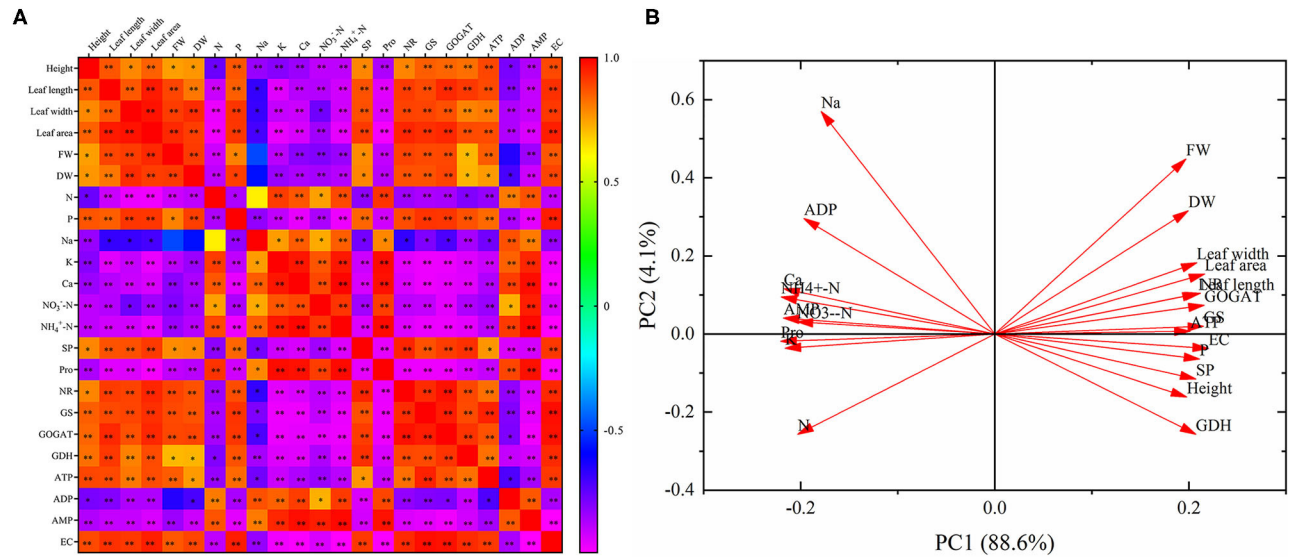

FIGURE 7 | Correlation analysis **(A)** and principal component analysis **(B)**.

study, the effect of melatonin on the morphological parameters in alfalfa (**Figure 1**) was consistent with the previous results (Zhang R. et al., 2017; Bose and Howlader, 2020). It indicates that melatonin can improve the growth and development of alfalfa under high-nitrate stress.

Ion uptake and compartmentalization are vitally important not only for improving normal growth but also for sustaining plant performance under high-salinity conditions because such adversity disturbs ion homeostasis (Adams et al., 1992). Excessive nitrogen is harmful to plants, even though nitrogen is essential for growth, development and metabolisms (Hao et al., 2015, 2017; Zhang R. et al., 2017; Wen et al., 2020). When nitrogen uptake exceeds plant assimilation capacity, plants will accumulate excessive NO_3^--N and NH_4^+-N, which will lead to soil secondary salinization and plant salt toxicity, thus affecting growth and development (Britto and Kronzucker, 2002; Roosta and Schjoerring, 2007; Zhang R. et al., 2017). In the present study, exogenous melatonin can enhance the capacity for nitrate reduction and ammonia assimilation, thereby alleviating the damage caused by high-nitrate stress. What's more, SP was rapidly degraded by high-nitrate stress both on days 0 and 10 (**Figure 3C**). Studies have revealed that Pro concentration of plants increases obviously under salinity, drought, heavy metals, high temperature, and pathogen infection (Krishnan et al., 2008; Liu et al., 2020). Because it can protect cells from damage by acting as both an osmotic agent and a radical scavenger. As a multi-functional molecule, Pro accumulated during a stress episode is degraded to provide a supply of energy to drive growth once the stress is relieved (Hayat et al., 2012; Kishor and Sreenivasulu, 2014). Furthermore, exogenous Pro plays an important role in improving and enhancing plant stress resistance (Hayat et al., 2012; Hussain et al., 2021). Hence, the Pro concentration increased in the HN treatment and decreased as melatonin alleviates high-nitrate stress. The results further suggest that excessive nitrogen severely affected nitrogen concentrations and plant growth of alfalfa, and the application of melatonin reversed these changes. That is, melatonin can improve the growth of alfalfa by inhibiting and controlling the excess nitrogen under high-nitrate stress.

Phosphorus is not only a constituent of many plant substances but also provides anion equivalents and is responsible for the charge balance in plants, especially those that are salt-stressed (Roosta and Schjoerring, 2007; Zhang R. et al., 2017). The effect of melatonin on phosphorus concentration in alfalfa (**Figure 2B**) is consistent with the previous studies (Yuan et al., 2015; Zhang R. et al., 2017). The adverse effects of salt stress on plants include osmotic stress, ionic stress, and secondary stress (Munns and Tester, 2008; Qin and Duan, 2019). Potassium is an indispensable inorganic ion in osmotic regulation (Golldack et al., 2003). Nitrates can cause osmotic stress and potassium can mitigate such stress (Zhang R. et al., 2017). Studies have shown that calcium provides intermolecular linkages and protects cell walls and membranes (Xu et al., 2014). Melatonin also plays a vital role in protecting cell membranes (Li et al., 2012; Zhang et al., 2013; Arnao and Hernández-Ruiz, 2019; Zhao et al., 2021). What's more, melatonin increases calcium concentration and works synergistically together in plants under stress (Zhang R. et al., 2017; Vafadar et al., 2020). In the present study, the treatment with KNO_3 and $Ca(NO_3)_2$ dramatically increased the concentrations of sodium, potassium, and calcium while melatonin limited their uptake under high-nitrate stress (**Figures 2C-E**). Melatonin likely regulates intracellular ion balance by controlling sodium and potassium, thus enabling

alfalfa to respond to osmotic stress caused by high-nitrate stress (Zhang R. et al., 2017). In addition, calcium intake exceeds the normal requirements of alfalfa, and melatonin can suppress excessive calcium intake to a certain extent. Thus, melatonin, along with appropriate calcium, plays a positive role in protecting the cells of alfalfa.

NR, GS, GOGAT, and GDH are the key enzymes involved in nitrogen metabolism. NO_3^--N is catalyzed to nitrite (NO_3^--N) by NR. NR is the rate-limiting enzyme in nitrogen metabolism. NR is also a substrate-inducing enzyme, which mainly occurs at the transcriptional level, and it is induced by NO_3^--N, carbohydrates and light, etc. Plant cells quickly transfer NO_3^--N from the cytoplasm to the chloroplasts of leaf cells or the cytoplasm of roots, where nitrite reductase (NiR) reduces NO_3^--N to NH_4^+ (Wen et al., 2020). Then, NH_4^+ from nitrate assimilation or photorespiration is transferred to amino acids to avoid the toxic effects of NH_4^+ accumulation (Liu et al., 2013; Zhang R. et al., 2017). NiR activity in plants is very strong, generally does not accumulate in plants, but NiR is very sensitive to salt stress. And salt stress reduces the activity of NR, so nitrate ions could not be converted into nitrite ions and accumulated in plants. GS, GOGAT, and GDH play crucial roles in NH_3 assimilation (Liu et al., 2013; Wen et al., 2020). GS catalyzes the synthesis of glutamine from NH_4^+ and glutamic acid. Furthermore, increased glutamine levels stimulate GOGAT and transfer the amides of glutamine to α-ketoglutarate, and produce glutamic acid. What's more, NH_4^+ can be assimilated via the deamination and synthesis of glutamate by GDH stimulation (Zhang R. et al., 2017; Wen et al., 2020). Although the activities of nitrogen metabolism enzymes are induced by NO_3^--N and NH_4^+-N, the high accumulations are toxic to higher plants because excessive accumulation of NO_3^--N and NH_4^+-N can cause strong feedback inhibition (Britto and Kronzucker, 2002; Zhang R. et al., 2017; Wen et al., 2020). Hence, high-nitrate stress inhibited the enzymes' activities, simultaneously inhibited protein synthesis and enhanced the hydrolase activities, and led to SP degradation; while melatonin significantly increased the enzymes' activities under high-nitrate stress (**Figures 3B**, **4**). The increased activity of these enzymes is related to melatonin's enhanced antioxidant properties because GS and GOGAT proteins can be oxidatively modified (Balestrasse et al., 2006; Reiter et al., 2016; Zhang R. et al., 2017; Bose and Howlader, 2020; Zhao et al., 2021). What's more, as an endogenous metabolite that can be degraded by plants, melatonin itself can promote plant development during different stages (Turk and Erdal, 2015; Arora and Bhatla, 2017). On the other hand, melatonin directly up-regulates *NR*, *GS*, and *GOGAT* to change the enzymes' activities under high-nitrate stress. It further illustrates the ability of melatonin to enhance enzyme activities and promote nitrogen metabolism in alfalfa.

As a universal intracellular energy currency, ATP directly provides energy for gene expression, metabolism, transport, and signal transduction pathways in all organisms (Cao et al., 2014; De Col et al., 2017; Tripathi et al., 2018). It is widely distributed in mitochondria, chloroplasts, and cytoplasm in cells. Itis also an important signaling molecule in the communication

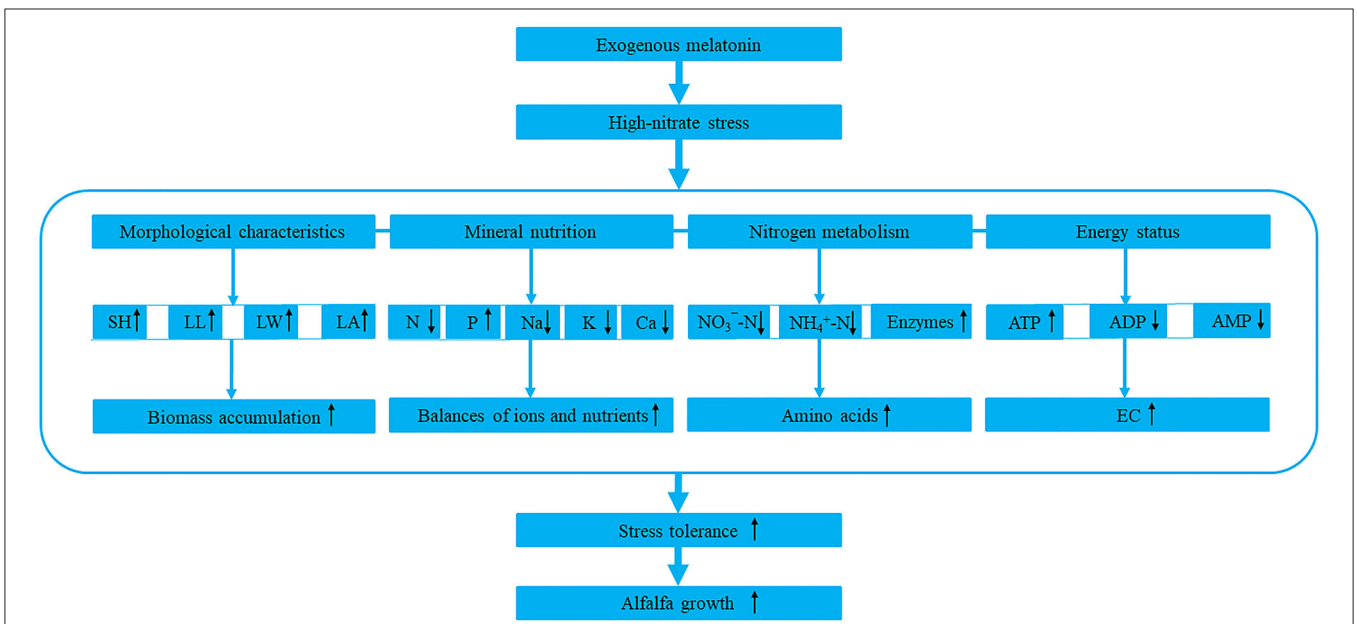

FIGURE 8 | The model of melatonin response to high-nitrate stress in alfalfa. SH, LL, LW, and LA represent shoot height, leaf length, leaf width, and leaf area, respectively. N, P, Na, K, and Ca represent nitrogen, phosphorus, sodium, potassium, and calcium, respectively. NO_3^--N and NH_4^+-N represent nitrate-nitrogen and ammonium-nitrogen, respectively. Enzymes include NR (nitrate reductase), GS (glutamine synthetase), GOGAT (glutamate synthase), and GDH (glutamate dehydrogenase). ATP, ADP, AMP, and EC represent adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, and energy charge. The upward or downward black arrows represent promotion or inhibition, respectively. The blue dash indicates that the four components are closely related to each other.

between cells. ATP in eukaryotic cells is synthesized via photosynthesis and respiration in plants (De Col et al., 2017; Gao et al., 2019). ATP is synthesized in the mitochondria, chloroplasts, and cytoplasmic stroma of plant cells and then transferred to the extracellular matrix in a variety of ways. Plant cytolysis occurs when cells are injured, which provides a passive pathway for the release of ATP. De-phosphorylation to ADP and AMP, and rephosphorylation to ATP allow high energy fluxes based on relatively small pool sizes in the cell (Rich, 2003; De Col et al., 2017). In addition, EC modulates the metabolisms related to energy utilization and regeneration because many physiological and molecular responses in plant cells are associated with energy state (Zhu et al., 2012). Studies have demonstrated that stresses such as anoxia (Huang et al., 2005), extreme temperature (Stupnikova et al., 2006), low pH (Messerli et al., 2005), and salt (Wu et al., 2019) deregulate the physiology of the plant cell and cause ATP overconsumption. In the present study, alfalfa under high-nitrate stress consumed more ATP and produced more AMP and ADP, thus resulting in a decrease in EC. Nevertheless, melatonin can protect alfalfa from high-nitrate stress, thereby enhancing ATP regeneration systems and reducing ATP-utilizing systems. What's more, correlation analysis and principal component analysis showed that the parameters of morphology, mineral nutrition, nitrogen metabolism, and energy status were closely correlated with each other, and they were divided into two principal components to explain the mechanisms. Namely, these parameters jointly regulate the growth of alfalfa under high-nitrate stress.

Based on the above, we summarized a model of melatonin response to high-nitrate stress (**Figure 8**). However, as a hormone, antioxidant and signaling molecule, melatonin has complex interactions with other hormones, and they jointly regulate and control the growth and development of plant. Therefore, the specific interactions and mechanisms in alfalfa need further study.

CONCLUSION

Melatonin inhibited the accumulations of NO_3^--N and NH_4^+-N by increasing the activities of enzymes involved in nitrogen metabolism and up-regulating the expression of their related genes in alfalfa. Melatonin also modulated mineral nutrition and energy status to alleviate the damage of high-nitrate to alfalfa. Thus, melatonin improved the growth of alfalfa under high-nitrate stress. Furthermore, the parameters correlated with each other and were divided into two principal components. It indicates that melatonin has a positive effect on modulating the morphology, mineral nutrition, nitrogen metabolism, and energy status of alfalfa under high-nitrate stress.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

ZC and JN conceived the ideas designed the methodology. ZC, XC, and JN conducted the experiments and analyzed the data. JN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Enhancement of *Nicotiana tabacum* Resistance Against Dehydration-Induced Leaf Senescence via Metabolite/Phytohormone-Gene Regulatory Networks Modulated by Melatonin

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Melatonin (MEL) is a pleiotropic agent with crucial functions reported in a variety of stress responses and developmental processes. Although MEL involvement in plant defense against natural leaf senescence has been widely reported, the precise regulatory mechanisms by which it delays stress-induced senescence remain unclear. In this study, we found that foliar spraying of melatonin markedly ameliorated dehydration-induced leaf senescence in *Nicotiana tabacum*, accompanied by attenuated oxidative damage, expression of senescence-related genes, and reduced endogenous ABA production. Metabolite profiling indicated that melatonin-treated plants accumulated higher concentrations of sugars, sugar alcohol, and organic acids, but fewer concentrations of amino acids in the leaves, than untreated plants after exposure to dehydration. Gene expression analysis revealed that the delayed senescence of stressed plants achieved by melatonin treatment might be partially ascribed to the upregulated expression of genes involved in ROS scavenging, chlorophyll biosynthesis, photosynthesis, and carbon/nitrogen balances, and downregulated expression of senescence-associated genes. Furthermore, hormone responses showed an extensively modulated expression, complemented by carotenoid biosynthesis regulation to achieve growth acceleration in melatonin-treated plants upon exposure to dehydration stress. These findings may provide more comprehensive insights into the role of melatonin in alleviating leaf senescence and enhancing dehydration resistance.

Keywords: dehydration stress, melatonin, reactive oxygen species, multi-omics, hormone profiling, carotenoid

INTRODUCTION

Drought is one of the most prominent environmental limitations restricting agricultural sustainability and food security worldwide (Tardieu et al., 2018). As sessile organisms, plants are inevitably challenged to detect and respond to water deprivation to mitigate negative effects on their development and yield (Antoniou et al., 2017). Drought stress triggers excessive accumulation

of reactive oxygen species (ROS), which can cause oxidative damage to DNA, proteins, membrane lipids, and chlorophyll, leading to an imbalance in cellular metabolism (Corpas and Barroso, 2013; Sun et al., 2018). This redox imbalance decreases the photosynthetic performance of plants by interfering with the chloroplast structure and photosystem reaction centers (Shao et al., 2016; Sharma et al., 2020). Thus, improving the ability of crop plants to cope with reduced water availability has emerged as one of the most urgent aims for maintaining agricultural productivity under climate change conditions.

Plants have evolved highly sophisticated strategies to mediate the detrimental effects of drought, such as stomatal closure to decrease transpiration rates and accumulation of osmoprotectants and antioxidants to cope with ROS imbalance (Mundada et al., 2021; Wang et al., 2021). Particularly, during water deficit, the abscisic acid (ABA) production of plants is markedly increased and activates a complex signaling network that induces massive reprogramming of ABA-dependent gene expression, subsequently modulating cellular and physiological acclimation responses (Zhu, 2016; Kuromori et al., 2018). Additionally, plant tolerance to drought involves complex regulatory networks that control global gene expression, protein modification, and metabolite composition (Urano et al., 2010; Kim et al., 2017). At the transcriptional level, several different types of transcription factors (TFs), such as bHLH, bZIP, MYB, dehydration-responsive element-binding (DREB), and WRKY families, regulate drought responses by activating downstream defense genes (Sakuraba et al., 2015; Wang et al., 2020; Xiang et al., 2020). These adaptation responses constitute a critical part of the core advantages in sustaining plant survival under water-deficit conditions, although other unidentified metabolic adjustment could also be involved in stressed plant survival. However, if stress conditions are prolonged, the internal defense system of plants is insufficient to regulate ROS balance, ultimately causing oxidative damage to cellular constituents (Lee et al., 2012).

As the final and inevitable stage of leaf development, leaf senescence is controlled by highly coordinated action at the cell, tissue, organ, and organism levels (Lin et al., 2015). Plants initiate leaf senescence to facilitate the remobilization of nutrients and energy from senescing leaves into growing organs and optimize growth fitness at the end of the growing season (Zhang et al., 2018; Jalil et al., 2019), which is a developmental process mediated by aging and can also be triggered by unfavorable environmental conditions, such as nitrogen limitation, darkness, excessive light, high salinity, and desiccation (Yang et al., 2015; Zhao et al., 2016; Kamranfar et al., 2018). Moreover, leaf senescence is promoted by multiple plant hormones, such as ABA, ethylene, salicylic acid (SA), and jasmonic acid (JA), but repressed by cytokinin (Gan and Amasino, 1995; Cutler et al., 2010; Jiang et al., 2014; An et al., 2019). Stress-induced leaf senescence affects the extended photosynthesis duration and ultimately compromises plant growth and development. For example, drought stress results in premature leaf senescence, characterized by a reduction in chlorophyll synthesis owing to chloroplast disassembly and membrane ion leakage (Ma et al., 2018). The senescence processes are often associated with the upregulated expression of genes for several senescence-associated enzymes, such as chlorophyllase, pheophytinase, and Chl-degrading peroxidase, and downregulated expression of photosynthetic genes, including RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN (RBCS) (Jalil et al., 2017; Ma et al., 2018; Zhang et al., 2018; He et al., 2021). In nature, accelerated leaf abscission and senescence are characteristics of drought tolerance because these processes contribute to the maintenance of water balance in the whole plant body, thereby improving the chance of plant survival under drought conditions (Pic et al., 2002). However, in crop species, leaf senescence has been linked to yield reduction because it decreases the accumulation of photosynthetic-assimilates. As transgenic plants with delayed leaf senescence have been shown to exhibit enhanced drought resistance associated with higher photosynthetic efficiency (Rivero et al., 2007), intervention of the senescence process by agents could confer dehydration stress tolerance in plants.

Melatonin (MEL, *N*-acetyl-5-methoxytryptamine), a well-known multifunctional biomolecule, was discovered over 60 years ago and has been widely applied in physiology and medicine (Reiter et al., 2014). In mammals, MEL exerts significant biological activities in regulating circadian rhythm, sleep–wake cycles, mood, sexual and reproductive behavior, seasonal photoperiod, immunological system, and detoxification of free radicals (Hernández-Ruiz et al., 2005; Calvo et al., 2013; Shi et al., 2015a). Plant MEL was initially identified in 1995 and has shown great potential in the regulation of plant growth, immunity, phytoremediation, and plant–rhizomicrobial interactions (Dubbels et al., 1995; Li et al., 2021). In the past 20 years, MEL has generated considerable interest, with hundreds of articles relating to MEL in plants published in PubMed (**Supplementary Figure 1**). As reviewed by Zhang et al. (2015), MEL biosynthesis in plants occurs through a biosynthesis pathway similar to that in animals, with four consecutive enzymes, including tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), arylalkylamine *N*-acetyltransferase (AANAT), and *N-acetylserotonin* methyltransferase (ASMT), identified to participate in MEL biosynthesis. In transgenic experiments, transgenic plants overexpressing ovine *AANAT* and ovine *ASMT* genes exhibited MEL levels significantly higher than that of the wild type (Park et al., 2013; Wang et al., 2014). In 2018, the first MEL plant receptor (CAND2/PMTR1) involved in MEL signaling perception was identified in *Arabidopsis* (Wei et al., 2018), and is thought to be a potential candidate phytohormone.

MEL is implicated in regulating various biological processes in plant systems, such as seed germination, growth, floral transition, rooting induction, photosynthesis, ripening/senescence, and cell protective responses (Arnao and Hernández-Ruiz, 2019). As an emerging plant growth regulator, MEL plays a prominent role in enhancing plant tolerance to stress stimuli, and at appropriate concentrations, most abiotic stresses causing oxidative damage could be alleviated by MEL (Arnao and Hernández-Ruiz, 2014). As several other classic antioxidants, MEL functions as a free radical scavenger to protect plants against a variety of biotic and abiotic stressors, such as pathogen infection, high salinity, alkalinity, extreme temperature, radiation, and heavy metal exposure (Yamamoto and Mohanan, 2001; Meng et al., 2014;

Gong et al., 2017; Siddiqui et al., 2019, 2020; Li et al., 2021), and it can directly detoxify ROS and indirectly regulate oxidative homeostasis in plants by activating cellular enzymatic and non-enzymatic antioxidants (Arnao and Hernández-Ruiz, 2014). Furthermore, MEL has been shown to protect plants from acid rain (Debnath et al., 2018), paraquat (Wang et al., 2018), and senescence (Wang et al., 2013a), and herbicide-induced oxidative stress was significantly ameliorated in MEL-rich transgenic plants (Park et al., 2013). In recent years, MEL has been found to be effective in preserving chlorophyll content in several plants, including barley (Arnao and Hernández-Ruiz, 2009), perennial ryegrass (Zhang et al., 2016), and rice (Liang et al., 2015). Although tremendous progress has been achieved in unraveling MEL mitigation effects in plant responses to abiotic stress, little is known about the detailed mechanisms of leaf senescence regulation by MEL to enhance dehydration resistance.

Tobacco is not only a model plant, but also an economically influential crop worldwide. While tobacco's development is highly susceptible to water stress, drought resulted in decreases of the biomass and reducing sugar content, and increases of total nitrogen and nicotine contents in flue-cured tobacco plants. Here, we studied the response of tobacco (*Nicotiana tabacum* L.) pretreated with MEL to long-term dehydration stress, with the aim of elucidating MEL-mediated metabolite/phytohormone-gene regulatory networks. To this end, an integrated analysis of the transcriptome and metabolome was employed to investigate the effects of external MEL application on dehydration-induced leaf senescence. The findings from this study not only provide a crucial therapeutic strategy to mitigate the extreme water deficit, but also reveal the protective role of MEL in repressing dehydration-induced leaf senescence.

MATERIALS AND METHODS

Plant Materials and Treatments

Sterilized tobacco seeds were germinated in the dark and then transferred to pots containing a mixture of peat and vermiculite (1:1, v/v) for culture in a greenhouse, as previously described (Chen et al., 2019). For MEL treatment, half of the 3-week-old potted seedlings were sprayed with 100 μM MEL solution (10 ml) every day for 1 week. In a previous trial, this treatment was shown to be quite effective in alleviating abiotic stress (Ye et al., 2016; Wang et al., 2019). Prior to dehydration treatment, the seedlings were bottom-irrigated with Hoagland's nutrient solution three times a week. After 4 weeks in the glasshouse, both control and MEL-treated plants were exposed to the cessation of watering. Dehydration stress was imposed by withholding watering for 15 days, when tobacco seedlings exhibited extensive phenotypic foliar injury (in the form of severe wilting and leaf chlorosis). In the experimental design (**Figure 1A**), healthy and uniform seedlings were subjected to four treatments: CK (non-MEL/well-watered treatment), MEL (100 μM MEL-applied/well-watered treatment), D (non-MEL/dehydration treatment), and MEL_D (100 μM MEL-applied/dehydration treatment). The fourth plant leaves were sampled at the indicated time points and kept at −80°C until further processing. Non-stressed controls were sampled at 4 weeks + 15 days in parallel with the drought treatment.

Histochemical Assays

In situ accumulation of O_2^- and H_2O_2 was visually detected using nitroblue tetrazolium (NBT) and diaminobenzidine (DAB) as substrates, respectively (Sen et al., 2017). Intracellular ROS levels were evaluated by dihydroethidium and then visualized using a Nikon Eclipse E600 epifluorescence microscope. For programmed cell death, the samples were assessed with a TUNEL apoptosis assay, as previously described (Li et al., 2018).

Electron Microscopy

Observation of leaf stomata was performed using an SU8010 scanning electron microscope (SEM, Hitachi, Tokyo, Japan), and its aperture was determined using ImageJ software (National Institutes of Health, Bethesda, MD, United States). Additionally, leaf ultrastructure was observed and imaged at 80 kV using a Hitachi H-7650 transmission electron microscope.

Physiological and Biochemical Detection

Relative water content (RWC) was measured following the method of Xiao et al. (2009), and photosynthetic rate (Pn) was determined as described by Huo et al. (2015). The maximal photochemical efficiency (F_v/F_m) was determined using a PAM-2100 Chl fluorometer (Walz, Effeltrich, Germany). Electrolyte leakage (EL) was determined according to Shi et al. (2012). Chlorophyll content was estimated as reported by Gang et al. (2010). Malondialdehyde (MDA) content, superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) levels, and the activities of antioxidant enzymes (SOD [E.C. 1.15.1.1], POD [E.C. 1.11.1.7], and CAT [E.C. 1.11.1.6]) were determined using the corresponding detection kits (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. Determination of ascorbic acid (AsA) and dehydroascorbate (DHA) levels was performed according to the method of Xing et al. (2019).

Metabolome Analysis

Metabolite extracts were analyzed using a Vanquish UHPLC system (Thermo Fisher, United States) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher, United States). The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud[1] and ChemSpider[2] database to obtained the accurate qualitative and relative quantitative results. We applied univariate analysis (*t*-test) to calculate the statistical significance (*P*-value). The metabolites with VIP $>$ 1 and *P*-value $<$ 0.05 and fold change $\geq$ 2 or FC $\leq$ 0.5 were considered to be

[1]www.mzcloud.org/

[2]www.chemspider.com/

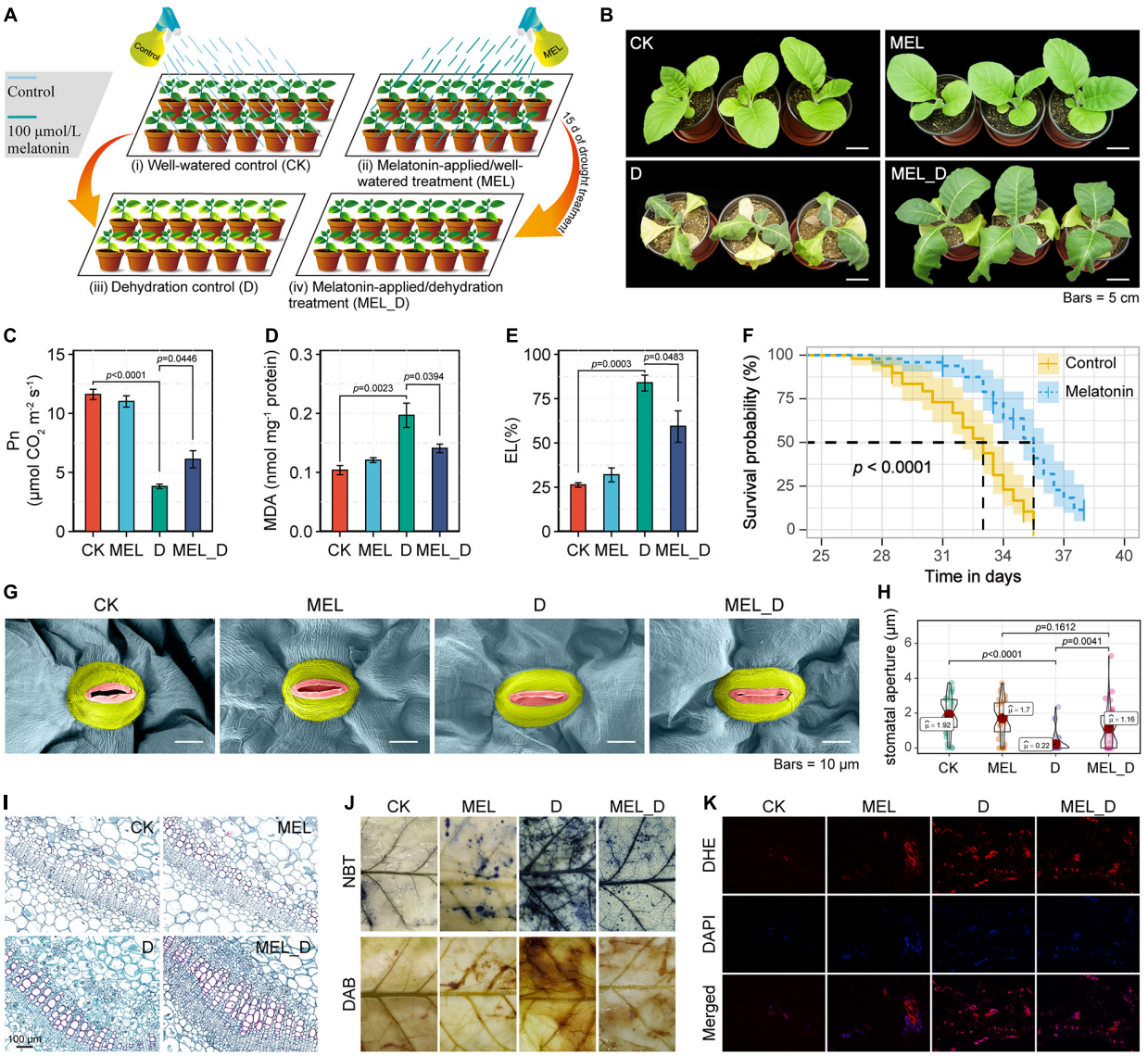

FIGURE 1 | Mitigation effects of exogenous melatonin on dehydration stress in *Nicotiana tabacum* seedlings. **(A)** Diagrammatic representation of the experimental design. CK, well-watered control; MEL, melatonin (MEL)-applied/well-watered treatment; D, dehydration control; MEL_D, melatonin-applied/dehydration treatment. The schematic was created using Adobe Illustrator software. **(B)** Phenotypic effects of melatonin pretreatment on *N. tabacum* seedlings exposed to dehydration stress for 15 days. **(C–E)** Measurement of photosynthetic rate (Pn), MDA concentration, and electrolyte leakage (EL). Error bars represent the SE calculated from three independent biological replicates. **(F)** Survival analysis of control and melatonin (100 μmol)-pretreated 2-week-old seedlings subjected to prolonged drought stress, by withholding watering (n = 50). **(G)** Scanning electron microscope (SEM) images of stomata from the fourth leaves of plants at 15 days of dehydration treatment. **(H)** Statistical analysis of stomatal aperture. **(I)** Images of transverse sections of the stems of seedlings after 15 days of dehydration stress. Red indicates degree of lignification. **(J)** Histochemical staining of control and MEL treatment leaves exposed to dehydration stress for 15 days. **(K)** Fluorescence microscopic analysis of leaf ROS levels. Results of dihydroethidium (DHE) staining (upper), 4,6-diamidino-2-phenylindole (DAPI) staining (middle), and overlapping images (lower) have been presented.

differential metabolites. The functions of metabolites and metabolic pathways were studied using the KEGG database. The reproducibility of untargeted analysis was assessed using six biological replicates.

Transcriptomic Analysis

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, United States) following manufacturer's protocol and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced using

an Illumina Novaseq platform and 150 bp paired-end reads were generated. After removing the adapters and low-quality sequence reads from the raw reads by Trimmomatic, the clean reads were mapped to the tobacco genome[3] using Hisat2 v2.0.5. The gene expression value was calculated as fragments per kilobase of transcript per million mapped reads (FPKM). Functional assignment of genes was conducted using Gene Ontology (GO) enrichment analysis. The reproducibility of the RNA-seq analysis was assessed using three biological replicates. The raw RNA-sequencing data were deposited in the NCBI database under the accession number PRJNA691642.

qPCR Assay

Total RNA extraction and first-strand cDNA synthesis were performed as previously described (Xia et al., 2018). qPCR analysis was performed using a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States) according to the manufacturer's recommendation. The relative gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and the tobacco *actin* gene was used as an internal control. Primer sequences were listed in **Supplementary Table 8**.

Detection of Plant Hormone Content

Fresh tobacco leaves (50 mg) were frozen in liquid nitrogen, ground into powder, and extracted with 1 mL methanol/water/formic acid (15:4:1, V/V/V). The combined extracts were evaporated to dryness under nitrogen gas stream, reconstituted in 100 μL 80% methanol (V/V), and filtered through 0.22 μm filter for further LC-MS analysis. The sample extracts were analyzed using an LC-ESI-MS/MS system (UHPLC, ExionLC^{TM} AD[4]; MS, Applied Biosystems 6500 Triple Quadrupole, see Text Footnote 4). AB 6500 + QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in both positive and negative ion modes and controlled by Analyst 1.6 software (AB Sciex).

Determination of Carotenoid Composition

Given that carotenoids are precursors of ABA synthesis, the carotenoid composition in the leaves of the four treatments was detected. Carotenoid extracts were analyzed using an LC-APCI-MS/MS system (UHPLC, ExionLC^{TM} AD; MS, Applied Biosystems 6500 Triple Quadrupole). A YMC C30 column (3 μm, 100 mm × 2 mm) was used for HPLC analysis. The experiments were performed at 28°C with a flow rate of 0.8 mL/min. The mobile phase consisted of solvent A, i.e., methanol: acetonitrile (1:3, v/v) containing 0.01% BHT and 0.1% formic acid, and solvent B (methyl tert-butyl ether with 0.01% BHT). The gradient program was as follows: 0% B (0–3 min), increased to 70% B (3–5 min), then increased to 95% B (5–9 min), and finally ramped back to 0% B (11–12 min). MS analysis was carried out using the API 6500 + Q TRAP LC-MS/MS System, equipped with an APCI Turbo Ion-Spray interface, operating in positive ion mode and controlled by Analyst 1.6.3 software (AB Sciex).

Statistical Analysis

Statistical analyses were performed using the statistical software R (R version R-4.0.2). Physiological and biochemical measurements were subjected to one-way ANOVA, and then, significant differences between individual means were assessed using Tukey's test. Three developmentally identical leaves were pooled for each biological replicate. At least nine tobacco plants are required for three biological replicates.

RESULTS

Exogenous Application of Melatonin Improves Dehydration Stress Resistance in *Nicotiana tabacum*

Phenotypic analysis revealed that exogenous MEL improved the tolerance of tobacco plants to water deprivation (**Figure 1B**), which was supported by positive changes in Pn, RWC, and biomass accumulation (**Figure 1C** and **Supplementary Figure 2**). With water deprivation, MEL_D-treated plants experienced less cell membrane damage than D controls, as verified by MDA content and EL in MEL_D being lower than those in D (**Figures 1D,E**). MEL and CK treatments displayed no differences for all examined parameters. Moreover, drought survival analysis showed that 2-week-old seedlings pretreated with MEL displayed higher survival rates relative to untreated seedlings after long-term dehydration stress (**Figure 1F**). Scanning electron microscopy showed that the stoma area in D- or MEL_D-treated leaves was smaller than that in well-watered leaves, while the stomata of stressed leaves were almost closed (**Figures 1G,H**), indicating that both D and MEL_D treatments dramatically induced stomatal closure. Subsequent anatomical observations revealed that MEL accelerated lignin biosynthesis and Casparian strip formation in tobacco plants compared to those of non-treated stressed plants (**Figure 1I** and **Supplementary Figure 2**).

Additionally, O_2^- and H_2O_2 were observed *in situ* using NBT and DAB staining, respectively, with CK- and MEL-treated leaves being similarly and lightly stained without water deprivation. The staining became darker with dehydration, but more intense staining was seen in D than in MEL_D (**Figure 1J**), which was further confirmed by quantitative measurement (**Supplementary Figure 3**). Consistently, fluorescence microscopic analysis of ROS level also showed ROS toxicity in MEL-pretreated leaves being lower than that in untreated leaves under dehydration conditions (**Figure 1K**). Furthermore, MEL treatment increased the expression of several stress-responsive genes (*NtSOD*, *NtCAT*, *NtAPX*, *NtGST*, *NtLTP1*, *NtRD29A*, *NtERD10C*, *NtERD10D*, *NtSAMDC*, and *NtSPSA*) (**Supplementary Figure 3**) and accumulated higher antioxidant levels (SOD, POD, CAT, AsA, and DHA) (**Supplementary Figure 4**) compared with those of non-pretreated stressed plants.

[3] ftp://ftp.solgenomics.net/genomes/Nicotiana_tabacum/

[4] https://sciex.com.cn/

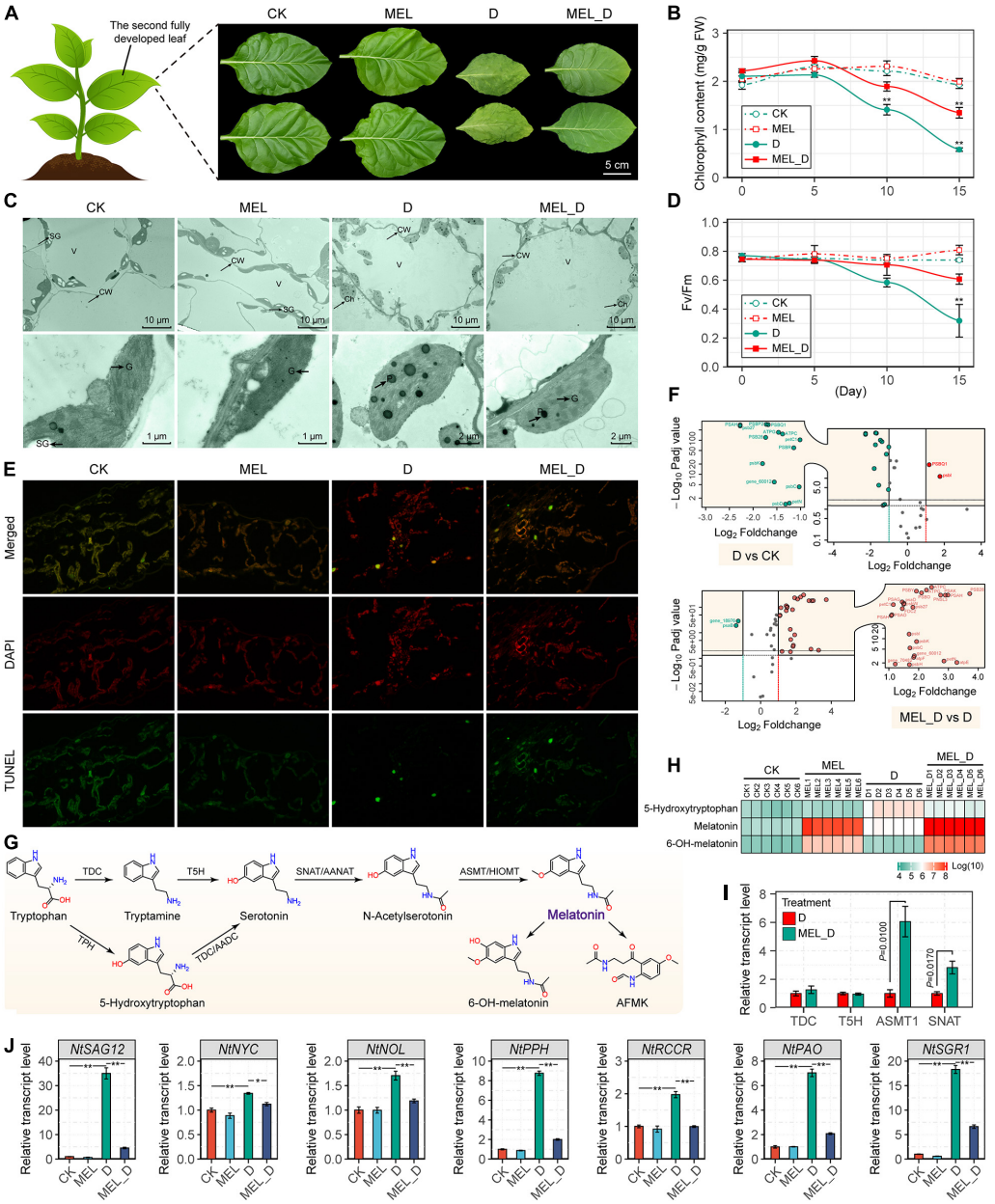

FIGURE 2 | Melatonin application delays leaf senescence of *N. tabacum* subjected to dehydration stress. **(A)** Senescence phenotypes of the fourth leaves of control and melatonin-treated plants after the 15-day dehydration treatment. **(B,C)** Changes in F_v/F_m and total chlorophyll contents in response to dehydration stress ($n = 3$; $^*P < 0.05$ and $^{**}P < 0.01$ compared with those in CK, according to Tukey's test). **(D)** Ultrastructure of chloroplasts in leaves of control and melatonin-treated seedlings. Ch, chloroplast; CW, cell wall; SG, starch grain; V, vacuole; G, granal thylakoids; P, plastoglobuli. **(E)** TUNEL assay of leaf cell death. **(F)** Volcano plot showing the expression profiles of photosynthetic genes. The *X*-axis shows the log2 fold change expression; the *Y*-axis show significant differences at *P*-values (log10 transformation) < 0.05. Red and green dots represent upregulated expression of genes (multiples vary more than two times and *P*-value < 0.05) and downregulated expression of genes, respectively. **(G)** Melatonin biosynthesis pathway. **(H)** Changes in the levels of endogenous melatonin and derivatives in *N. tabacum* leaves. **(I)** Transcript levels of melatonin biosynthetic genes under water-deficit conditions. **(J)** Expression of genes responsible for leaf senescence. The vertical bars indicate SE ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$.

Melatonin Ameliorates Dehydration-Induced Premature Senescence in *Nicotiana tabacum*

After water was withheld for 15 days, the leaves showed obvious yellowing (**Figure 2A**) and the total chlorophyll content in those plants was dramatically lower than that in the well-watered controls (**Figure 2B**). MEL-treated plants clearly showed retardation of dehydration-inducible leaf senescence, as shown by a decrease in chlorophyll and F_v/F_m on days 10 and 15 (**Figures 2B,C**). Additionally, the anatomical observation of

the control and MEL-treated leaves indicated that mesophyll cell density was lower in D than in MEL_D (**Supplementary Figure 5**), resulting in a stay-green phenotype in MEL_D-treated leaves. Further, transmission electron microscopy showed that treatments with dehydration severely disrupted the thylakoid membranes, reduced the starch grain quantities, and increased the osmiophilic granules in the chloroplast. However, the chloroplast ultrastructure in the MEL_D-treated leaves was similar to that in the unstressed leaves (**Figure 2D**), showing that MEL protected chloroplasts. TUNEL staining indicated that dehydration-inducible cell death was relieved by exogenous MEL application (**Figure 2E**). Using transcriptomic analysis, we found that the expression of most photosynthesis-associated transcripts was dramatically reduced in leaves of D compared with those of CK, whereas these genes displayed higher transcript levels in MEL_D than in D (**Figure 2F** and **Supplementary Figure 5**).

Moreover, we found that the endogenous levels of MEL and its derivative, 6-OH-MEL, were significantly elevated after treatment with 100 μM MEL (**Figures 2G,H**), and MEL supplementation also increased the expression of tobacco MEL biosynthetic genes, with *NtTDC*, *NtASMT1*, and *NtSNAT* expression in MEL-treated leaves being higher than those in non-treated leaves under dehydration stress (**Figure 2I**). Expression of a senescence marker (*NtSAG12*) and six chlorophyll catabolic genes (*NtNYC*, *NtNOL*, *NtPPH*, *NtRCCR*, *NtPAO*, and *NtSGR1*) was upregulated in response to dehydration stress, whereas their levels were decreased by MEL (**Figure 2J**).

Metabolites Accumulate in Melatonin-Treated Leaves Under Dehydration Conditions

To explore the impact of MEL supplementation on the metabolomic response of tobacco plants to dehydration stress, the relative abundance of metabolites was analyzed (**Supplementary Figure 6** and **Supplementary Tables 1–3**). Principal component analysis (PCA) of all the detected metabolites showed that CK was not separated from MEL but was clearly separated from the D or MEL_D groups (**Figures 3D,E**). Moreover, there was a clear separation between the D and MEL_D groups. The D and MEL_D treatments induced significant accumulation of various sugars, such as sucrose, fructose, raffinose, glucose, and hexose (**Figure 3A**). Conversely, the accumulation of most organic acids was dramatically decreased in both D- and MEL_D-treated seedlings, including citric acid, phosphoric acid, ketoglutaric acid, adipic acid, aminobutyric acid, and palmitoleic acid (**Figure 3B**). Among amino acids, the levels of valine, leucine, tyrosine, histidine, and lysine were elevated in the dehydration-treated seedlings, whereas the levels of alanine, serine, aspartic acid, glutamine, and pyroglutamic acid were reduced relative to those of the controls (**Figure 3C**).

Next, the associations between MEL levels and the identified metabolites were investigated using correlation analysis (**Figure 3F**). Five metabolites were strongly positively correlated with MEL induction, which included galactitol, glucose, hexose, proline, and arachidonic acid. Contrastingly, 12 metabolites were strongly negatively correlated with MEL induction, including trehalose, alanine, serine, aspartic acid, glutamine, L-pyroglutamic acid, citric acid, phosphoric acid, ketoglutaric acid, adipic acid, GABA, and palmitoleic acid (**Figure 3F**). Additionally, 18 identified metabolites were assigned to carbohydrate and tricarboxylic acid (TCA) metabolic pathways (**Figure 3G**). The carbohydrate levels and metabolites of the TCA cycle were reduced following dehydration treatment. Among them, 12 metabolites were increased in the MEL_D group relative to those in D, such as serine, threonine, succinate, citric acid, ketoglutaric acid, glutamic acid, and glutamine. These results further confirmed that tolerance to dehydration stress may be largely dependent on carbon and amino acid metabolism.

Comprehensive Sets of DEGs in Melatonin-Treated Leaves Under Dehydration Conditions

Transcriptomic analysis identified more than 30,000 differentially expressed genes (DEGs) in MEL-, D-, and MEL_D-treated leaves versus CK (**Supplementary Tables 4–6**). Compared to the transcripts of untreated leaves, D-treated leaves had 7,525 and 12,232 upregulated and downregulated genes, respectively. Compared to D samples, MEL_D-treated samples had 8,111 and 4,989 upregulated and downregulated genes, respectively. Venn diagrams revealed that the number of genes commonly downregulated in D was greater than that in MEL_D (**Figures 4A,B**). Although a wide array of GO terms were commonly or differentially enriched among the analyzed sets, photosynthesis was the most enriched GO term, mainly because of the large number of corresponding enzymes being downregulated upon dehydration treatment (**Figure 4C**). The qPCR analysis of six randomly selected genes (*NtBH0283*, *NtARR6*, *NtCAO*, *NtCIPK1*, *NtGDCSP*, and *NtLOG1*) confirmed the accuracy of RNA-seq (**Figure 4D**). The transcription of most genes involved in photosynthetic light reactions and the Calvin cycle in D was decreased compared to those in MEL_D (**Figure 4E**). *Cyt-INV* and sucrose synthase, which are responsible for sucrose degradation, were upregulated in the D and MEL_D treatments (**Figure 4F**), and the AGPase and starch synthase genes, which encode enzymes for starch biosynthesis, were highly expressed in MEL_D. However, the expression of five AGPase and four starch synthase genes was reduced in D. Compared to that in CK, the expression of most genes encoding alpha- and beta-amylases, which are key enzymes involved in the degradation of starch, was not changed in MEL_D, whereas the transcription of four alpha-amylases and four beta-amylases was significantly increased in D. The expression patterns of genes related to starch degradation agreed with the decrease in starch under D treatment and its accumulation under MEL_D treatment conditions.

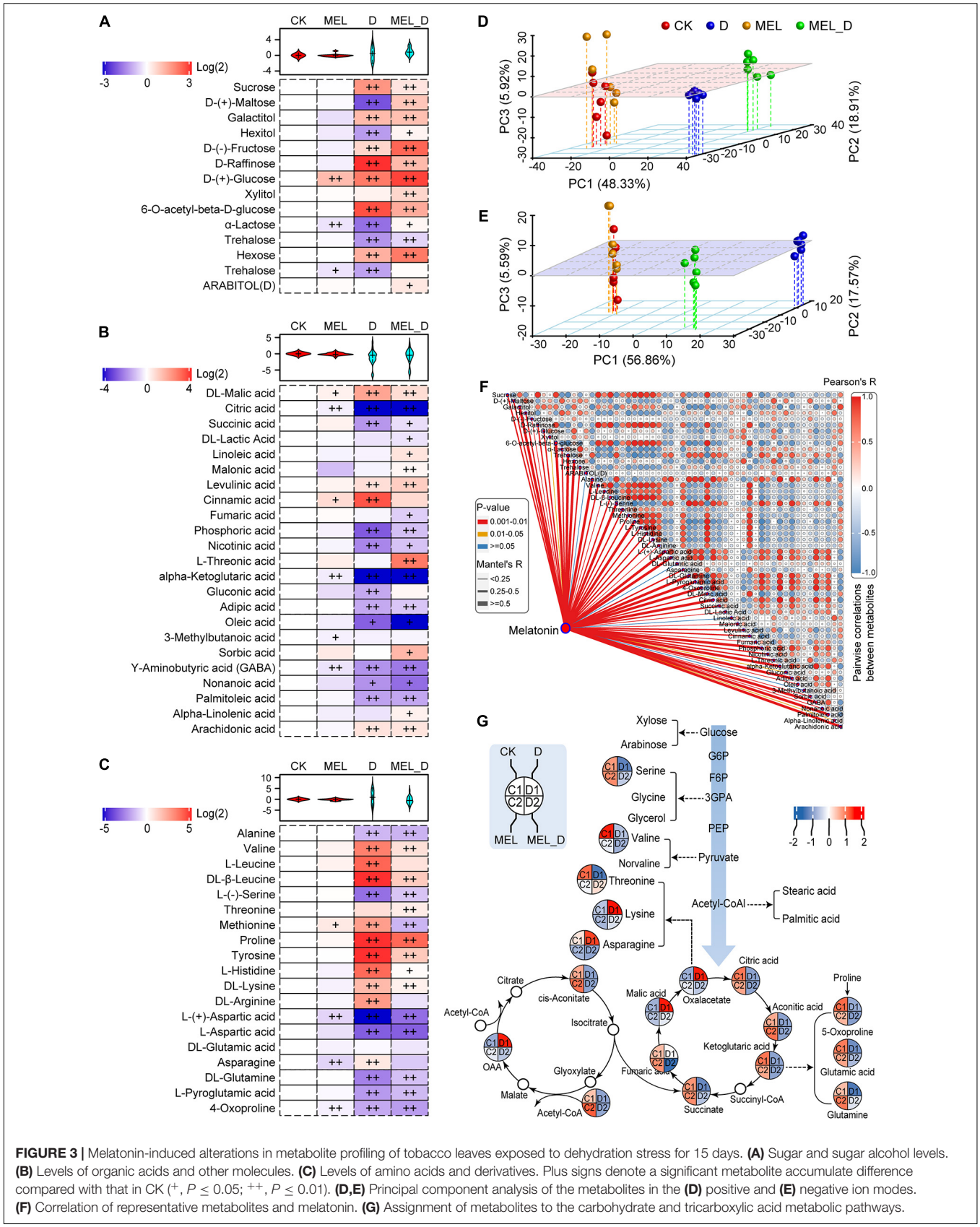

FIGURE 3 | Melatonin-induced alterations in metabolite profiling of tobacco leaves exposed to dehydration stress for 15 days. **(A)** Sugar and sugar alcohol levels. **(B)** Levels of organic acids and other molecules. **(C)** Levels of amino acids and derivatives. Plus signs denote a significant metabolite accumulate difference compared with that in CK ($^{+}$, $P \leq 0.05$; $^{++}$, $P \leq 0.01$). **(D,E)** Principal component analysis of the metabolites in the **(D)** positive and **(E)** negative ion modes. **(F)** Correlation of representative metabolites and melatonin. **(G)** Assignment of metabolites to the carbohydrate and tricarboxylic acid metabolic pathways.

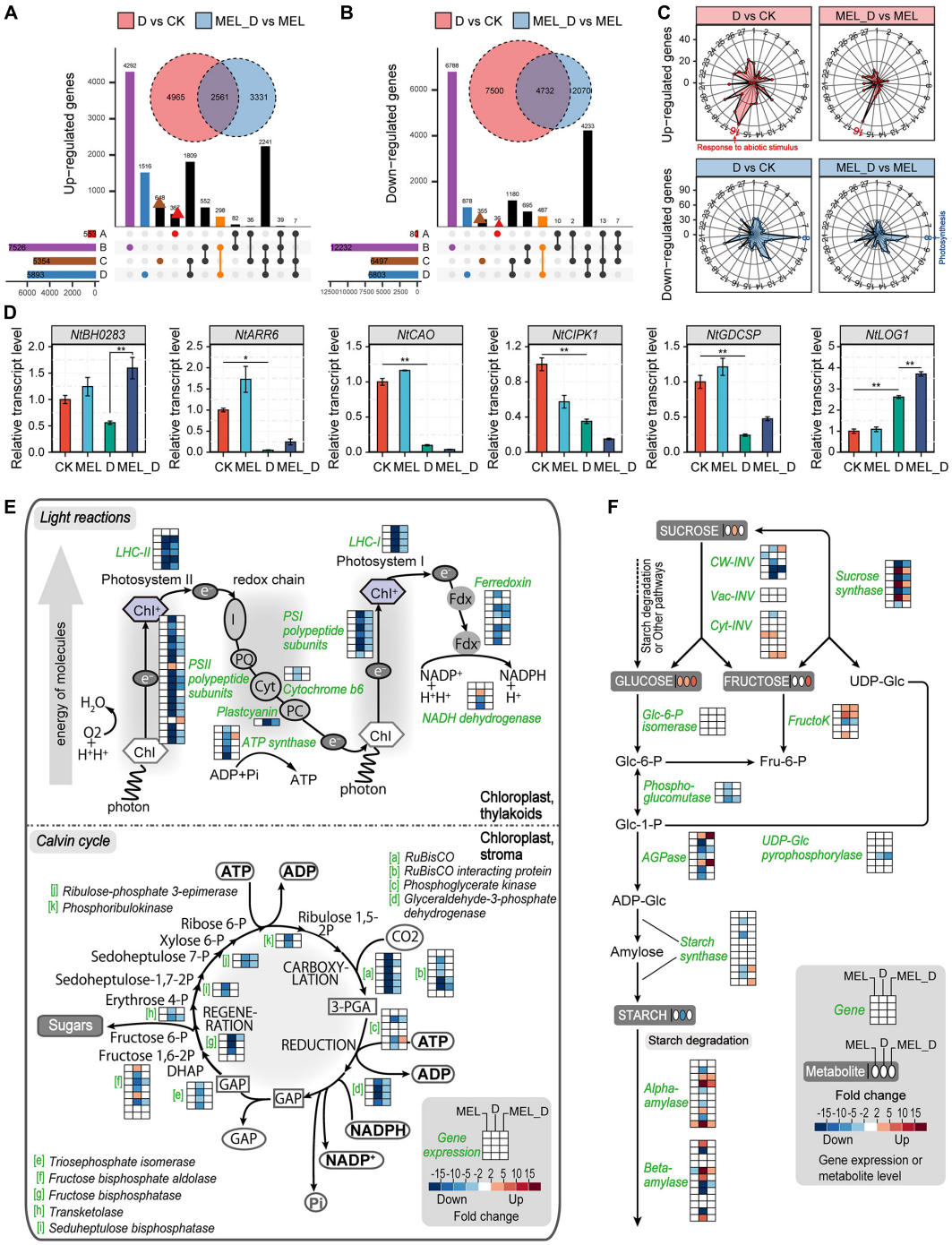

FIGURE 4 | Comprehensive expression profiles of genes in tobacco leaves after dehydration stress for 15 days. **(A,B)** Comparison of the numbers of differentially expressed genes (fold change ≥ 3.0) acquired through the transcriptome analysis. Four comparison groups were analyzed, containing treatments of melatonin (MEL) versus CK **(A)**, D (dehydration) versus CK **(B)**, MEL_D (dehydration plus melatonin) versus CK **(C)**, and MEL_D versus D **(D)**. **(C)** Enriched Gene Ontology (GO) terms of DEGs. Radar charts of 27 upregulated and downregulated GO classes showing the frequency of each class. 1, carbohydrate metabolism; 2, energy metabolism; 3, lipid metabolic process; 4, nucleotide metabolism; 5, amino acid metabolism; 6, organic acid metabolism; 7, signal transduction; 8, photosynthesis; 9, DNA replication; 10, cell redox homeostasis; 11, defense response; 12, cell wall organization; 13, response to hormone; 14, response to stress; 15, response to oxidative stress; 16, response to abiotic stimulus; 17, pigment metabolic process; 18, polysaccharide metabolic process; 19, small molecule catabolic process; 20, pectinesterase activity; 21, oxidoreductase activity; 22, helicase activity; 23, peroxidase activity; 24, ribonuclease activity; 25, hydrolase activity; 26, protein ubiquitination; 27, vitamin binding. **(D)** Expression levels of the selected genes using qPCR analysis (n = 3). **(E)** Alterations in the expression of genes related to light and dark reactions of photosynthesis. **(F)** Alterations in the expression of genes related to starch metabolism. Pathway diagrams in **(E,F)** were constructed using MapMan.

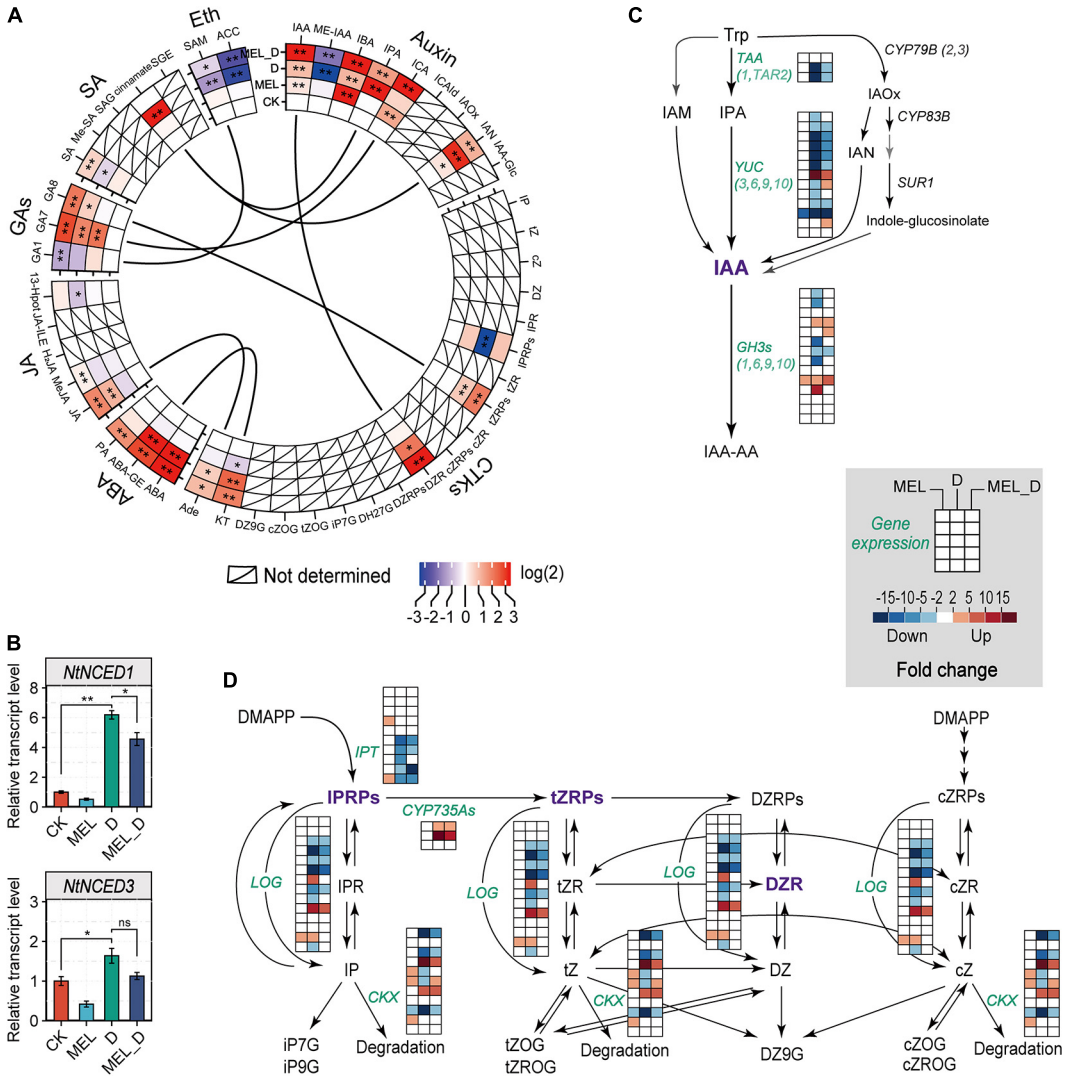

FIGURE 5 | Melatonin effects on the expression of phytohormone biosynthetic genes in tobacco leaves after exposure to dehydration. **(A)** Relative levels of phytohormones based on the untargeted analysis. CTKs, cytokinins; ABA, abscisic acid; JA, jasmonates; GAs, gibberellins; SA, salicylic acid; Eth, ethylene. Asterisks symbols represented statistically significant differences (*$P < 0.05$, **$P < 0.01$). **(B)** ABA biosynthesis and the expression levels of genes encoding NCED, as determined by qPCR. Values have been presented as mean ± SE ($n = 3$). **(C)** Transcript levels of genes involved in IAA biosynthetic pathway. **(D)** Expression levels of genes related to cytokinin biosynthesis and metabolism.

Expression of Genes Involved in Phytohormone Biosynthesis in Melatonin-Treated Leaves Under Dehydration Conditions

To determine whether phytohormones were involved in the regulation of MEL-mediated dehydration tolerance, we analyzed the endogenous levels of various phytohormones based on the metabolome results. The levels of auxin-related molecules, specifically IAA, IBA, IPA, ICA, and IAN, were increased in the leaves under D and MEL_D conditions (**Figure 5A**). Similar to those of auxin-related molecules, the levels of tZRPs, DZR, and KT accumulated specifically in the D- and MEL_D-treated plants. Marked increases in ABA accumulation were observed in the D- and MEL_D-treated leaves, and the JA, GA7, and GA8 levels were also elevated in the leaves treated with D- and MEL_ D. Contrastingly, the level of 1-aminocyclopropane-1-carboxylic acid (ACC), i.e., a precursor in ethylene synthesis, was decreased in D- and MEL_D. Then, we analyzed the transcript levels of *NtNCED1* and *NtNCED3*, which encode the key enzymes involved in ABA biosynthesis, using qPCR. The expression of both genes was upregulated in leaves under D- and MEL_D conditions (**Figure 5B**), showing a correlation between the accumulation of ABA and the transcription of these genes. Conversely, the expression of most genes encoding the key enzyme for IAA biosynthesis, YUC, was downregulated upon D- and MEL_D treatments (**Figure 5C**). We also

examined the alterations in expression of genes involved in cytokinin degradation (cytokinin oxidase/dehydrogenases, CKXs) and those responsible for the formation of IAA amino acid conjugates (GH3s). The expression of most CKX and GH3 genes was downregulated in D compared to that in MEL_D (**Figure 5D**). Further, extensively modulated expression occurred in the GA, SA, and ethylene biosynthesis pathways (**Supplementary Figure 7**). These data indicated that the alterations in phytohormonal levels in the MEL-, D-, and MEL_D-treated leaves were partially caused by the transcriptional regulation of genes involved in their biosynthesis and catabolism.

Expression of Genes Involved in Senescence-Related Pathways in Melatonin-Treated Leaves Under Dehydration Conditions

To examine the expression profiles of genes related to senescence progress under dehydration stress, several enriched metabolic pathways were analyzed, including hormone signaling, porphyrin and chlorophyll metabolism, and regulation of autophagy. Among the genes involved in hormone transduction, 62 DEGs were enriched, including 0, 55, and 35 genes dramatically expressed in MEL, D, and MEL_D relative to those in CK, respectively (**Figure 6**). Except for some irregular gene expression, most genes showed downregulated expression from CK to D, such as *ARF16*, *SAUR50*, *PYL2*, *LAX5*, *IAA27*, *CYCD3*, whereas few genes showed upregulated expression upon dehydration treatment, and of special concern were the ABF and ERF genes. Furthermore, the expression of 32 and 28 genes associated with porphyrin and chlorophyll metabolism, respectively, was suppressed after D and MEL_D treatment, respectively (**Supplementary Figure 8A**). Additionally, 18 out of 23 genes participating in the regulation of autophagy were highly expressed in D-treated leaves, whereas the transcription of these genes in MEL_D was more downregulated than those in D (**Supplementary Figure 8B**), indicating that the autophagy increase induced foliar senescence. The significant genes mostly existed in D-treated leaves, and a part of them in MEL_D, indicating the extent of senescence.

Phytohormone Profiles of Melatonin-Treated Leaves Under Dehydration Conditions

We identified 15 phytohormones in tobacco leaves and performed PCA to analyze their responses (**Figures 7A–C**). The cumulative contribution ratio of the first two PCA axes was 82.28%. PC1 represented increases in phytohormones levels upon dehydration treatment, and the highest PC1 score was found in MEL_D-treated samples. Meanwhile. PC2 reflected the differences in MEL action under different growth conditions, and the PC2 values were positive for D, negative for MEL, and near zero for CK. We focused on representative phytohormones by comparing PCA loadings (**Figure 7B**). The highest PC1 loading was for ABA or ABA-GE, and the levels of both compounds were markedly increased in response to dehydration relative to those in well-watered leaves, while being higher in D than in MEL_D. The lowest PC1 loading was observed for IPR. IPR content in MEL-treated leaves was higher than that in untreated leaves under both normal and dehydration conditions (**Figure 7C**). The first- and second-highest PC2 loadings were for GA15 and IP, respectively. The accumulation of both compounds in MEL_D was increased compared with that in D. ACC displayed the second-lowest PC2 loading, without differences among the four treatments. Furthermore, we investigated the MEL-hormone relationships in plants at metabolic levels using correlation analysis (**Figure 7D**), with five phytohormones being strongly correlated with MEL accumulation, which included DZR, ICA, IBA, IAA, and PA. These data suggested that MEL played a prominent role in the regulation of phytohormone levels in tobacco seedlings under dehydration conditions.

Carotenoid Profiles of Melatonin-Treated Leaves Under Dehydration Conditions

Data demonstrated that carotenoid synthesis was suppressed after the onset of dehydration, with accumulation of mainly phytoene, phytofluene, zeaxanthin, antheraxanthin, and violaxanthin dibutyrate (**Figures 8B,C**). Upon dehydration treatment, MEL-treated leaves exhibited higher levels of α-carotene, lutein, β-cryptoxanthin, violaxanthin, neoxanthin, and echinenone than untreated leaves. Specifically, phytoene accumulation in the four treatments reached 73.3–383.0 μg/g FW, with the highest content found in D and MEL_D. After phytoene, the carotenoid synthesis is divided into two branches: δ- and γ-carotene. In the δ-carotene branch, the levels of α-carotene and lutein in CK and MEL_D were markedly higher than those in MEL and D. Relative to those of the δ-carotene branch, the carotenoids in the γ-carotene branch displayed more complex accumulation patterns in the four treatments. β-carotene levels in CK and MEL_D were slightly higher than those in MEL and D, respectively. Meanwhile, yellow xanthophylls (β-cryptoxanthin, violaxanthin, and neoxanthin) mainly accumulated in CK and MEL_D. Intriguingly, we detected capsanthin only in unstressed leaves, whereas canthaxanthin was only detected under dehydration conditions (**Figure 8B**). Accordingly, expression profiling revealed that MEL selectively induced a different set of carotenoid biosynthesis genes from those mediated by dehydration (**Figure 8A**).

Then, we performed PCA to compare carotenoid levels, which indicated that neoxanthin was positively associated with violaxanthin and lutein on PC1 and negatively associated with phytofluene on PC2. Three of these traits were found with higher magnitude because of their longest vector length among all of the traits, and hence contributed the most to overall variation (**Figure 8D**). These changes suggested that MEL could induce the differential accumulation of carotenoids in tobacco, which contributed to MEL-treated and untreated plants displaying varying differences in the senescence process under dehydration conditions.

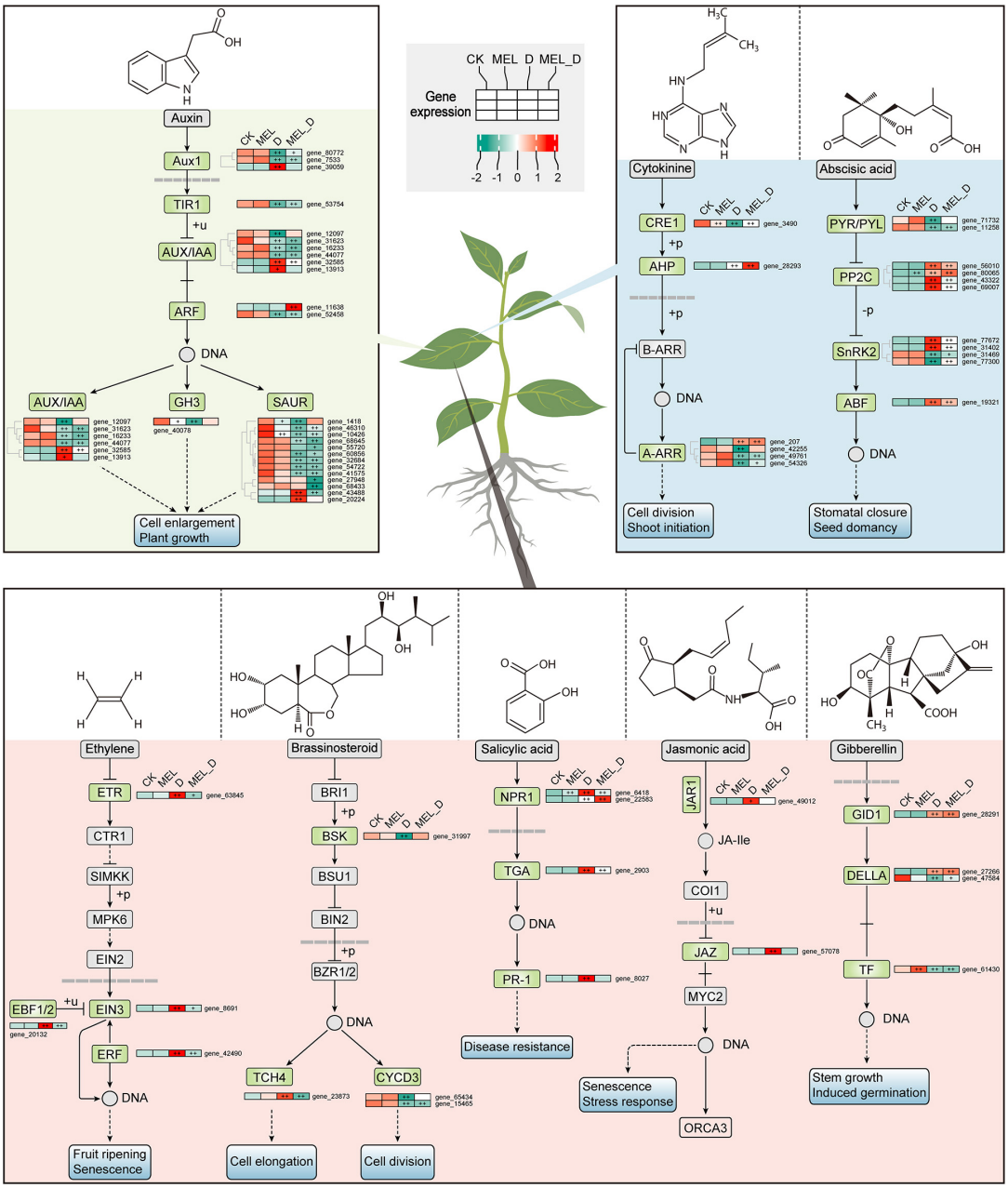

FIGURE 6 | Melatonin effects on expression of genes involved in hormone signaling pathways under dehydration stress. Plus signs denote a significant gene expression difference compared with that in CK ($^{+}P \leq 0.05$, $^{++}P \leq 0.01$).

Melatonin-Mediated Transcriptional Reprogramming in Response to Dehydration Stress

To further elucidate the underlying mechanism by which MEL regulates the dehydration response, we analyzed the transcriptional regulatory networks. In our transcriptome data, 5,741 TF genes were differentially expressed in response to dehydration stress, including AP2, WRKY, bZIP, NAC, PP2C, bHLH, and MYB family members (**Figure 9A**). The heat map showed that the 10 ABA-responsive element-binding (AREB)/ABF and DREB members exhibited varying expression patterns (**Figure 9B**). As a first step to understand the networks of MEL-mediated transcriptional reprogramming, we first constructed a correlation network based on the expression of genes and metabolites (**Figure 9C**). In this network analysis, differentially expressed TF genes and key transcripts were mainly selected from clusters 1 and 4 (**Supplementary Figure 9** and **Supplementary Table 7**) and the metabolites were screened. The correlated pairs were filtered using a correlation coefficient of $r > 0.99$. A transcript–metabolite

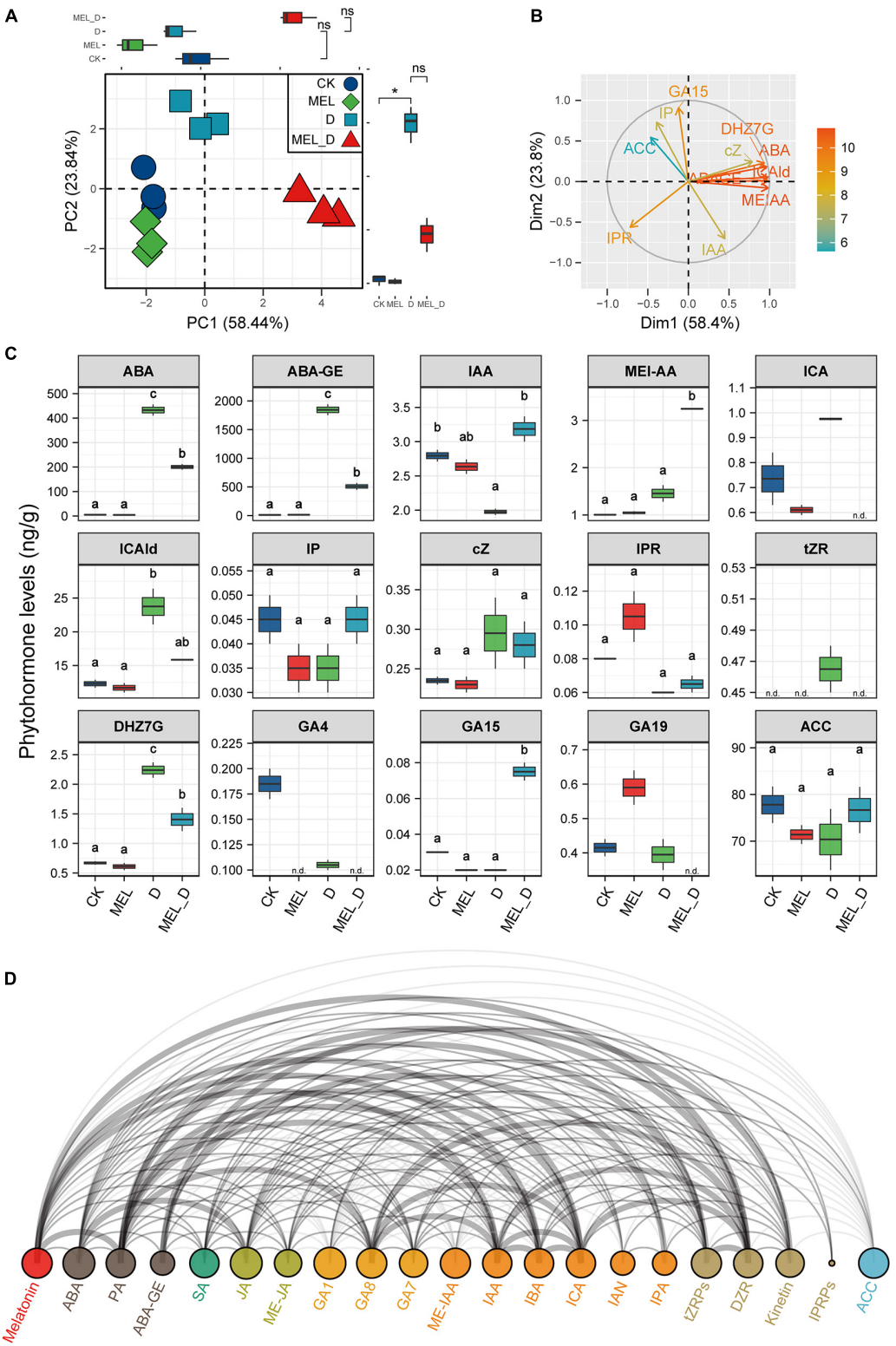

FIGURE 7 | Melatonin supplementation effect on endogenous phytohormone levels. Plant hormone contents were measured in tobacco leaves subjected to four treatments: CK, control; MEL, melatonin; D, drought, and MEL_D, melatonin and drought. **(A,B)** Principal component analysis of phytohormones. PC1 and 2 have been represented in the *x*- and *y*-axis, respectively. **(C)** Statistical analysis of representative phytohormones. n.d., not detected. Data were obtained from three replicated experiments ($n = 3$), and different small letters indicated significant differences ($P < 0.05$) among the treatments. **(D)** Arc diagram illustrating the correlation between representative phytohormones.

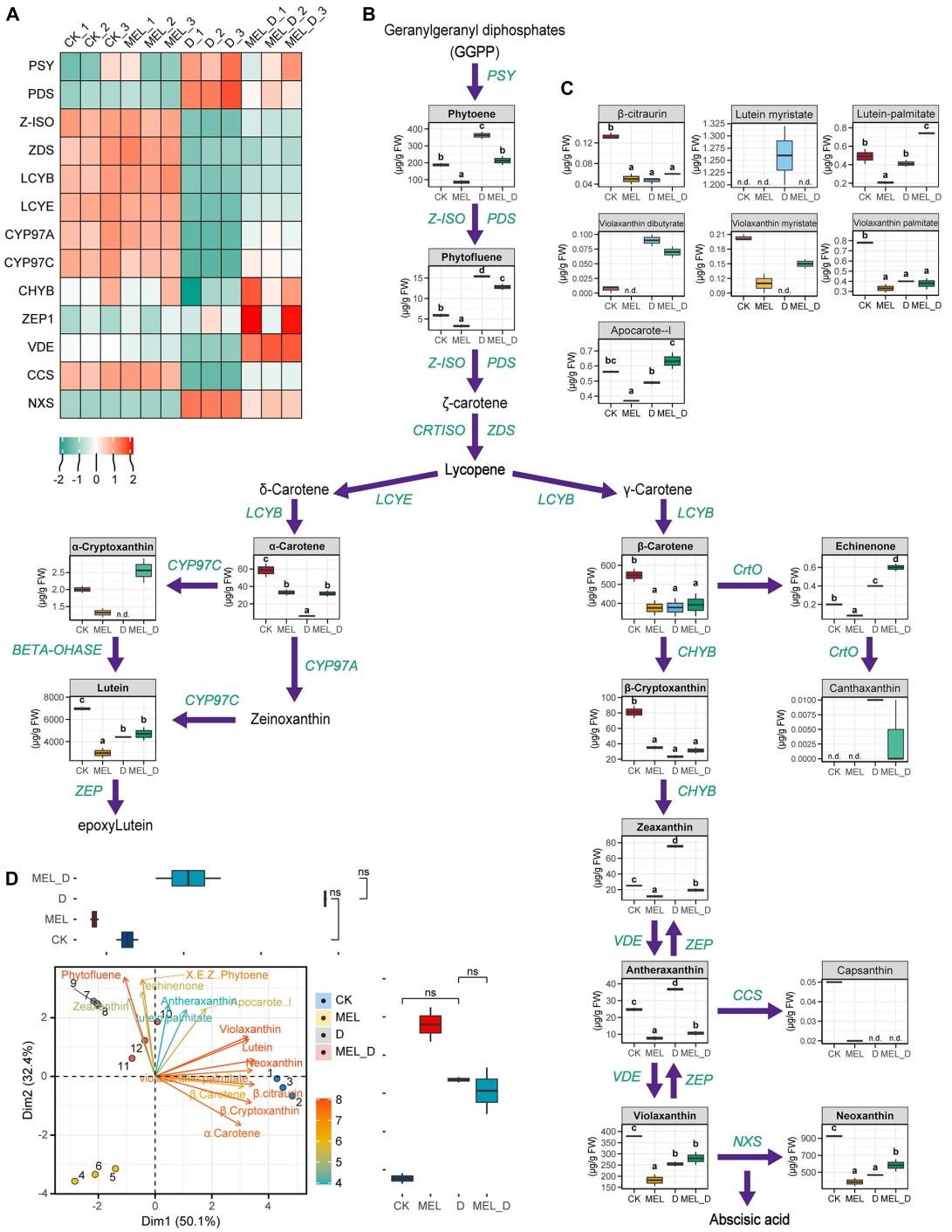

FIGURE 8 | Melatonin supplementation effects on the carotenoid levels and expression of carotenoid synthesis genes. **(A)** Expression of genes involved in carotenoid biosynthesis. Green and red represent low and high expression, respectively. **(B,C)** Carotenoid identification in the leaves under four treatment conditions (CK, control; MEL, melatonin; D, drought, and MEL_D, melatonin and drought). n.d., not detected. Different letters mentioned above columns denote significant differences at *P*-values < 0.05. **(D)** Principal component analysis for carotenoids levels. Values on the *x*- and *y*- axes represent PC1 and PC2, respectively.

correlation network including 173 nodes and 3,920 edges was visualized in Cytoscape, indicating that numerous core TF genes play a central role in the regulation of dehydration responses. Additionally, gene co-expression network analysis (WGCNA) identified seven modules, as shown in the gene dendrogram (**Supplementary Figure 10**). Furthermore, we associated each of the co-expression modules with 12 treatment samples using correlation analysis (**Figure 9D**), showing that

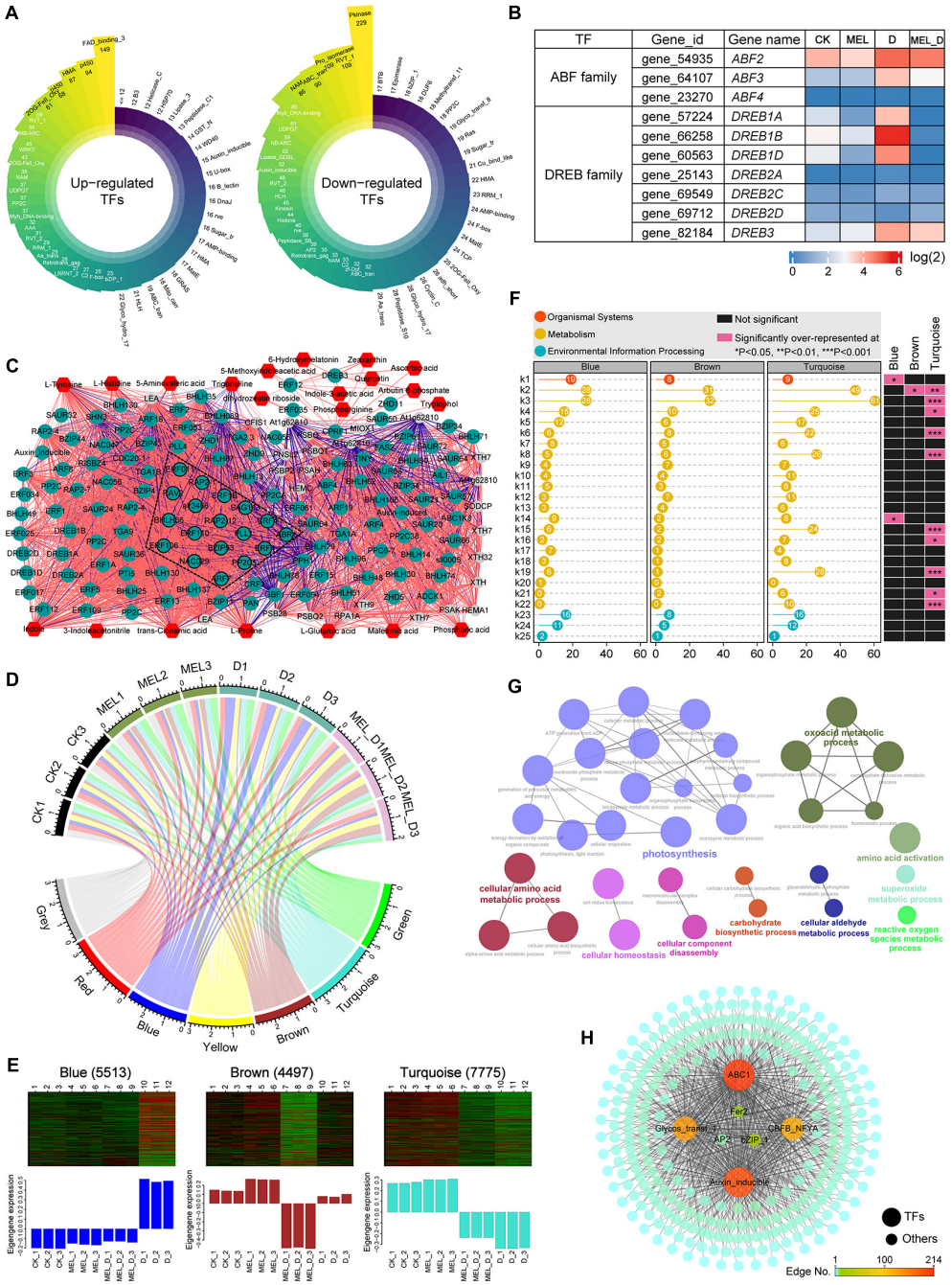

FIGURE 9 | Transcriptional modulation regulated by melatonin in tobacco plants exposed to dehydration conditions. **(A)** Classification of transcription factor families in upregulated and downregulated genes expressed under dehydration stress. **(B)** Expression levels of ABA-responsive element-binding (AREBs) and dehydration-responsive element-binding (DREBs) genes. **(C)** Transcript–metabolite correlation network. Different node colors represent TF genes (green), metabolic genes (gray) and metabolites (red). Red and blue edges indicate positive and negative correlations, respectively. Edge thickness indicates the correlation coefficient value for each correlated pair. **(D)** Module-sample associations. Edge thickness represents the correlation coefficient between modules and samples. **(E)** Eigenprotein expression profile for three specific modules (blue, brown, and turquoise) in different samples. The number of genes for selected modules is mentioned in parenthesis. **(F)** KEGG pathway analysis of gene lists from the key modules. k1, plant-pathogen interaction; k2, biosynthesis of amino acids; k3, carbon metabolism; k4, glycolysis/Gluconeogenesis; k5, starch and sucrose metabolism; k6, carbon fixation in photosynthetic organisms; k7, arginine and proline metabolism; k8, glyoxylate and dicarboxylate metabolism; k9, phenylpropanoid biosynthesis; k10, alanine, aspartate and glutamate metabolism; k11, fatty acid biosynthesis; k12, phenylalanine, tyrosine and tryptophan biosynthesis; k13, phenylalanine metabolism; k14, ascorbate and aldarate metabolism; k15, porphyrin and chlorophyll metabolism; k16, carotenoid biosynthesis; k17, tryptophan metabolism; k18, nitrogen metabolism; k19, photosynthesis; k20, zeatin biosynthesis; k21, nicotinate and nicotinamide metabolism; k22, photosynthesis-antenna proteins; k23, plant hormone signal transduction; k24, MAPK signaling pathway-plant; k25, ABC transporters. **(G)** Enriched GO terms for the turquoise module. **(H)** Co-expression network analysis of the turquoise module with edge weight over 0.6.

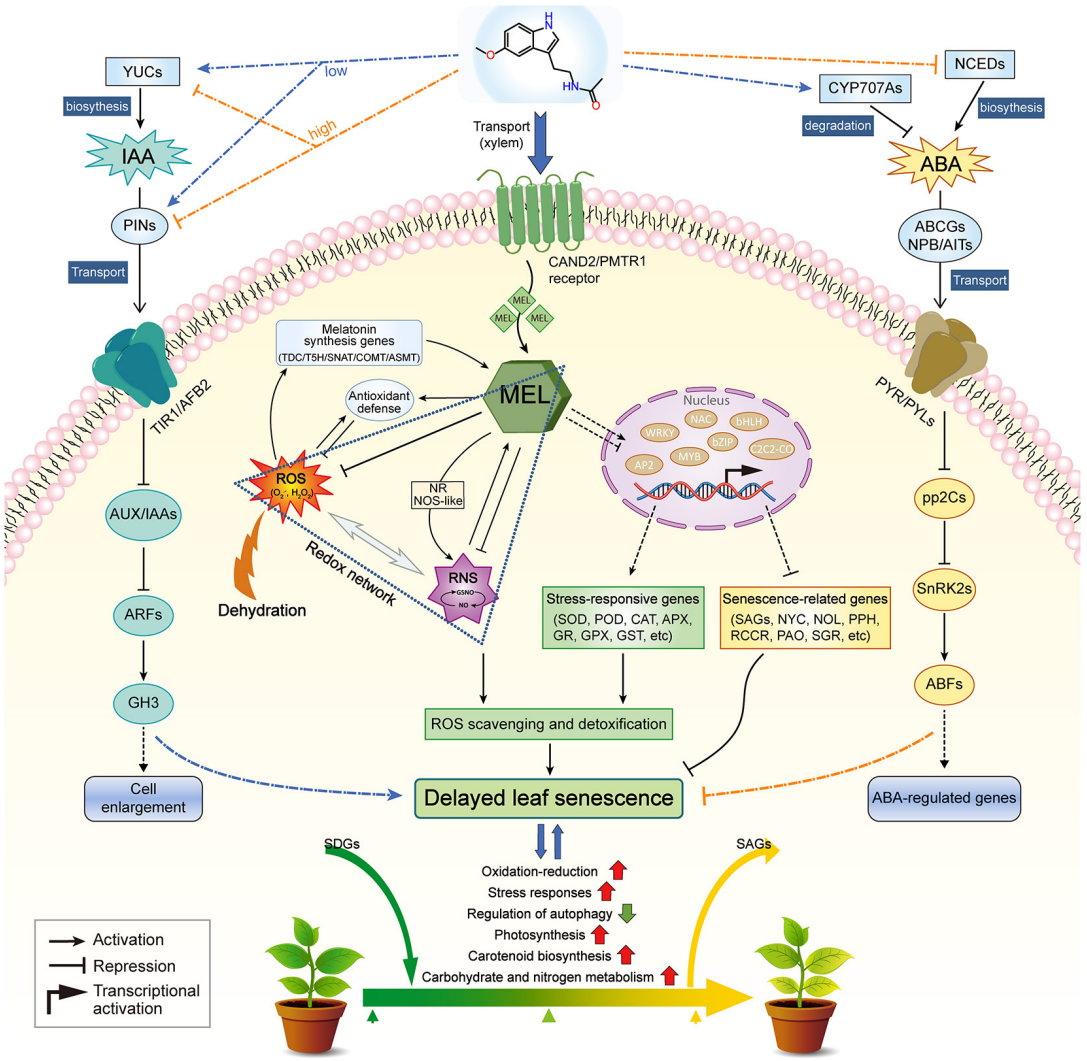

FIGURE 10 | A working model depicting the role of melatonin in regulating dehydration-induced leaf senescence. This model has been proposed based on our own observations and findings reported in literature references. Red and green arrows indicate increased and decreased levels of components, respectively.

the turquoise module showed a relatively high correlation with the dehydration treatment. The eigengene expression for the co-expression modules revealed that several turquoise module genes were weakly expressed in the D and MEL_D treatment groups (**Figure 9E**). In the turquoise module, KEGG pathway and GO enrichment analyses indicated that gene sets related to photosynthesis, porphyrin/chlorophyll metabolism, carbon metabolism, glyoxylate/dicarboxylate metabolism, organic acid metabolic process, amino acid metabolism, and cellular homeostasis were over-represented (**Figures 9F,G**). The gene sets for the turquoise module were primarily downregulated upon dehydration treatment and were highly correlated with ABC1, auxin inducible, CBF, bZIP, AP2, and Fer2 (**Figure 9H**), which appear to be candidate genes that may regulate tobacco defenses to dehydration stress. These results indicate that MEL-mediated transcriptional networks regulate leaf senescence and the dehydration response in tobacco.

DISCUSSION

In this study, we discovered that MEL application promoted tobacco seedling resistance to prolonged dehydration exposure through alterations in the metabolic flux into photosynthesis, phytohormone biosynthesis, carbohydrate metabolism, and carotenoid synthesis, subsequently mitigating leaf senescence. Our results also reveal that the molecular basis by which MEL delays leaf senescence involves the translational regulation of stress response, chlorophyll degradation, and hormone signaling in stressed plants.

Melatonin Confers Enhanced Resistance to Dehydration by Modulating Redox Homeostasis

Plant stress tolerance is tightly associated with the activation of internal ROS-scavenging systems, consisting of low-molecular

weight antioxidants and antioxidant enzymes (Mittler, 2002). Under drought conditions, elevated oxidation levels would lead to either enhanced sustainability or ultimately cell death, depending on the intensity of the oxidative signal activated. High ROS concentrations that surpass the capacity of the detoxifying machinery may activate apoptosis-inducing factors, thereby causing cell death (Miller et al., 2010; Khan et al., 2021). The initial MEL function in organisms is to act as a free radical agent to eliminate reactive oxygen and nitrogen species and protect plant cells from oxidative damage (Arnao and Hernández-Ruiz, 2019). Exogenous application of MEL significantly reduces ROS accumulation and lipid peroxidation, as well as enhances the antioxidant system in apple (Wang et al., 2013a), cucumber (Zhang et al., 2014), grape (Meng et al., 2014), bermudagrass (Shi et al., 2015b), and *Carya cathayensis* (Sharma et al., 2020). Similar results were obtained in this study, which showed that dehydration stress increased the occurrence of ROS-induced oxidative stress in tobacco plants, but MEL treatment reduced ROS accumulation and alleviated plant growth inhibition (**Figure 1**). Accordingly, MDA and EL, as important indicators of oxidative damage, were relatively less pronounced in MEL-treated stressed plants (**Figures 1D,E**), further implying that MEL might protect cell membranes against oxidative stress. Moreover, MEL-activated antioxidant defenses play key roles in tobacco resistance to water deficit. This study suggested that MEL treatment increased the enzymatic and non-enzymatic antioxidant contents and upregulated mRNA levels of anti-stress genes in stressed tobacco leaves (**Supplementary Figures 3**, **4**), which was consistent with the decrease in ROS levels. In this context, the coordinated regulation of multiple antioxidative enzymes and antioxidants efficiently scavenged excessive ROS, thereby maintaining the cellular redox state in stressed plants. Thus, MEL-treated plants achieve high tolerance to dehydration-induced oxidative damage through the development of a powerful detoxifying capacity, which in turn results in better growth behavior under stress conditions.

Melatonin Delays Early Leaf Senescence and Inhibits Chlorophyll Breakdown

As a potent anti-senescent compound, MEL plays a critical role in inhibiting premature leaf senescence, as reported by Wang et al. (2013b), who demonstrated that long-term MEL treatment induced a marked delay in leaf senescence of *Malus hupehensis* by regulating metabolic status and protein degradation. In this study, we found that MEL-treated tobacco leaves exhibited a delayed senescence phenotype, including an increase in photosynthetic rate (**Figure 2**). Moreover, leaf MEL content was slightly elevated with dehydration, and MEL addition had a significant effect in maintaining high levels of *in vivo* MEL concentration (**Figure 2H**). Accordingly, the expression of MEL biosynthetic genes, such as *NtTDC*, *NtT5H*, *NtASMT1*, and *NtSNAT* was upregulated by MEL treatment (**Figure 2I**). Similarly, a recent study in citrus trees also showed that the transcript levels of MEL biosynthetic genes, *CsTDC1*, *CsT5H*, and *CsASMTs* were upregulated by exogenous MEL supplementation (Nehela and Killiny, 2020). Further, endogenous MEL level is remarkably induced in MEL-pretreated *Medicago sativa* plants subjected to prolonged drought stress (7 days) (Antoniou et al., 2017). Chlorophyll degradation progresses throughout senescence but is promoted upon drought stress. Our data clearly demonstrated that external administration of MEL restored the dehydration-stressed plants' leaf yellowing phenotype to levels comparable to those of controls, while maintaining a higher photosynthetic efficiency and chlorophyll level, and preventing the breakdown of chloroplast ultrastructure (**Figure 2**). These changes are in line with previous reports suggesting that drought-induced chlorophyll degradation was attenuated by MEL application in apples (Wang et al., 2013b) and Chinese hickory (Sharma et al., 2020). Similarly, our work indicated cell death levels were decreased in MEL-treated stressed seedlings (**Figure 2E**), implying that MEL can ameliorate dehydration-induced foliar senescence by decreasing cell death. Upon induction of dehydration stress, the expression of several senescence-related genes is modified. In this experiment, exogenous MEL strongly mitigated the upregulated expression of senescence markers and chlorophyll catabolic genes (**Figure 2J**), which are responsible for the degradation of chlorophyll (Wang et al., 2012). These findings suggest another mechanism by which MEL preserves chlorophyll levels, increases leaf longevity, and alleviates dehydration-induced photoinhibition, in addition to ROS detoxification.

Melatonin Regulates Leaf Senescence via Modulation of Phytohormonal Biosynthesis and Signaling

In plants, previous studies on ABA signaling cascades showed that ABA induces premature leaf senescence via activating a series of senescence-associated genes (Sakuraba et al., 2020). Specifically, the ABA receptor *PYL9* was shown to accelerate leaf senescence in *Arabidopsis* (Zhao et al., 2016). Recently, overexpression of *ABFs* in tobacco plants not only induced leaf senescence and ABA production, but also activated genes that code for ABA biosynthesis and chlorophyll degradation (Tan et al., 2019). Similarly, our plant hormone profiles of tobacco leaves showed that the stress hormone ABA was the phytohormone most inducible by dehydration (**Figure 7**). The expression of key genes (*NtNCED1*, *NtNCED3*, and *NtABF*) involved in ABA biosynthesis and signaling was increased upon dehydration treatment (**Figures 5**, **6**), which supported the suggestion that prolonged dehydration stress gates ABA signaling to induce leaf senescence in tobacco plants. In contrast, the expression of genes responsible for auxin biosynthesis and signaling was suppressed by dehydration stress. These results suggested that auxin and ABA antagonistically regulate dehydration-induced senescence.

There is a close relationship between phytohormone levels and MEL concentration. Accumulating evidence supports that MEL acts synergistically or antagonistically

with other phytohormones such as auxin, CTK, GA, ABA, ethylene, and JA during biological processes, particularly in stress responses (Zhang et al., 2014; Arnao and Hernández-Ruiz, 2015, 2018; Fan et al., 2018; Tan et al., 2019; Nehela and Killiny, 2020), with ABA and auxin being particularly interesting. Previous studies have shown that MEL decreases the anabolic capacity for ABA biosynthesis and increases catabolic capacity for ABA metabolism, thereby regulating the functions of stomata in dehydration-treated *Malus* plants (Li et al., 2015). In drought-stressed apple leaves, MEL selectively downregulates *MdNCED3* and upregulates ABA catabolic enzymes (*MdCYP707A1* and *MdCYP707A2*), thus halving endogenous ABA production (Li et al., 2015). In agreement with these reports, our results revealed that the accumulation of ABA in stressed tobacco plants was attenuated by exogenous application of MEL, but not that of auxin, which was remarkably higher in MEL-treated stressed plants than in non-treated stressed plants (**Figure 7**). Similar findings were obtained in Chinese flowering cabbage, with MEL attenuating leaf senescence by inhibiting ABFs (*BrABF1*, *BrABF4*, and *BrABI5*)-mediated ABA accumulation and reducing expression of chlorophyll catabolic genes (Tan et al., 2019). The MEL-dependent defense against dehydration stress might also be associated with other hormones, including CTK, GA, JA, and ethylene, as MEL affects key gene expression involved in these signaling pathways, such as ARR3 (response regulator 3), GID1C (gibberellin receptor GID1), JAR1 (jasmonic acid-amino synthetase) and ERF1 (ethylene response factor 1) (**Figure 6**). These findings demonstrate that MEL supplementation decreases ABA accumulation and maintains high IAA levels by simultaneously inhibiting and activating ABA and IAA biosynthetic genes, respectively, under dehydration conditions. Thus, MEL-triggered delayed leaf senescence appears, at least in part, by modulating hormone biosynthesis and signaling. In the future, it will be important to unravel the MEL-ABA-auxin interactions and their roles in the control of plant senescence to improve our understanding of hormone networks regulating plant defense.

Melatonin Participates in Plant Response to Dehydration Stress by Modulating the Transcriptional Network

Transcriptional regulation comprises a dynamic gene network involving the interaction of TFs with the *cis* elements of the target genes (Breeze et al., 2011). Multiple TF families have been implicated in the regulatory network underlying leaf senescence, including MYBs, WRKYs, NACs, GRAS, and NF-Ys (Balazadeh et al., 2011; Wu et al., 2012; Tian et al., 2020). Recently, NAC (NAM, ATAF1/2, and CUC2) proteins have been identified as key players in the induction of leaf senescence (Fang et al., 2008; Balazadeh et al., 2011; Takasaki et al., 2015Mao et al., 2017). In *Arabidopsis*, NAP plays a key role in regulating both senescence and hormone signaling, promoting ABA biosynthesis and chlorophyll degradation by activating the expression of *AAO3* and *NYC1* (Yang et al., 2015). Similarly, *OsNAP* and *OsNAC2* accelerate leaf senescence in rice by directly activating ABA biosynthetic genes and chlorophyll degradation genes (Liang et al., 2014; Mao et al., 2017). Interestingly, several TFs such as DREB/CBF, HSF, ZFPs, WRKY, NAC, and MYB have previously been proposed as putative targets of MEL involved in stress responses (Liang et al., 2015; Shi et al., 2015c, 2018). Consistently, our transcriptomic profiling analysis revealed that the expression of a significant proportion of the senescence-controlling TFs, including 41 NACs, 4 DREBs, and 9 HSFs, as well as two ABF proteins, were transcriptionally induced in leaves by dehydration treatment but repressed by MEL (**Figure 9**). Furthermore, co-expression network analysis indicated that TFs such as ABC1, auxin inducible, CBF, bZIP, AP2, and Fer2 were strongly associated with leaf senescence in stressed tobacco plants by modulating photosynthesis and other metabolic processes. These results demonstrated that MEL-mediated transcriptional regulators are likely to play prominent roles in the control of leaf senescence caused by dehydration stress. Considering that MEL can regulate ROS homeostasis and that elevated ROS production triggers senescence signals, these key regulatory genes should be excellent candidates for dissecting MEL-mediated leaf longevity and stress resistance. Thus, elucidating the crosstalk between MEL and key TFs that control plant senescence under dehydration conditions is essential. Transcripts are only one part of the regulatory networks, and it is also possible that MEL regulates the progression of senescence through other molecular targets of currently unknown functions. Thus, further research focusing on defining the detailed mechanisms by which MEL controls leaf longevity during dehydration is required.

CONCLUSION

In summary, the protective effect of MEL against dehydration-induced leaf senescence points to its physiological roles as a powerful radical scavenger, promoting ROS detoxification, or as a more specific agent associated with the regulation of senescence-related genes. MEL-treated plants clearly exhibited delayed dehydration-inducible leaf senescence, as shown by less water loss, decreased lipid peroxidation, and higher photochemical efficiency, relative to that of untreated plants. In the presence of MEL, MEL regulates the transcription of TFs, such as NAC, bHLH, AP2, bZIP, MYB, WRKY, and ABF, which in turn modulates the expression of genes responsible for ROS scavenging and chlorophyll degradation. The validity of this function is manifested by our observation that MEL addition suppresses intracellular ROS production during dehydration stress. Consequently, the expression of genes involved in diverse biological functions, such as oxidation–reduction, stress responses, autophagy, photosynthesis, carotenoid biosynthesis, and carbohydrate/nitrogen metabolism, are modified by MEL, resulting in delayed leaf senescence and elevated endurance to dehydration stress (**Figure 10**). These findings provide evidence of MEL functioning as a positive factor in mitigating dehydration-promoted oxidative damage and leaf

senescence, and highlights the exciting potential of MEL for crop improvement.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are publicly available. This data can be found here: NCBI repository, accession: PRJNA691642.

AUTHOR CONTRIBUTIONS

JX and ZX conceived the experiments. ZC drafted the manuscript. WJ participated in the main experiments in this work, with assistance from SL. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.686062/full#supplementary-material

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ORIGINAL RESEARCH
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Physiological and Transcripts Analyses Reveal the Mechanism by Which Melatonin Alleviates Heat Stress in Chrysanthemum Seedlings

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Heat stress limits the growth and development of chrysanthemum seedlings. Although melatonin (MT) has been linked to the heat stress response in plants, research on the underlying molecular mechanisms is scarce. In this study, the regulatory networks of MT on heat stress in chrysanthemum seedlings were explored. Physiological measurements suggested that MT not only reduced malondialdehyde accumulation, hydrogen peroxide content, and superoxide anion free radical generation rate, but also significantly promoted osmotic regulation substance synthesis (proline and soluble protein), antioxidant accumulation (GSH and AsA), and the antioxidant enzyme activities (SOD, POD, CAT, and APX) in chrysanthemum leaves under heat stress. Furthermore, MT increased the fresh weight, dry weight, chlorophyll content, photosynthesis rate, and gas exchange indexes. Further, RNA-seq results revealed 33,497 and 36,740 differentially expressed genes in the S/Con and SMT/ConMT comparisons, respectively. The differences in the comparisons revealed that MT regulated heat shock transcription factors (HSFs) and heat shock proteins (HSPs), and the genes involved in Ca^{2+} signal transduction (*CNGCs* and *CAM/CMLs*), starch and sucrose metabolism (*EDGL, BGLU, SuS*, and *SPS*), hormone (*PP2Cs*, *AUX/IAAs*, *EBFs*, and *MYC2*), chlorophyll metabolism (*HEMA* and *PORA*), flavonoid biosynthesis (*CHS*, *DFR*, and *FNS*), and carotenoid biosynthesis (*DXPS*, *GGDP*, and *PSY*). MT effectively improved chrysanthemum seedling heat-resistance. Our study, thus, provides novel evidence of a gene network regulated by MT under heat stress.

Keywords: chrysanthemum, melatonin, high temperature, physiology, RNA-seq

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INTRODUCTION

High temperature stress restricts plant growth and development, thereby, severely reducing crop yields (Wilson et al., 2014; Lesk et al., 2016). Heat damage includes leaf curling and yellowing, whole leaf wilting, and leaf edge scorching (Sharma et al., 2016). Moreover, reactive oxygen species (ROS) are produced in excess under heat stress, which in turn causes a series of complex metabolic alterations, including, changes in enzyme activity, in proteins and nucleic acids, and in cell membrane and cytoskeleton stability (Ahammed et al., 2016). Plants have their own antioxidant systems that can effectively scavenge ROS (Baxter et al., 2014). However, excessive accumulation of ROS causes

severe disruption of ROS homeostasis, resulting in the oxidation of lipids, DNA, and proteins (Vanderauwera et al., 2011; Baxter et al., 2014). High ambient temperatures enhance transpiration, causing tissue dehydration and even plant death (Locato et al., 2009). Urgent measures are required for the amelioration of heat-induced plant damage; a research area that has received widespread attention (Baninasab and Ghobadi, 2010), particularly, because the plant heat-response system can be overwhelmed by conditions of chronic and severe heat stress.

Acting as an antioxidant and growth regulator (Fleta-Soriano et al., 2017), melatonin was first discovered in the pineal gland of bovines (Arnao and Hernandez-Ruiz, 2014). It plays a vital role in the growth and development of plants, and their resistance to stress (Arnao and Hernandez-Ruiz, 2015), assisting plants to survive and thrive (Reiter et al., 2015). Endogenous regulation and exogenous spraying of melatonin can improve plant resistance to biotic and abiotic stress (Hasan et al., 2015; Shi et al., 2015; Xu et al., 2016). A recent study in *Lolium perenne* revealed that exogenous spraying of melatonin reduced abscisic acid content under heat stress, as well as increased the concentration of endogenous melatonin and cytokinin (Zhang et al., 2017). Heat stress causes misfolding of proteins in plant cells; in tomato, melatonin reduced the ratio of insoluble protein to total protein, thereby protecting plant proteins against heat-induced denaturation (Xu et al., 2016). The application of melatonin increased superoxide dismutase (SOD) (Zhang et al., 2017), and ascorbic acid (AsA)-GSH cycle-related enzyme activities, such as that of ascorbate peroxidase (APX). Melatonin can modulate Ca^{2+} influx through a non-selective Ca^{2+} permeable cation channel (Celik and Nazıroğlu, 2012), and stimulate Ca^{2+} transport across the cellular membranes (Santofimia-Castaño et al., 2014). In kiwifruit seedlings, melatonin can effectively modulate carbon fixation and improve photosynthesis under heat stress by regulating the transcription of triosephosphate isomerase (*TIM*), ribose 5-phosphate isomerase A (*RPI*), and phosphoenolpyruvate carboxykinase (*PCK*) genes (Liang et al., 2019). Melatonin also increase the biosynthesis of polyphenols such as total phenols, flavonoids, and anthocyanins in grape berries (Meng et al., 2019). In addition, melatonin was shown to induce the transcription of heat shock proteins (HSPs) and to promote the degradation of denatured proteins in response to abiotic stress (Shi et al., 2015; Wang et al., 2015; Xu et al., 2016). In plants, melatonin affects root architecture (Pelagio-Flores et al., 2012), organ development (Arnao and Hernandez-Ruiz, 2014), photosynthesis (Arnao and Hernandez-Ruiz, 2015), defense (Weeda et al., 2014), senescence (Byeon et al., 2012; Wang et al., 2013), and stress responses (Kostopoulou et al., 2015; Zhang et al., 2015). Specifically, melatonin effectively maintains photosynthesis in tomato plants growing under heat stress (Ahammed et al., 2018). In maize seedlings, melatonin enhances thermotolerance by modulating antioxidant defense, methylglyoxal detoxification, and osmoregulation systems (Li et al., 2019). In wheat seedlings, melatonin suppressed the heat stress-induced damage by modulating the antioxidant machinery (Buttar et al., 2020). However, whether MT could enhance the thermotolerance of chrysanthemum and the underlying mechanisms is not known.

Chrysanthemum is one of the most widely cultivated cut flowers in the world, thus having high ornamental and economic values. In summer, under heat stress conditions, chrysanthemum seedlings grow slowly, and the leaves curl, turn yellow, and wither. In severe cases, chrysanthemum seedlings can die. If chrysanthemum encounters extreme heat stress during the reproductive growth period, its flowers will die prior to propagation, which will seriously restrict the development of the chrysanthemum industry and the value of ornamental chrysanthemum. Therefore, it is important to identify methods for improving the resistance of chrysanthemum to heat stress conditions. In this study, we explored the melatonin-mediated enhancement of chrysanthemum seedling-stress resistance through its regulation of the physiological and molecular responses involved. Physiologically, our main analyses focused on osmotic regulation substances, peroxides, antioxidant contents, and antioxidant enzymes. Moreover, we highlighted the genes involved in the ROS, the heat shock transcription factor (HSF)-HSP, Ca^{2+} signal transduction, carbon fixation, the starch and sucrose metabolism pathways, hormone signal transduction, and the chlorophyll, flavonoid, and carotenoid metabolic pathways. We elucidated the gene regulatory networks involving melatonin under heat stress; furthermore, our study provides a sound theoretical basis for research on melatonin to improve heat tolerance in plants.

Abbreviations: ABI5, ABA-insensitive; AGPase, ADP-glucose pyrophosphorylase; ARF, auxin response factor; ARG7, IAA-induced protein; APX, ascorbate peroxidase; AsA, ascorbic acid; AUX/IAAs, auxin/indole acetic acid protein; BGLU, β-glucosidase; BXL, β-D-xylosidase; CAT, catalase; CAM/CMLs, calmodulin/calmodulin-like protein; CHI, chalcone isomerase; CHLH, magnesium-chelatase subunit ChlH; CHS, chalcone synthase 2; Con, Control; ConMT, control with melatonin; CLH, hydroxymethyl chlorophyll a reductase; CNGCs, cyclic nucleotide gated channel; DEGs, differentially expressed genes; DFR, dihydroflavonol 4-reductase; DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; EBFs, EIN3-Binding F-Box protein; EDGL, endoglucanase; EGLC, endoglucane-1, 3-β-glucosidase; ERF25, ethylene-responsive transcription factor 25; FDR, false discovery rate; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; GCL, glutamate cysteine ligase; GGDP, geranylgeranyl diphosphate synthase; GME, GDP-D-mannose 3′, 5′-epimerase; GR, glutathione reductase; GSH, glutathione; HEMA, glutamyl-tRNA reductase; HEMF, coproporphyrinogen III oxidase; H_2O_2, hydrogen peroxide; HSFs, heat shock transcription factors; HSPs, heat shock proteins; MAPKKKs, mitogen-activated protein kinase kinase kinases; MDA, malondialdehyde; MT, melatonin; NYC1/NOL, chlorophyllide a oxygenase; $O_2^{\bullet-}$, superoxide anion free radicals; PCK, phosphoenolpyruvate carboxykinase; PIF3, photosensitive interaction factor 3; POD, peroxidase; PORA, protochlorophyllide oxidoreductase; PP2Cs, protein phosphatase 2C; PPH, pheophytinase; Pro, proline; PSY, phytoene synthase; RBOH, respiratory burst oxidase; ROS, reactive oxygen species; RPI, ribose 5-phosphate isomerase A; S, stress; SAUR, small auxin-up RNA; SOD, superoxide dismutase; S/Con, stress/control; SMT, stress with melatonin; SMT/ConMT, stress with melatonin/control with melatonin; SPS, sucrose phosphate synthase; SuS, sucrose synthase; TIM, triosephosphate isomerase; TIR1, transport inhibitor response 1; TPP, trehalose-phosphate phosphatase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

The chrysanthemum cultivar "Jinba" was obtained from the Chrysanthemum Germplasm Resource Preserving Center (Nanjing Agricultural University, China). Rooted seedlings

were transplanted into pots filled with a 1:1 soil/vermiculite mixture and placed in a greenhouse under a 16:8 h light:dark regime; a 25/15°C day/night temperature regime, and 70% relative humidity, until they had formed approximately ten fully expanded functional leaves, excluding young leaves at the same developmental phase. To screen for a suitable melatonin concentration, five concentrations (0, 50, 100, 200, and 400 μM) were tested in a preliminary experiment. The results of preliminary experiments indicated that 200 μM solution was selected as the treatment concentration, owing to it had a stronger effect on the physiological indexes, including growth indicators (i.e., fresh and dry weights), osmotic regulators [i.e., malondialdehyde (MDA), and proline content], and antioxidant enzyme activities [SOD and peroxidase (POD)]. Then chrysanthemum seedlings were uniformly sprayed with 200 μM melatonin every other day for 6 days until the leaves and stems were fully moistened, without the occurrence of dripping. The total volume of melatonin solution sprayed per plant per day was approximately 10 mL with spraying prevention of soils. Control plants (Con) were sprayed with distilled H_2O (no melatonin). Melatonin was first dissolved in a small amount of alcohol and then formulated to 200 μM melatonin in distilled water. Each treatment was applied to 30 chrysanthemum seedlings and the experiment was performed in triplicates. Subsequently, the chrysanthemum seedlings and controls pre-treated with melatonin or water were subjected to the following treatments: Con, 25°C/15°C with water; stress (S) plants, 40°C/30°C with water; stress with melatonin (SMT), 40°C/30°C with MT, and control with melatonin (ConMT), 25°C/15°C with MT. At 0, 6, 12, 24, and 48 h, leaf samples were collected from the four treatments and stored at −80°C to determine physiological indicators. After 6 days of heat stress, we observed the phenotype of chrysanthemum seedlings pre-treated with melatonin or water, and measured their fresh weight, dry weight, chlorophyll content, photosynthesis rate, and gas exchange indexes.

Physiological Measurements

Malondialdehyde content was measured by the thiobarbituric acid method after Schmedes and Hølmer (1989). The proline content (Pro) was established by the acid ninhydrin method (Li, 2000), and the Coomassie Brilliant Blue G-250 method was used to determine the content of soluble protein (Bradford, 1976). The soluble sugar content was identified by anthrone colorimetry (Li, 2000), and the hydrogen peroxide (H_2O_2) level was determined according to Willekens et al. (1997). The presence of superoxide anion free radicals ($O_2^{\bullet-}$) was detected using nitroblue tetrazolium staining (Kamrul et al., 2015). Reduced glutathione (GSH) and reduced AsA levels were measured using the method of Ma and Cheng (2003). The activity of SOD was determined based on the method of Giannopolittis and Ries (1997), peroxidase (POD) by the method of Scebba et al. (2001), and catalase (CAT) was determined using the improved method of Kato and Shimizu (1987). APX activity was determined as described by Nakano and Asada (1981). The above measurements were repeated three times, and the average value was used as the representative value for each treatment.

Measurement of Growth, Photosynthesis Rate and Gas Exchange

Three chrysanthemum seedlings were randomly selected 0 and 6 days after treatment. The seedlings were subsequently cut, rinsed with water, their surface wiped clean with filter paper, and weighed (g), after which, they were oven dried at 105°C for 10 min and then at 80°C to constant weight prior to measurement of dry-mass weight (g). Additionally, three chrysanthemum seedlings were randomly selected, and 0.2 g of fresh-leaf samples were obtained, wiped clean of surface dirt, and then the midrib was removed; the foliar blades were mixed and placed in a test tube, added 10 mL of 95% ethanol and incubated for 12 h in the dark. Absorbance was measured at 665 and 649 nm, and used to calculate chlorophyll a and chlorophyll b, respectively, and total chlorophyll. Rubisco enzyme activity was determined according to the method described by Xia et al. (2009). A portable LI-6800 IRGA (Li-COR, Lincoln, NE, United States) was used to determine net photosynthetic rate (Pn), stomatal conductance (g_s), intercellular CO_2 concentration (Ci), and transpiration rate (Tr). Three plants were randomly selected for each treatment and mature leaves at plant mid height were selected for the determination of photosynthesis and gas exchange. The open air path and red and blue light sources were selected; quantum flux density was set to 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and the airflow rate in the sample chamber was set to 500 $\mu mol \cdot s^{-1}$; CO_2 concentration, relative humidity, and temperature were set to 390–410 $\mu mol \cdot mol^{-1}$, 30–40%, and 25°C, respectively.

RNA Sequencing and Bioinformatics Analysis

According to the measured physiological indicators, we found that the difference between SMT and S was most significant at 24 h after treatment; therefore, we chose 24 h samples for transcriptomic analysis. Total RNA was extracted from Con-24 h, ConMT-24 h, S-24 h, and SMT-24 h using an RNA isolation kit (Waryong, Beijing, China). The leaves collected from five potted plants were considered as one biological replicate, and samples of three such biological replicates were subjected to the DNBSEQ platform for RNA sequencing. Adaptor-polluted, low-quality, and high-content unknown base (N) reads were removed from the raw data (Grabherr et al., 2011). Trinity software (Pertea et al., 2003) was used for *de novo* assembly of clean reads (removed PCR duplication to improve assembly efficiency), and the obtained unigenes were assigned a presumptive function according to homolog deposits in the NR, NT, Swiss-Prot, KEGG, COG, and Pfam databases. The transcriptome datasets are available in the NCBI repository[1], Accession No. for library PRJNA732569. We identified differentially expressed genes (DEGs) using a false discovery rate (FDR) ≤ 0.05, and $|\log_2 \text{FoldChange}| \geq 1.0$. The heat map of the DEGs was generated using the MeV software.

Quantitative Real-Time PCR Assay

Eight genes were randomly chosen for quantitative verification to confirm the accuracy of the transcriptome data. Primers

[1]https://submit.ncbi.nlm.nih.gov/subs/sra/

used for qRT-PCR were designed using the Primer5 software (**Supplementary Table 1**). The reference gene *EF-1α* (GenBank: KF305681) was selected as an expression control (Wang et al., 2015). Each sample was represented by three biological replicates and three technical replicates. The specific steps of qRT-PCR were as described previously (Ren et al., 2014). The relative transcriptional expression of DEGs was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Using Duncan's test for data analysis, differences among Con, ConMT, S, and SMT groups were identified as significant at $P < 0.05$. SPSS v17.0 software (SPSS Inc., Chicago, IL, United States) was used for statistical analyses.

RESULTS

Physiological Changes of Heat Treatment

To investigate the effects of MT on the resistance of chrysanthemum seedlings to heat stress, the physiological alterations were measured. First, the MDA content was detected, and showed a significant increase in S of 137.5, 144, 178.26, and 152.08% at 6, 12, 24, and 48 h, respectively, and a significant decrease in S + MT of 35.79, 35.25, 25.78, and 17.36% over the same time periods, respectively (**Figure 1A**). Second, the content of osmotic adjustment substances in chrysanthemum seedlings under heat stress was measured, and compared with control, the proline content of S significantly increased by 154.58, 252.4, 313.31, and 552.32% at 6, 12, 24, and 48 h, respectively. Additionally, the proline content of S + MT increased significantly compared to S by 56.16, 49.88, 65.61, and 10.74% over the same time frames, respectively (**Figure 1B**). The soluble protein content of S increased by 50.23 and 58.74% compared with control at 24 and 48 h, respectively, whereas that of S + MT began to accumulate at 12 h, increasing by 51.98, 18.17, and 30.1% compared with S at 12, 24, and 48 h, respectively (**Figure 1C**). The soluble sugar content of S exceeded that of control by 52.22, 87.99, and 42.71% at 12, 24, and 48 h, respectively, while S + MT remained at a higher level than S at 48 h, increasing 29.19% (**Figure 1D**).

Third, the influence of melatonin treatment on peroxide content in chrysanthemum seedlings under heat stress was investigated. The H_2O_2 content of S increased by 14.99, 34.62, 62.35, and 74.16% compared with control at 6, 12, 24, and 48 h, respectively, whereas that of S + MT decreased when compared to S by 8.30, 20.89, 26.60, and 4.57% at the respective time periods (**Figure 1E**). The generation rate of superoxide anion free radicals of S was significantly increased by 13.55% (6 h), 16.08% (12 h), 33.52% (24 h), and 29.73% (48 h) compared with control, while the generation rate of S + MT was significantly lower than that of S at all times measured and maintained at the control level at 6–24 h (**Figure 1F**). The antioxidant content of chrysanthemum seedlings under heat stress was measured, and compared with control, the reduced glutathione (GSH) content of S was 87.91, 120.22, 226.19, and 270.89% higher than the control at 6, 12, 24, and 48 h, respectively. Compared with S, the GSH content of S + MT increased by 20.47 and 19.39% at 6 and 12 h, respectively (**Figure 1G**). The AsA content of S + MT increased when compared to S by 14.46% (12 h), 15.43% (24 h), and 16.91% (48 h) (**Figure 1H**). Antioxidant enzyme activities were also detected. The SOD activity of S + MT was significantly higher than that of S, which increased by 6.73, 17.41, 4.74, and 7.94% at 6, 12, 24, and 48 h, respectively (**Figure 1I**). Similarly, POD of S + MT showed greater activity than that of S at all times measured, with increases of 5.06, 7.76, 19.18, and 17.7% compared with S at the four respective times (**Figure 1J**). The CAT activity of S + MT was increased by 6.65% (6 h), 15.15% (12 h), 7.14% (24 h), and 3.37% (48 h), respectively, compared with S, but there was a significant difference only at 12 h (**Figure 1K**). Compared with control, the APX activity of S increased by 205.00, 132.14, 261.54, and 333.33% at 6, 12, 24, and 48 h, respectively. At 12 and 48 h, APX was significantly more active in S + MT than S by 53.85 and 19.78%, respectively (**Figure 1L**). The chrysanthemum seedlings were subjected to heat stress for 6 day; those of Con + MT and CK grew vigorously, followed by S + MT, whereas the S seedlings were the most wilted (**Figure 1M**). Collectively, these results show that melatonin can effectively alleviate the damage aroused by ROS in chrysanthemum seedlings under heat stress, and is beneficial to the synthesis of osmotic regulation substances and antioxidant contents. Meanwhile, melatonin treatment can effectively reduce the content of MDA and peroxide, and improve the resistance of chrysanthemum seedlings to heat stress.

Heat-Induced Changes in Growth and Photosynthetic Parameters

Melatonin treatment alleviated the damage caused by heat stress in chrysanthemum seedlings, whose fresh and dry weights were reduced by 42.27 and 28.77%, respectively, compared with control, after 6 days in the S treatment, while those corresponding to the seedlings under the S + MT treatment were significantly increased by 30.97 and 22.12%, respectively (**Figures 2A,B**). Similarly, chlorophyll a, chlorophyll b, and total chlorophyll content decreased by 44.12, 38.08, and 42.44%, respectively, in S, compared with the control, while the three increased by 36.79, 40.88, and 38.01%, respectively, under the S + MT treatment, compared to the corresponding values measured under the S treatment (**Figures 2C–E**). Rubisco is the rate-limiting enzyme in photosynthesis. In this study, Rubisco activity was significantly reduced by 45.28% relative to control after 6 days of heat stress. However, melatonin significantly increased Rubisco activity by 48.28% under S + MT, compared to S (**Figure 2F**). Additionally, photosynthesis rate (Pn) and gas exchange parameters including, Pn, g_s, Ci, and Tr, all decreased by 42.32, 78.28, 54.79, and 71.45%, respectively, compared with control, while they increased by 29.11, 114.08, 25.0, and 60.32%, respectively, under the S + MT treatment compared with the S treatment (**Supplementary Table 2**).

Transcriptome Sequencing and Analysis

To explore the crucial genes and regulatory network at play in chrysanthemum seedlings in response to heat stress

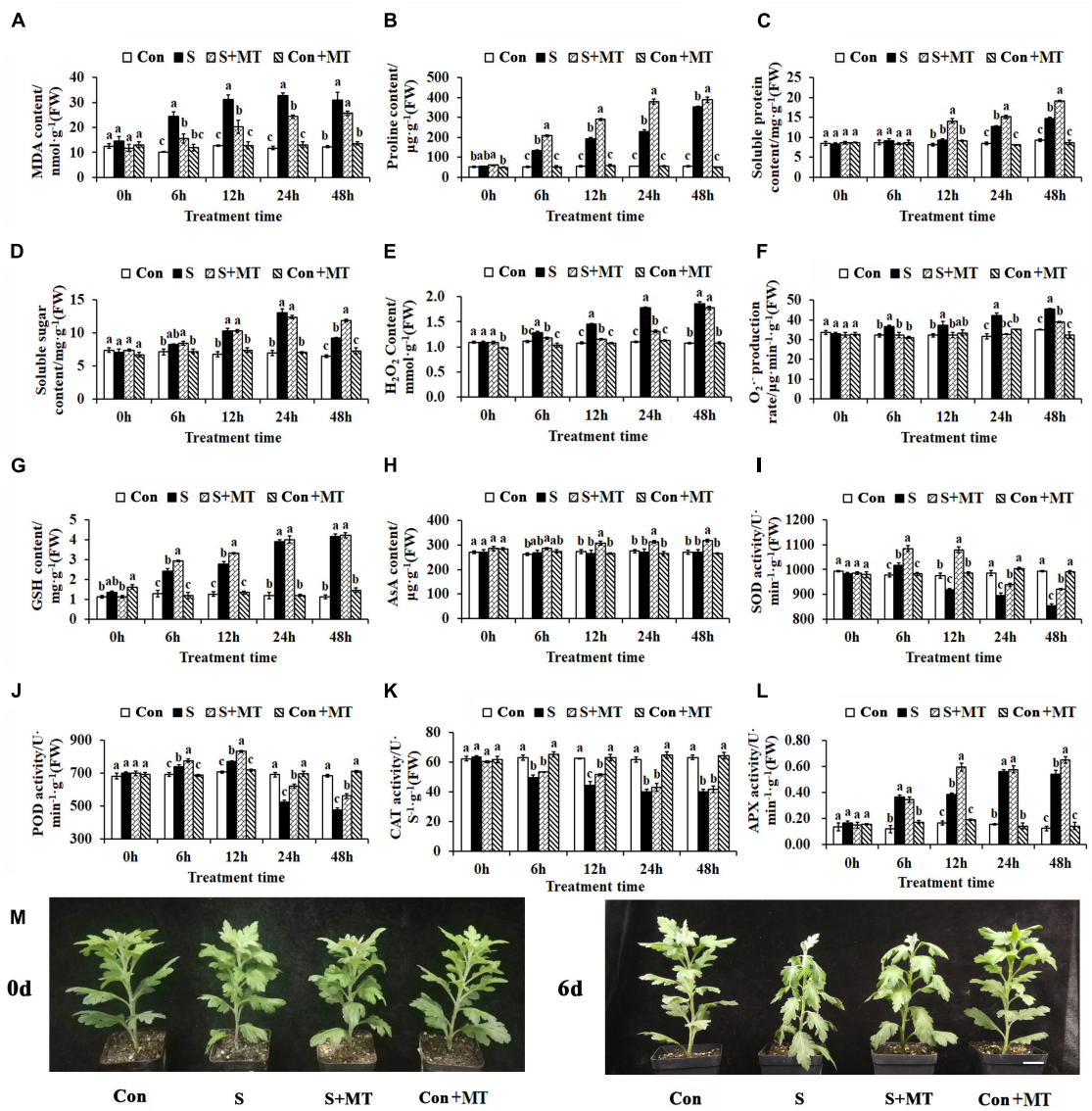

FIGURE 1 | Physiological responses of chrysanthemum seedlings under heat stress. **(A–E)** The content of MDA **(A)**, proline **(B)**, soluble protein **(C)**, soluble sugar **(D)**, and hydrogen peroxide **(E)**. **(F)** The generation rate of superoxide anion free radicals. **(G)** The content of reduced glutathione. **(H)** The ascorbic acid content. **(I–L)** The activities of SOD **(I)**, POD **(J)**, CAT **(K)**, and APX **(L)**. **(M)** Phenotypes after 6 days of heat stress treatment. Con, control; S, heat stress; S + MT, heat stress with exogenous melatonin treatment; Con + MT, control with exogenous melatonin treatment. Error bars indicate SE (n = 3). Duncan's multiple range test was used to analyze significant differences. Different letters indicate significant differences at $P < 0.05$.

and melatonin, we performed transcriptome sequencing on chrysanthemum leaves. Twelve cDNA libraries were sequenced on the DNBSEQ platform, and the total raw reads, total clean reads, and total clean bases obtained were a minimum of 40.48 Mb, 37.58 Mb, and 5.64 Gb (Q20 values > 95.53%, Q30 values > 89.86%, and clean reads > 91.73%), respectively (**Supplementary Table 3**). After assembly and de-redundancy, 145,639 unigenes were obtained. The total length, average length, N50, N70, N90, and GC content were 174,088,211 bp, 1195 bp, 1667 bp, 1193 bp, 616 bp, and 39.58%, respectively (**Supplementary Table 4**).

The results showed 17,262 downregulated and 16,235 upregulated DEGs in the stress/control (S/Con) comparison; in contrast, in the stress with melatonin/control with melatonin (SMT/ConMT) comparison, downregulated and upregulated DEGs were 18,785 and 17,955, respectively (**Figure 3A** and **Supplementary Table 5**). Furthermore, 4378 and 830 DEGs were generated in the comparisons of ConMT/Con and SMT/S, respectively, suggesting that melatonin may control more genes to cope with heat stress. Among the different comparisons, overlapping DEGs were further analyzed. The results showed 329 overlapping DEGs between ConMT/Con and SMT/S, and 25,226

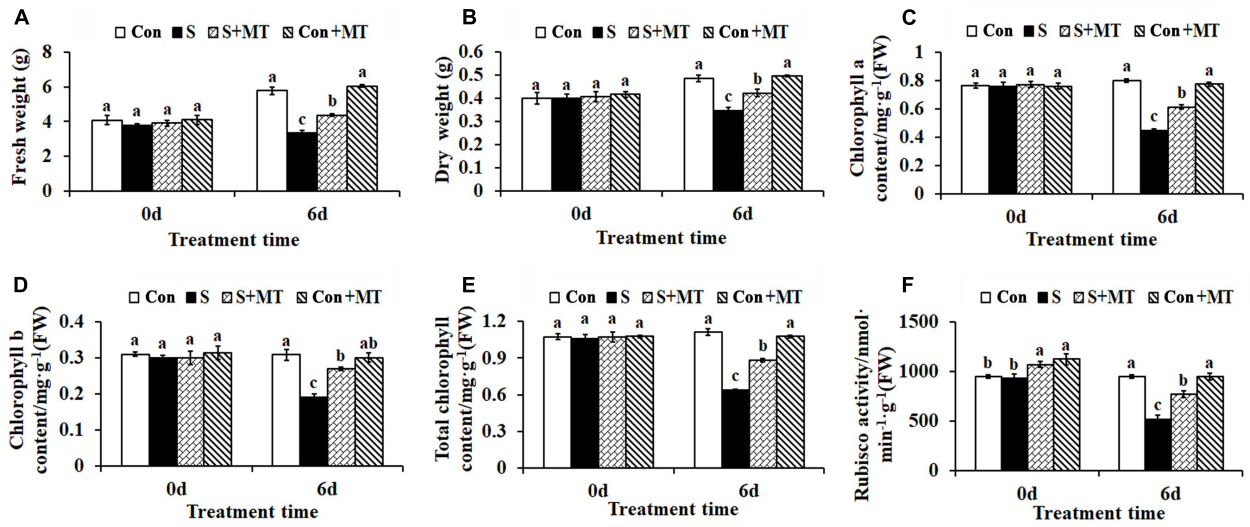

FIGURE 2 | Effects of melatonin on chrysanthemum seedling growth, chlorophyll content and Rubisco enzyme activity under heat stress. **(A)** Fresh weight. **(B)** Dry weight. **(C)** Chlorophyll a. **(D)** Chlorophyll b. **(E)** Total chlorophyll. **(F)** Rubisco activity. Con, control; S, heat stress; S + MT, heat stress with exogenous melatonin treatment; Con + MT, control with exogenous melatonin treatment. Error bars indicate SE (n = 3). Duncan's multiple range test was used to analyze significant differences. Different letters indicate significant differences at $P < 0.05$.

were found between S/Con and SMT/ConMT (**Figure 3B**). To verify the veracity of our transcriptome data, we randomly selected eight genes for qRT-PCR, and the results proved that RNA-seq was reliable (**Figure 4**).

In addition, KEGG enrichment analysis was conducted to explore the potential functions of DEGs in response to melatonin and heat stress (**Supplementary Table 6**). The KEGG pathway in **Figure 3C** indicates that the DEGs in S/Con and SMT/ConMT were enriched in global and overview maps, carbohydrate metabolism, translation, and signal transduction. In addition, DEGs involved in carbohydrate metabolism and biosynthesis of other secondary metabolites were observed in the ConMT/Con and SMT/S comparisons. Annotation results suggested that highly representative pathways might be indispensable for chrysanthemum survival rate and melatonin regulation under heat stress.

Figure 3D and **Supplementary Table 7** list 56 families of TFs. Among them, the number of differentially expressed transcription factors of HSF, MADS, MYB, NAC, TCP, WRKY, and bHLH were 63, 85, 301, 117, 30, 239, and 200, respectively. These results revealed that exogenous melatonin regulated the differential expression of many transcription factors, thus confirming that melatonin plays a vital role in heat stress.

Genes Involved in the Metabolism of ROS-Scavengers, Heat Shock Transcription Factors and Heat Shock Proteins

Based on the FPKM value, we analyzed the transcriptome data to further clarify the molecular mechanism of exogenous melatonin treatment of chrysanthemum leaves under heat stress conditions, and found 12 enzymes, 3 HSFs, and 4 HSPs related genes (**Figure 5** and **Supplementary Table 8**). The expression of glutamate cysteine ligase (*GCL*) and GDP-D-mannose 3′, 5′-epimerase (*GME*) were significantly more active in SMT than in S (**Figure 5**, #1 to #2). The DEGs encoding antioxidant enzymes 1 glutathione reductase (*GR*), 1 *SOD*, 3 *POD* (peroxidase), 3 *CAT* (catalase), and 2 *APX* (**Figure 5**, #3 to #12) respectively, were significantly upregulated in SMT than in S. The expression of the heat-response-related genes *HSFB3*, *HSFA1a*, *HSFA2b*, *HSP23*, *HSP70*, *HSP80*, and *HSP90* was the greatest in the SMT treatment, followed by S (**Figure 5**, #13 to #19). This shows that spraying chrysanthemum leaves with melatonin under intense heat conditions can effectively improve the stress resistance of the plants.

Genes Involved in Ca^{2+} Signal Transduction, Carbon Fixation, and Starch and Sucrose Metabolism

We explored different genes related to calcium signal transduction, photosynthetic biological carbon sequestration, and starch and sucrose metabolism (**Figure 6**). In S/Con and SMT/ConMT comparisons, heat stress induced *WRKYs* and decreased the expressions of cyclic nucleotide gated channel (*CNGCs*) and mitogen-activated protein kinase kinase kinases (*MAPKKKs*). In S/Con, the expression of respiratory burst oxidase (*RBOH*) was increased, whereas, in SMT/S, it was reduced (**Supplementary Table 9**). In the SMT/S comparisons, six DEGs involved in the Ca^{2+} signaling pathway were upregulated (**Figure 6A**). There were four genes in the calmodulin/calmodulin-like protein (CaM/CML) gene family, including two *CML*, one *CAM2*, and one *CAM4*, while two genes (*CNGC4* and *CNGC20*) belonged to the CNGC gene family, and the remaining gene was in the *RBOH* group.

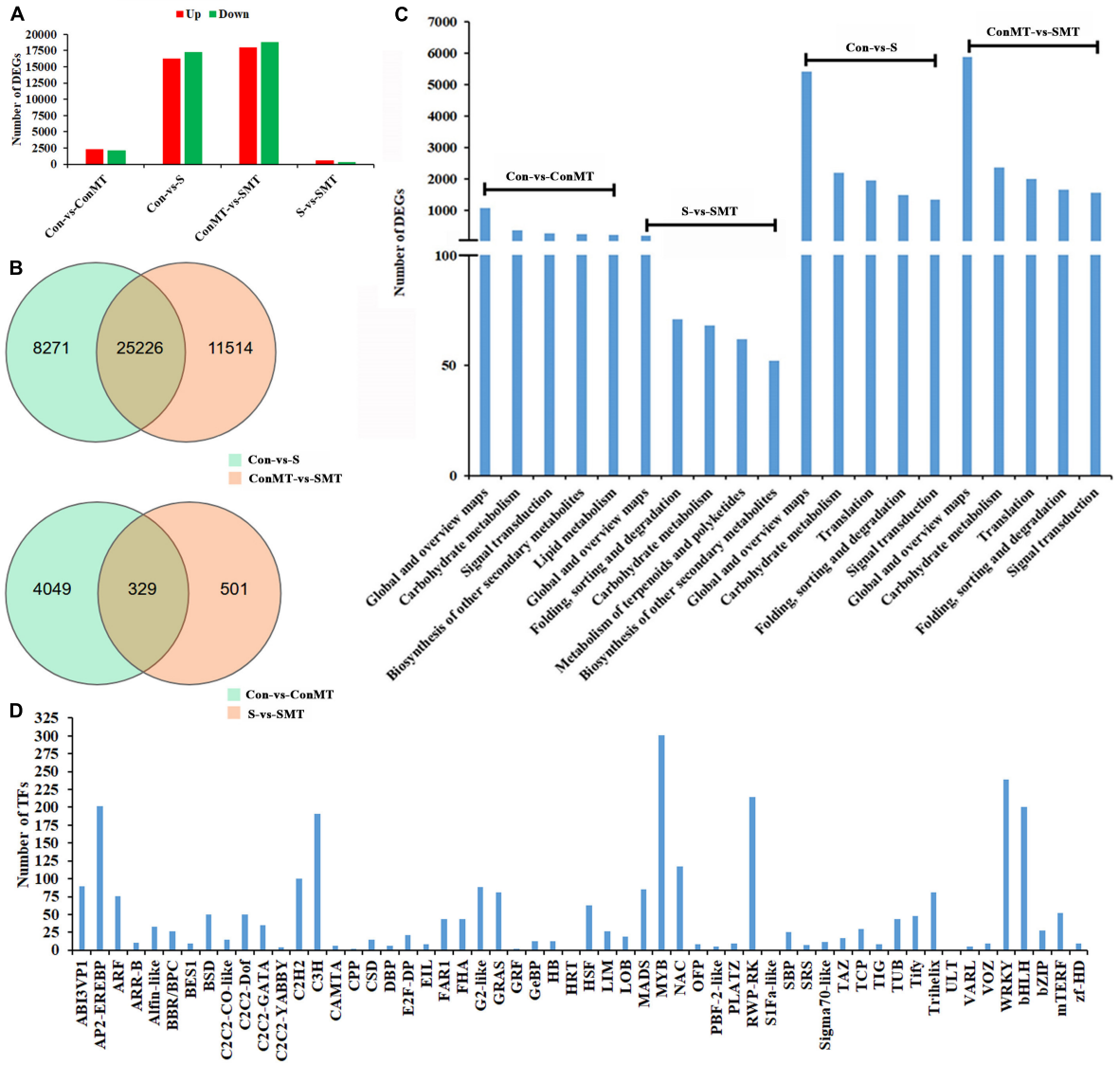

FIGURE 3 | Effects of exogenous melatonin and heat stress on gene expression. **(A)** Number of differentially expressed genes in different comparisons. **(B)** Overlapped differentially expressed genes in different comparisons. **(C)** KEGG analysis in different comparisons (top 5). **(D)** Number of TFs families. Con, control; S, heat stress; SMT, heat stress with exogenous melatonin treatment; ConMT, control with exogenous melatonin treatment.

The majority of the genes in the carbon fixation pathway had inhibited expression in relation to heat stress. We emphasized the DEGs in SMT/S. Specifically, the transcription of TIM, PCK, and RPI were upregulated by melatonin (**Figure 6B** and **Supplementary Table 10**). Our results (**Supplementary Table 11**) showed that in ConMT/Con, five genes were induced, including one β-D-*xylosidase* (*BXL*), one *endoglucanase* (*EDGL*), one *trehalose-phosphate phosphatase* (*TPP*), and two *pectinesterase* genes. One sucrose synthase (SuS) encoding gene was reduced, while *polygalacturonase*, *TPP*, and *pectinesterase* were significantly increased in SMT/S. The expression of DEGs encoding ADP-glucose pyrophosphorylase (AGPase), β-glucosidase (BGLU), EDGL, and endoglucane-1, 3-β-glucosidase (EGLC) was reduced under heat stress; conversely, the expression of genes encoding sucrose phosphate synthase (SPS), *TPP*, α-amylase, and β-amylase were induced both in S/Con and in SMT/ConMT comparisons (**Supplementary Tables 11, 12**).

Genes Involved in Plant Hormone Signal Transduction

Aiming to clarify the regulatory networks involving melatonin in the plant responses to heat stress, we analyzed the differential expression of genes related to plant-hormone signal transduction (**Figure 7** and **Supplementary Table 13**).

In S/Con and SMT/ConMT, ABA signaling related genes protein phosphatase 2C (PP2C1, PP2C3, PP2C5, PP2C6, PP2C10, PP2C11, PP2C16, PP2C25, PP2C29, PP2C47, PP2C55,

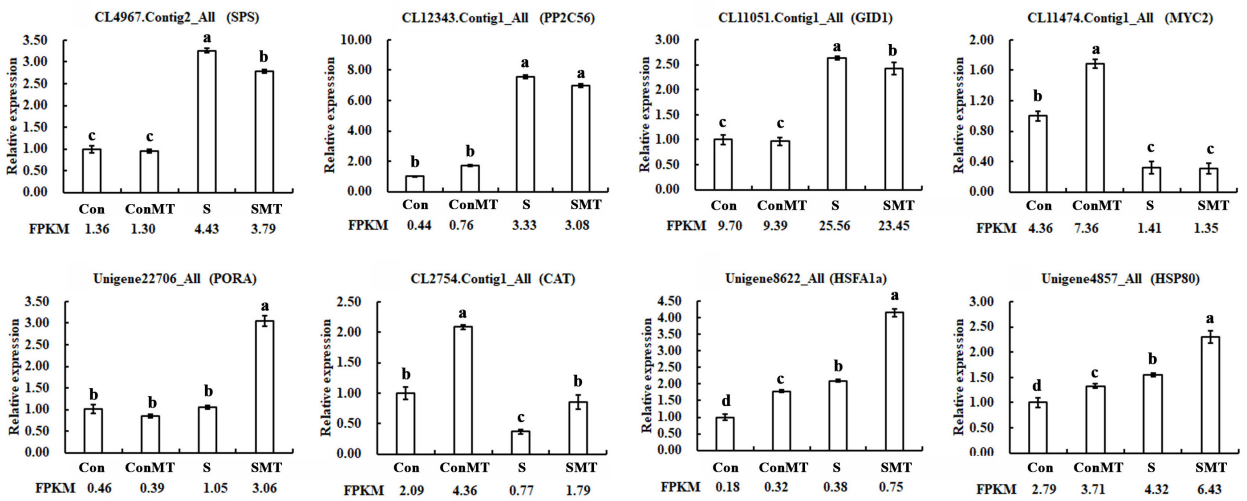

FIGURE 4 | Use of qRT-PCR to verify the accuracy of RNA-Seq. Con, control; S, heat stress; SMT, heat stress with exogenous melatonin treatment; ConMT, control with exogenous melatonin treatment. Error bars represent SE (n = 3). Duncan's multiple range test was used for significant difference analysis, and different letters indicate a significant difference at $P < 0.05$.

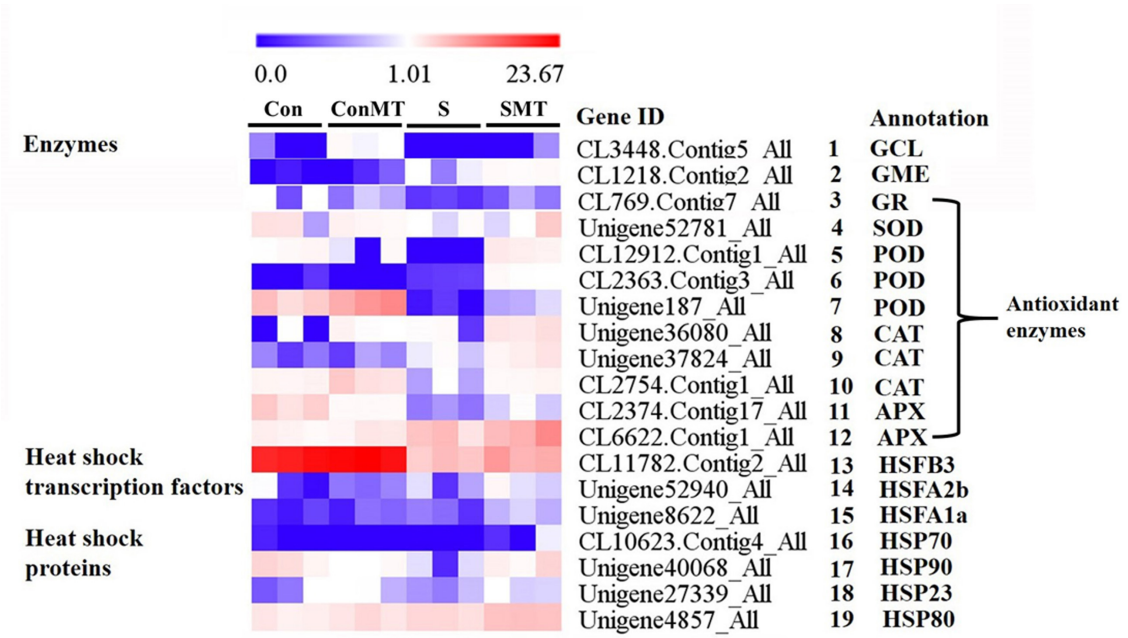

FIGURE 5 | Differentially expressed genes related to enzymes, heat shock transcription factors and heat shock proteins under heat stress in four comparisons. The bar indicates the expression (FPKM) of each gene in Con, ConMT, S, SMT as indicated by blue, white, and red squares. Blue and red represent low and high expression, respectively. Con, control; S, heat stress; SMT, heat stress with exogenous melatonin treatment; ConMT, control with exogenous melatonin treatment. More detailed information is shown in **Supplementary Table 8**.

and PP2C56) (**Figure 7**, #3 to #14), the ABA-insensitive (*ABI5*) (**Figure 7**, #15) gene and *ABF2* (**Figure 7**, #16) were induced under heat stress, whereas the expression of *PYL4* and *PYL5* (**Figure 7**, #1 to #2) were inhibited.

Five DEGs including, one *IAA-induced protein* (*ARG7*) (**Figure 7**, #29), one *AUX28* (**Figure 7**, #27), one *IAA7* (**Figure 7**, #28), and two small auxin-up RNA (SAUR) (**Figure 7**, #25 to #26), were detected in ConMT/Con as induced and related to auxin signal transduction. Heat stress-induced auxin response factors (ARFs) and the expression trends of genes encoding SAURS were inconsistent. In SMT/S, the expression of the auxin receptor *transport inhibitor response 1* (*TIR1*) (**Figure 7**, #17

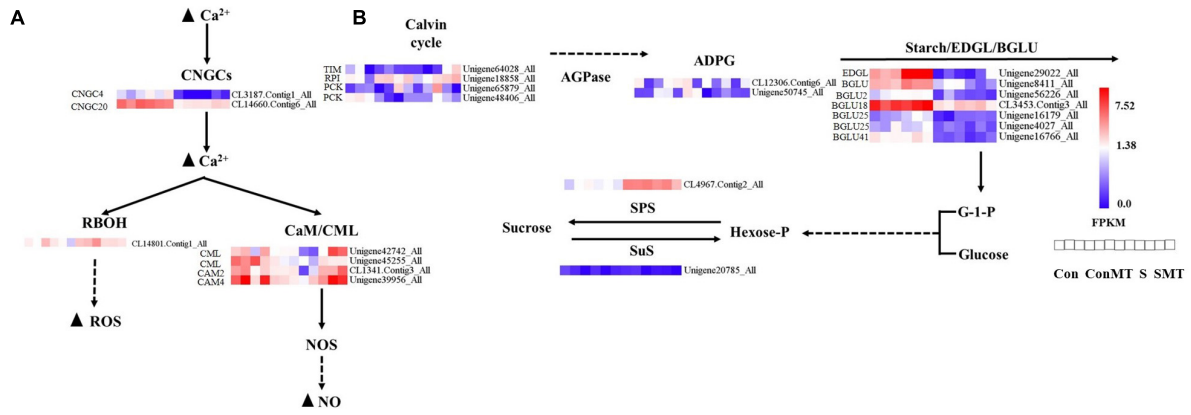

FIGURE 6 | Differentially expressed genes caused by heat stress. **(A)** Calcium signal transduction. **(B)** The metabolism of starch and sugars. The bar represents the expression (FPKM) of each gene in Con, ConMT, S, SMT as indicated by blue, white, and red squares. Blue and red represent low and high expression, respectively. Con, control; S, heat stress; SMT, heat stress with exogenous melatonin treatment; ConMT, control with exogenous melatonin treatment. More detailed information is shown in **Supplementary Table 12**.

to #18) decreased significantly, whereas in S/Con, there was no change in the expression of *TIR1*. Therefore, auxin/indole acetic acid protein (AUX/IAAs) (**Figure 7**, #19 to #21), *ARFs* (*ARF16* and *ARF18*) (**Figure 7**, #22 to #24), *SAUR*, and the auxin-responsive proteins *IAA7* and *ARG7* were expressed as hubs in S/Con, whereas in the SMT/ConMT comparison, *TIR1*, *AUX/IAAs*, *ARF18*, *SAUR*, and *ARG7* were pivotal genes.

In terms of GA signaling, heat stress induced the GA receptor (GID1) (**Figure 7**, #30 to #31), whereas the DELLA proteins (GAI) (**Figure 7**, #32) were reduced in S/Con and SMT/ConMT, and the expression levels of GID2 (F-box proteins) (**Figure 7**, #33) and photosensitive interaction factor 3 (PIF3) (**Figure 7**, #34) were repressed in the latter.

The center gene ethylene-responsive transcription factor 25 (*ERF25*) plays an important role in the heat stress response. In SMT/ConMT and S/Con, EIN3-Binding F-Box protein (*EBF1* and *EBF2*) (**Figure 7**, #35 to #36) were inhibited. In the SMT/S comparison, *ERF25* was downregulated (**Figure 7**, #37).

In terms of jasmonic acid signaling, in both S/Con and SMT/ConMT comparisons, heat stress repressed the expression of *TIFY10C* (**Figure 7**, #41) and *MYC2* (**Figure 7**, #39 to #40) but there was a similar upregulation of *TIFY6B* (**Figure 7**, #42) in the latter comparison, whereas *TIFY10B* (**Figure 7**, #43) and *TIFY10C* were induced in SMT/S.

To reveal how melatonin biosynthesis-related genes were altered in response to heat stress or exogenous melatonin, we analyzed the expression of *SNAT*, *COMT*, and *TDC* genes. In all, ConMT/Con, S/Con, and SMT/S comparisons, *SNAT* (**Figure 7**, #44), *COMT* (**Figure 7**, #45), and *TDC* (**Figure 7**, #46) genes were all upregulated.

Genes Involved in Chlorophyll, Flavonoid, Carotenoid Metabolism

The differential expression of genes involved in chlorophyll, flavonoid, and carotenoid metabolism (**Figure 8** and **Supplementary Table 14**) was identified, which is essential for determining the potential regulatory network of melatonin under heat stress. There were nine differential transcriptions associated with chlorophyll, five genes involved in chlorophyll biosynthesis (**Figure 8**, #1 to #5), two in the chlorophyll cycle (**Figure 8**, #6 to #7), and two genes involved in chlorophyll degradation (**Figure 8**, #8 to #9). The chlorophyll biosynthesis [*HEMA*, *HEMF*, *CHLH*, and protochlorophyllide oxidoreductase (*PORA*)] and chlorophyll cycle (*NYC1/NOL* and *HCAR*) genes had increased abundance in the SMT/S comparison, whereas they remained unchanged in the ConMT/Con comparison. Two transcripts associated with chlorophyll degradation [*CLH* and pheophytinase (*PPH*)] were down-regulated in SMT/S comparison, whereas they were upregulated in the S/Con comparison and remained unchanged in the ConMT/Con comparison. The transcripts associated with flavonoid biosynthesis were (**Figure 8**, #10 to #14) chalcone isomerase (*CHI*), chalcone synthase (*CHS*), dihydroflavonol 4-reductase (*DFR*), flavanone 3-hydroxylase (*F3H*), and flavone synthase (*FNS*). In SMT/S comparison, these genes were all significantly upregulated, but there was no consistent trend in the other comparisons. In addition, under heat stress conditions, genes related to carotenoid synthesis were affected. In this study, we found nine (**Figure 8**, #15 to #23) key genes for carotenoid synthesis, two 1-deoxy-D-xylulose-5-phosphate synthase (*DXPS*) (**Figure 8**, #15 to #16), two geranylgeranyl diphosphate synthase (*GGDP*) (**Figure 8**, #17 to #18), one phytoene synthase (*PSY*) (**Figure 8**, #19), one violaxanthin de-epoxidase (*VDE*) (**Figure 8**, #20), one zeaxanthin epoxidase (*ZEP*) (**Figure 8**, #21), and two 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*DXR*) (**Figure 8**, #22 to #23), all of which were significantly upregulated in SMT/S comparisons. In contrast, the expression of *DXPS*, *PSY*, *VDE*, and *ZEP* were significantly downregulated in S/Con comparisons, but there was no significant difference between *GGDP* and *DXR*.

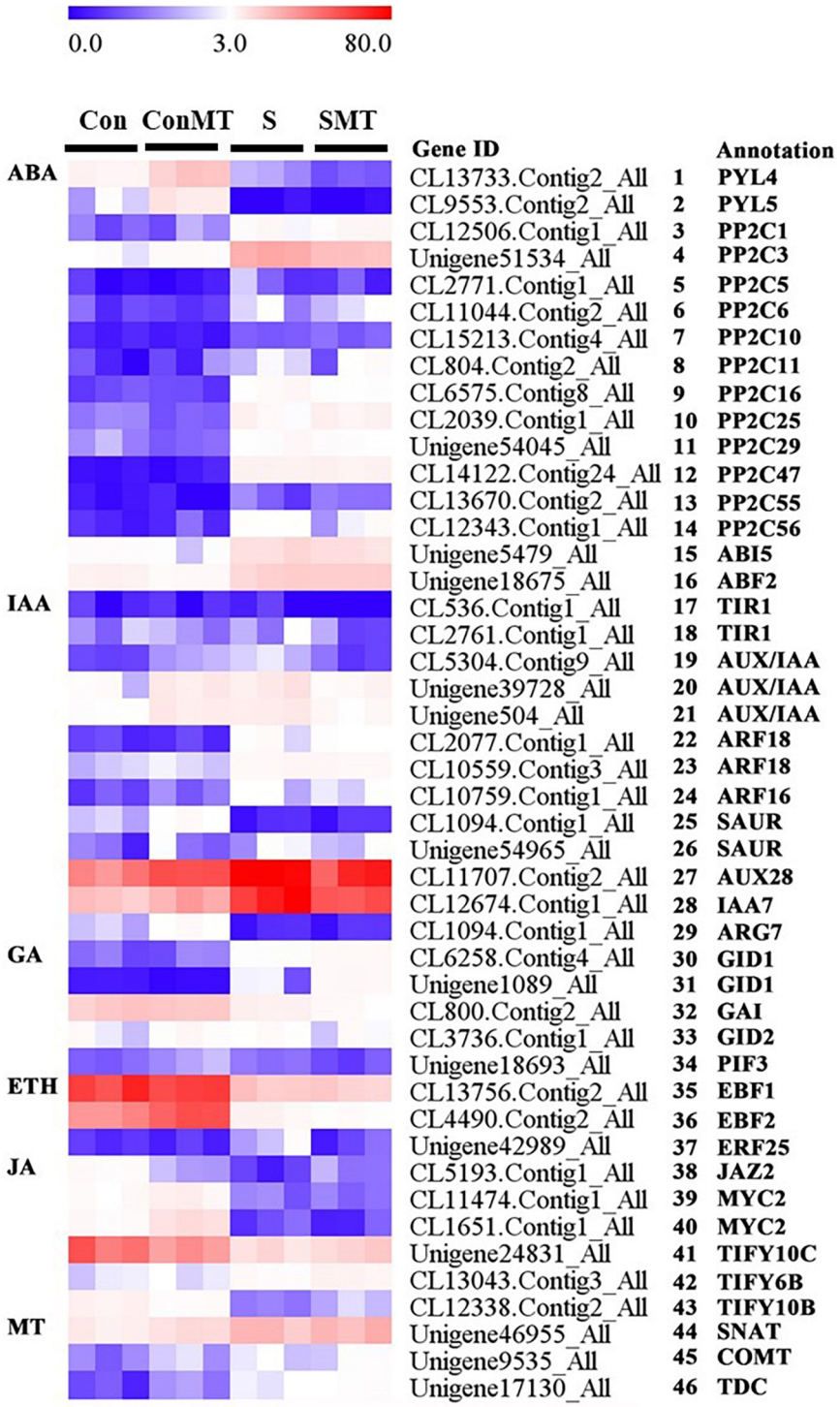

FIGURE 7 | Differentially expressed genes related to plant hormone under heat stress. The bar represents the expression (FPKM) of each gene in Con, ConMT, S, SMT, as indicated by blue, white, and red squares. Blue and red represent low and high expression, respectively. Con, control; S, heat stress; SMT, heat stress with exogenous melatonin treatment; ConMT, control with exogenous melatonin treatment. More detailed information is provided in **Supplementary Table 13**.

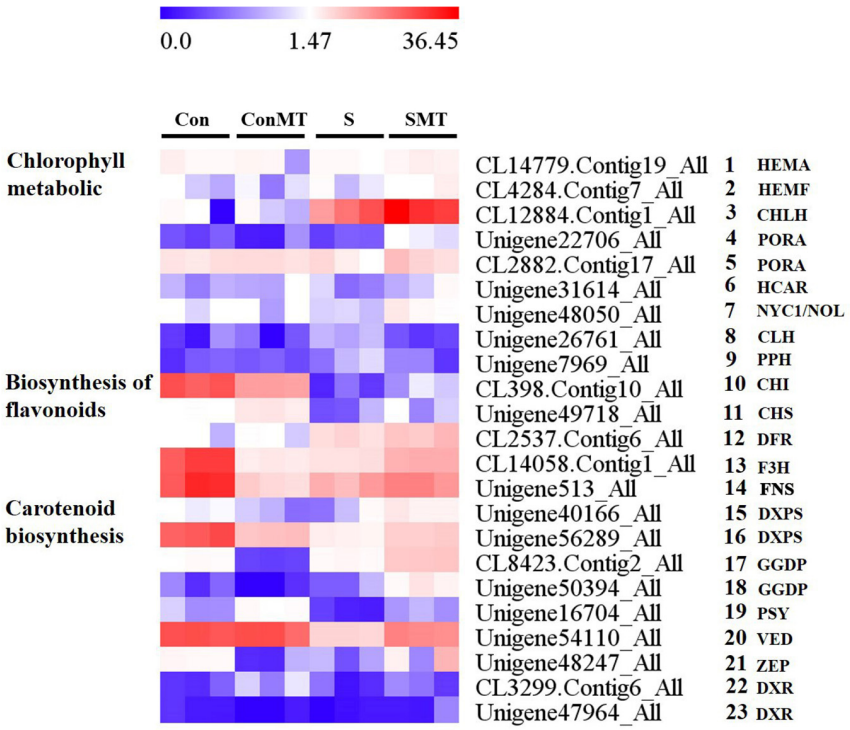

FIGURE 8 | Heat stress modified the expression profiles of chlorophyll, flavonoids, and carotenoid metabolic related genes in four comparisons. The bar represents the expression (FPKM) of each gene in Con, ConMT, S, SMT as indicated by blue, white, and red squares. Blue and red represent low and high expression, respectively. Con, control; S, heat stress; SMT, heat stress with exogenous melatonin treatment; ConMT, control with exogenous melatonin treatment. More detailed information was shown in **Supplementary Table 14**.

DISCUSSION

Effects of Exogenous Melatonin on Osmotic Regulation Substances and Reactive Oxygen Species in Chrysanthemum Seedlings Under Heat Stress

To avoid injury due to heat stress, plants have developed a series of strategies (Chen and Yang, 2019). Thus, for example, H_2O_2 plays a critical role in heat stress (Konigshofer et al., 2008; Banti et al., 2010). AtRbohB and AtRbohD are the main synthetases of $O_2^{\bullet-}$ production by heat stress (Chen and Yang, 2019). In our study, the expression of *RBOH* was significantly upregulated under S, compared to Con treatment; furthermore, we detected that H_2O_2 content and $O_2^{\bullet-}$·production rate increased significantly in chrysanthemum seedlings under heat stress. Melatonin can directly remove ROS to stabilize cell membranes and avoid lipid peroxidation under stress (Zhang et al., 2015). Here, the expression of *RBOH*, and H_2O_2 and $O_2^{\bullet-}$·levels were reduced in SMT/S. The SOD, POD, CAT, and APX activities of chrysanthemum seedlings enhanced the ability of chrysanthemum seedlings to remove ROS, which is consistent with the results of a related study on lupine (Garnczarska and Bednarski, 2004) and other previous studies (Qi et al., 2018). This could relate to the fact that MT in chrysanthemum heightens antioxidant enzyme activity by increasing the expression of the related genes and reducing the degradation of biological macromolecules, thereby enhancing the ability to remove ROS. When plants are subjected to oxidative stress, GSH is one of the effective scavengers produced by intracellular metabolism and the peroxide processes, which makes plants more resistant to environmental stress, therefore, when plants encounter adversity, the contents of AsA and GSH will change (Yin et al., 2008). Compared with SMT, AsA and GSH contents were lower under S, suggesting that MT enhanced GR activity to accelerate the process of regeneration, and AsA and glutathione synthesized these compounds to relatively high concentrations in chrysanthemum seedlings subjected to heat stress; thereby, improving plant resistance under these conditions.

Effects of Exogenous Melatonin on Heat Shock Transcription Factor-Heat Shock Protein in Chrysanthemum Seedlings Under Heat Stress

Heat stress increases endogenous melatonin production in Arabidopsis leaves; furthermore, exogenous melatonin enhances the heat resistance of this genus (Shi et al., 2015). Exogenous melatonin and heat stress remarkably induced the expression of A1 heat-shock factors (*HSFA1s*) in *Arabidopsis thaliana*, which are major regulators of the heat stress response (Shi et al., 2015).

Studies have shown that exogenous melatonin-enhanced heat resistance was notably attenuated in quadruple knockout *HSFA1* mutants, while *HSFA1*-activated the thermal response gene-transcripts (*HSFA2*, *HSP90*, and *HSP101*), implying they might participate in melatonin-mediated thermotolerance (Shi et al., 2015). HSPs are closely related to heat stress, and *Hsp70* gene overexpression can enhance plant tolerance to this condition (Wang et al., 2004). Consistent with previous reports, the transcription of heat response-related genes (*HSFB3*, *HSFA1a*, *HSFA2b*, *HSP23*, *HSP70*, *HSP80*, and *HSP90*) under SMT was significantly greater than that of S, suggesting that heat-responsive genes played a vital part in melatonin-mediated heat resistance of chrysanthemum leaves.

Effects of Exogenous Melatonin on Ca^{2+} Signal Transduction in Chrysanthemum Seedlings Under Heat Stress

In plants, calcium signal transduction is the basic mechanism responsible for sensing and responding to environmental stimuli (Duszyn et al., 2019). In signal transduction, CNGCs are involved in the absorption of Ca^{2+} ions as ligand-gated protein channels. A stress-induced Ca^{2+} increase can activate CNGC, leading to a CaM/CML response (Gao et al., 2020). When plants are subjected to heat stress, the specific Ca^{2+} channel on the cell membrane opens, allowing Ca^{2+} to flow along the concentration gradient and activate multiple calcium/calmodulin-binding protein kinases and Ca^{2+}-dependent protein kinases, which initiates the expression of downstream genes related to heat stress. In *Ganoderma lucidum*, plasma membrane-mediated extracellular calcium influx, intracellular calcium store release, and other calcium ions from different sources are involved in regulating the increase in intracellular calcium content under heat stress (Zhang et al., 2016), thereby improving the heat tolerance of plants. In our study, in the SMT/S comparisons, *CNGCs* and *CAM/CMLs* were upregulated. Therefore, MT enhanced the stress resistance of plants by activating the expression of *CNGCs* and *CAM/CMLs*.

Effects of Exogenous Melatonin on Carbon Fixation, and Starch and Sucrose Metabolism in Chrysanthemum Seedlings Under Heat Stress

Heat stress can cause changes in photosynthesis of plants, thereby shortening their life cycle and reducing their productivity (Barnabas et al., 2008). Increasing temperatures inhibit the activities of various enzymes in the Calvin cycle during photosynthesis (Morales et al., 2003). However, by regulating the transcription of *TIM*, *RPI*, and *PCK* genes, MT can effectively modulate carbon fixation and improve photosynthesis under heat stress (Liang et al., 2019). In SMT/S comparison, MT induced the expression of genes encoding *TIM*, *RPI*, and *PCK*.

The high utilization rate of carbohydrates under high-temperature stress is closely related to heat resistance (Roitsch and González, 2004). As sugars act as signaling molecules in the stress response pathway, sucrose and its metabolites can modulate the developmental processes of plants and their response to heat stress through changes in the distribution of carbon, and sugars signaling (Yang X. et al., 2019). Stress induction accelerates the conversion of starch to sugars, thereby, playing a protective role as it contributes to osmotic regulation and a quick energy supply (Dong and Beckles, 2019). When the endosperm develops at high temperatures, starch accumulation decreases (Zhao et al., 2008). SPS plays a vital role in the synthesis of sucrose (Winter and Huber, 2000), our results reveal that the decrease in starch content and induction of α-*amylase*, β-*amylase*, and *SPS* genes under heat stress may promote starch degradability. Exogenous melatonin induced the accumulation of starch and balanced the content of sugars. In line with this hypothesis, exogenous melatonin reportedly improves photosynthesis in plants, which in turn promotes starch accumulation (Sharma et al., 2020). Interestingly, *SuS* was downregulated in the SMT/S comparisons, and *EDGL* was downregulated in S/Con and SMT/ConMT comparisons. Moreover, *BGLU* (*BGLU2*, *BGLU12*, and *BGLU18*) genes were specifically downregulated in the S/Con and SMT/ConMT comparison. Studies have shown that the deficiency of *BGLU18* delays the accumulation of dehydration-induced ABA, indicating that melatonin can stabilize ABA content and abiotic stress responses by regulating BGLU18-mediated ABA-glucose ester hydrolysis (Han et al., 2020). In conclusion, heat stress activated sucrose metabolism and starch degradation, while melatonin enhanced resistance to heat stress by positively regulating the accumulation of carbohydrates and the ratio of starch to sucrose.

Effects of Exogenous Melatonin on Hormone Signal Transduction in Chrysanthemum Seedlings Under Heat Stress

Studies have shown that heat stress induces plant hormones, such as ABA, AUX, GA, and ETH, which are considered to play a vital role in plant heat tolerance (Kotak et al., 2007). In this study, the differential expression of hormone-related genes was involved in different treatments, further, these genes were related to the increase in heat resistance of chrysanthemum seedlings upon spray treatment with exogenous melatonin under heat stress.

PYR/PYL are ABA receptors that interact with PP2C to reduce the inhibitory effect on SnRK2, thereby regulating the downstream gene ABF (Yang X. et al., 2019; Yang Y. et al., 2019). In the present study, heat stress induced the expression of *PP2Cs*, *ABI5*, and *ABF2*. Studies have shown that overexpression of ABA receptors PYR/PYL might promote ABA signal transduction, thereby increasing plant resistance to abiotic and biotic stress conditions (Liu et al., 2020). Melatonin plays a vital part upstream of the ABA signal (Arnao and Hernandez-Ruiz, 2018), and in this study, melatonin induced the expression level of *PYL4* genes, while the effects of melatonin on the expression of *PP2Cs* did not involve DEGs; therefore, we hypothesized that the MT-mediated expression of *PYL4* enhanced heat stress resistance.

Auxin plays a negative regulatory role in the process of plant resistance to stress, and Aux/IAAs and TIR1/AFB

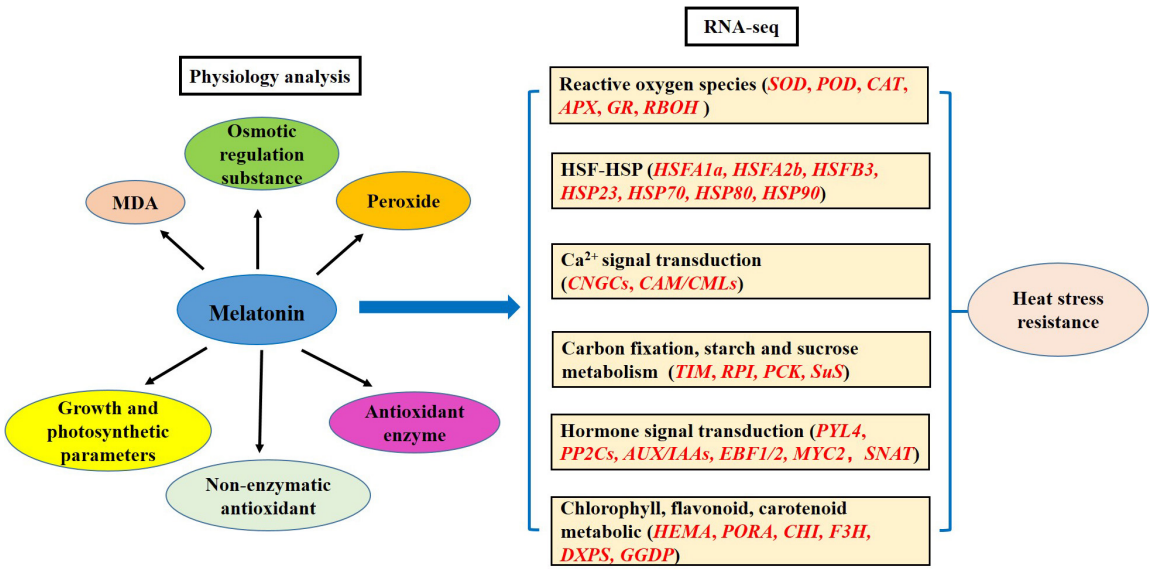

FIGURE 9 | Model of melatonin regulating the adaptability of chrysanthemum seedlings to heat stress.

receptors activate a complex regulatory network to regulate the expression of ARF genes (Kazan, 2013; Jung et al., 2015). In accordance with our results, melatonin significantly reduced the transcription of *TIR1* and *Aux/IAA* under heat stress. Studies have found that the IAA content increases under heat stress conditions (Franklin et al., 2011), and in our study, *IAA7* was significantly upregulated by heat stress treatment. In addition, melatonin can regulate the interaction between Aux/IAA and TIR1 to resist heat stress (Naser and Shani, 2016), which was consistent with our study results, suggesting that Aux/IAA multimers significantly inhibited auxin-signal transduction and might improve resistance to heat stress by affecting ROS metabolism. With an increase in GA content, GA interacts with DELLA protein (GAI) after binding to its receptor GID1, thereby causing it to be ubiquitinated and degraded, ultimately activating the GA response (Wang et al., 2021). Under salt stress, melatonin promoted the expression of GA biosynthesis genes *GA20ox* and *GA3ox* in cucumber seedlings, causing the upregulation of GA3 and GA4 (Zhang et al., 2014, 2016). Heat stress decreased GA levels by repressing the expression of GA biosynthetic genes, such as *GA20ox1*, *GA20ox2*, *GA20ox3*, *GA3ox1*, and *GA3ox2* (Toh et al., 2008). Although GA is normally identified as possessing an antagonistic effect on ABA, there is a strong interaction between DELLA proteins and ABF2 (Wang et al., 2020). In accordance with our results, GAI, GID1, GID2, and phytochrome interacting factor 3 were the core components of the GA signal pathway. In studies of tomatoes (Inaba and Chachin, 1988) and apples (Lurie and Klein, 2006), it was found that heat stress (above 38°C) reduced the rate of fruit ripening and the production of ethylene and enhanced respiration. The production of ethylene can improve the resistance of plants to heat stress. Exogenous melatonin slightly increased ethylene generation by inducing the expression of 1-aminocyclopropane-1-carboxylic acid synthase. In contrast, melatonin in etiolated lupine seedlings significantly inhibited ethylene synthesis (Arnao and Hernández-Ruiz, 2007). In the present study, the hub genes *EBF2* and *ERF25* were downregulated by MT under heat stress. *SNAT*, *COMT*, and *TDC* are the melatonin biosynthetic genes (Arnao and Hernandez-Ruiz, 2014). Exogenous melatonin application induced the accumulation of endogenous MT and upregulated the expression of *SNAT* and *COMT* genes in loquat seedlings during a stress period (Wang et al., 2021). In *Agaricus bisporus*, exogenous melatonin application promoted endogenous MT accumulation by increasing the expression levels of *TDC*, *T5H*, *SNAT*, and *ASMT*, which was helpful in protecting membrane integrity (Shekari et al., 2021). Consistent with the previous studies, here, an exogenous melatonin spray promoted the expression of melatonin biosynthesis-related genes *SNAT*, *COMT*, and *TDC* under heat stress.

Effects of Exogenous Melatonin on Chlorophyll, Flavonoid, and Carotenoid Metabolism in Chrysanthemum Seedlings Under Heat Stress

Heat stress negatively affects various physiological processes of plants, including photosynthesis, and flavonoid and carotenoid metabolism. Heat stress can lead to the degradation of plant chlorophyll, the decrease in photosynthetic rate, the hindrance of photosynthetic electron transfer, and the decrease of enzyme activity related to carbon assimilation (Zhou et al., 2016). Melatonin can improve plant photosynthesis under adversity and improve its resistance to stressors (Biswojit et al., 2018). Consistent with previous results, chlorophyll a, chlorophyll b, and total chlorophyll contents were significantly higher under the SMT than those under the S treatment. The chlorophyll

biosynthesis (*HEMA*, *HEMF*, *CHLH*, and *PORA*) and chlorophyll cycle (*NYC1/NOL*) genes were upregulated, and chlorophyll degradation (*CLH* and *PPH*) genes were down-regulated in SMT/S comparison.

Flavonoids are important secondary metabolites in plants. Flavonoids can eliminate various types of ROS, thus resulting in a strong antioxidant capacity (Hernandez et al., 2009). When plants encounter heat stress and other adversity stresses, and a large amount of ROS accumulates in their bodies, flavonoids can degrade this excess ROS and maintain the ROS metabolism balance in plants. Studies have found that melatonin up-regulates the biosynthesis of polyphenols such as total phenols, flavonoids, and anthocyanins in grape berries (Meng et al., 2019). Similar results were found in cabbage, tomato (Sun et al., 2015), and other plants. Melatonin can enhance the activity of phenylalanine ammonia lyase, cinnamic acid-4-hydroxylase, CHS, F3H, leucoanthocyanin reductase, and anthocyanin reductase, and enhance the transcriptional abundance of the corresponding genes, thereby promoting the production of flavonoids, such as anthocyanins in the leaves of kiwifruit, and delaying senescence (Liang et al., 2018). In our study, *CHI*, *CHS*, *DFR*, *F3H*, and *FNS* were all significantly upregulated in the SMT/S comparison, suggesting that exogenous melatonin can improve the stress resistance of chrysanthemum under heat stress conditions.

In addition, studies have found that lutein and some other terpenoids can stabilize and protect the thylakoid membrane from abiotic stress (Camejo et al., 2006). Meanwhile, after overexpressing the chyB gene in *Arabidopsis*, the resistance to heat stress was higher, which indicates that zeaxanthin can prevent oxidative damage of the membrane (Meiri et al., 2010). The survival rate of *Pinctada fucata* decreased with an increase in temperature from 26 to 34°C and with decreasing total carotenoid content. Conversely, a higher total carotenoid content was accompanied by a higher survival rate. This compound, along with and total antioxidant capacity reduced evidently at 30°C with increasing stress (Meng et al., 2016). In our study, the carotenoid synthesis-related genes, such as *DXPS*, *GGDP*, *PSY*, *VDE*, *ZEP*, and *DXR* were significantly upregulated in SMT/S comparisons, and the expression of *DXPS*, *PSY*, *VDE*, and *ZEP* were significantly downregulated in S/Con comparisons, but there was no significant difference between *GGDP* and *DXR*. The results showed that melatonin effectively alleviated the degradation of carotenoids under heat stress conditions and improved the stress resistance of chrysanthemums.

Overall, exogenous spraying of melatonin improves the resistance of chrysanthemum leaves under heat stress conditions, including physiological and transcription analyses. The physiological aspects mainly include MDA, osmotic regulation substances, peroxides, non-enzymatic antioxidant, and antioxidant enzymes. RNA-seq involves the ROS, HSF-HSP, calcium ion-calmodulin, carbon fixation, starch and sucrose metabolism, hormone, and chlorophyll, flavonoid, and carotenoid pathway-related genes. Based on the above results, we propose the model of the MT-regulated adaptive response to high temperature stress in chrysanthemum leaves (**Figure 9**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

WF, JfJ, and FC conceived and designed the project. XX and YD collected the materials. YD and JyJ carried out the lab work and measured the morphological traits. XX and AS performed the analysis. XX wrote the manuscript with the help from JfJ. FC and SC supervised the experiment. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.673236/full#supplementary-material

Supplementary Table 1 | Primer sequences of transcriptome qRT-PCR.

Supplementary Table 2 | Effects of melatonin treatment on photosynthesis and gas exchange parameters in chrysanthemum seedlings.

Supplementary Table 3 | Summary of sequencing reads after filtering in chrysanthemum leaves were treated with exogenous melatonin.

Supplementary Table 4 | Quality metrics of unigenes in chrysanthemum leaves were treated with exogenous melatonin.

Supplementary Table 5 | Differentially expressed genes in Con-vs.-ConMT, Con-vs.-S, ConMT-vs.-SMT, and S-vs.-SMT comparisons.

Supplementary Table 6 | KEGG analysis (top 5) in Con-vs.-ConMT, Con-vs.-S, ConMT-vs.-SMT, and S-vs.-SMT comparisons.

Supplementary Table 7 | Number of TFs families.

Supplementary Table 8 | Detailed information of the changes in the expression of enzymes, heat shock transcription factors, and heat shock proteins related genes in Con, ConMT, S, and SMT comparisons.

Supplementary Table 9 | Detailed information of the changes in the expression of WRKY, Ca^{2+} signal transduction, and MAPKKKs in Con, ConMT, S, and SMT comparisons.

Supplementary Table 10 | Detailed information of the changes in the expression of calvin cycle in Con, ConMT, S, and SMT comparisons.

Supplementary Table 11 | Detailed information of the changes in the expression of starch and sucrose in Con, ConMT, S, and SMT comparisons.

Supplementary Table 12 | Detailed information of the changes in the expression of the vital genes in the Ca^{2+} signal transduction, starch and sugars metabolism in Con, ConMT, S, and SMT comparisons.

Supplementary Table 13 | Detailed information of the changes in the expression of hormone related genes in Con, ConMT, S, and SMT comparisons.

Supplementary Table 14 | Detailed information of the changes in the expression of chlorophyll, flavonoids, and carotenoid metabolic related genes in Con, ConMT, S, and SMT comparisons.

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Melatonin Alleviates Low-Temperature Stress *via* ABI5-Mediated Signals During Seed Germination in Rice (*Oryza sativa* L.)

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With increasing areas of direct sowing, low-temperature (LT) stress drastically affects global rice production. Exogenous applications of melatonin (MT) serve as one of the effective ways to improve seed germination under various stress conditions. In this study, we found that MT treatment greatly improved the LT stress-induced loss of germination percentage and the weak performance of seedlings under LT of constant 20°C (LT20). This was largely dependent on the activated antioxidant system and enhanced activities of storage substance utilization-associated enzymes. Moreover, we also detected that exogenous feeding of MT significantly increased the biosynthesis of gibberellin (GA) and endogenous MT but simultaneously inhibited the accumulation of abscisic acid (ABA) and hydrogen peroxide (H_2O_2) under LT20 stress. These results suggested that MT had antagonistic effects on ABA and H_2O_2. In addition, MT treatment also significantly enhanced the expression of *CATALYSE 2* (*OsCAT2*), which was directly regulated by ABA-INSENSITIVE 5 (OsABI5), a core module of ABA-stressed signals, and thus promoting the H_2O_2 scavenging to reach reactive oxygen species (ROS) homeostasis, which consequently increased GA biosynthesis. However, in *abi5* mutants, *OsCAT2* failed in response to LT20 stress irrespective of MT treatment, indicating that OsABI5 is essential for MT-mediated seed germination under LT20 stress. Collectively, we now demonstrated that MT showed a synergistic interaction with an ABI5-mediated signal to mediate seed germination, partially through the direct regulation of *OsCAT2*.

Keywords: rice, seed germination, low temperature, melatonin, ABI5

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INTRODUCTION

Seed germination is vital for the subsequent establishment of seedlings in higher plants (Bewley, 1997). Some favorable environmental conditions, including at least optimal temperature, water, and oxygen, are required for the initiation of seed germination (Gutterman, 2002). However, due to their sessile lifestyle, plants may encounter various abiotic stress conditions and are forced to delay seed germination during the sowing seasons (Baskin and Baskin, 1998; Ahmad et al., 2012). It is well known that temperature plays a crucial role in the determination of germination under field conditions (Batlla and Benech-Arnold, 2010), whereas extreme temperature (i.e., cold and heat)

makes negative effects on the metabolic process and water absorption, and consequently leads to a huge loss of germination rates during seed germination (Lou et al., 2007). For example, the rate of germination was largely prohibited or reduced in wheat (Cheng et al., 2009) and *Triticum aestivum* (Yamamoto et al., 2008) under high-temperature conditions. At the same time, seeds failed to germinate at low temperatures (LTs) in melon (Edelstein and Kigel, 1990) and *Citrullus colocynthis* (El-Keblawy et al., 2019).

Due to its tropical and subtropic origins, *Oryza sativa* L. is very susceptible to LT during the developmental stages (Hussain et al., 2016), and seed germination would be greatly delayed when the temperature is less than 15°C in rice (Fujino et al., 2004). LT stress drastically threatens the rice output of over 15 Mha all over the world (Lou et al., 2007). Nowadays, the race between increasing areas of direct sowing and increasing unstable climate during rice seedling (Iwata et al., 2010; Hussain et al., 2016; Wang et al., 2018) emphasizes the urgency to solve the challenges of rice cultivation at LTs, especially in several temperate Asian countries.

Exogenous applications of melatonin (*N*-acetyl-5-methoxytryptamine, MT) have been determined as an effective way to improve seed germination under various stress conditions (Hernández et al., 2015; Xiao et al., 2019; Simlat et al., 2020). For example, exogenous MT enabled the promotion of seed germination under salinity (Zhang et al., 2014) and water stress (Zhang et al., 2013) in cucumber (*Cucumis sativus* L.) while high levels of germination under post-salt and toxic copper ion stress conditions were also observed in cotton (*Gossypium hirsutum* L.) (Chen et al., 2020) and in red cabbage (Posmyk et al., 2008), respectively. Moreover, MT treatments also protected seeds against heat stress in *Arabidopsis* (Hernández et al., 2015) and salt stress in *Stevia rebaudiana* Bertoni (Simlat et al., 2020). In addition, improvements of MT on seed germination have also been reported in maize under chilling stress (Cao et al., 2019).

Germination involves a series of phytohormones in abiotic stress signaling such as abscisic acid (ABA), gibberellin (GA), and MT (Ahmad et al., 2012; Kanwar et al., 2018). Notably, the cross talk of MT with other phytohormones and stress makers constitutes a very complex regulatory network (Kanwar et al., 2018). Among these, ABA and its downstream signal components, including ABSCISIC ACID-INSENTIVE3 (ABI3) and ABI5, have been established as the downstream signals of MT in the regulation of antioxidant response to adverse conditions (Kanwar et al., 2018). MT may also regulate seed germination by improving antioxidant systems and scavenging reactive oxygen species (ROS) (Posmyk et al., 2008; Zhang et al., 2013). Moreover, ABI5 has been revealed to regulate seed germination partially through the homeostasis of ROS in *Arabidopsis* (Bi et al., 2017) and in barely (Ishibashi et al., 2017). Recently, Lv et al. (2021) reported that high concentrations of MT suppressed seed germination through its interrelationships with ABA, GA, and auxin under normal conditions in *Arabidopsis*. These studies confirmed that MT was involved in regulating seed germination *via* ABA signals. However, the possible regulatory mechanism of MT on seed germination *via* the balance of ABA and ROS remains largely unknown at LT in rice. Therefore, we now focused on the balance between MT and ROS under LT stress during rice seed germination.

MATERIALS AND METHODS

Plant Materials

To generate *abi5* mutants, the 1st exon of *OsABI5* (*Os01g0859300*) was selected as a target (**Supplementary Figure 1**). The single-guide RNAs (sgRNAs) were designed by searching UniProt for precise positions[1], and the CRISPR-P program[2] was used to minimize the off-target effect (Lei et al., 2014). Rice calli was induced from mature seeds of the cultivar *O. sativa* L. *japonica* and was transformed with the pH-*osabi5* vector by *Agrobacterium*-mediated transformation. The cultivar *O. sativa* L. *japonica* was used as the wild type (WT).

The working MT solution was prepared as follows. About 0.175 g MT (Sangon Biotech, Shanghai, China) was firstly dissolved in 3 ml of ethyl alcohol, and then the fully dissolved solution was added with distilled water (DW) up to 500 ml, which served as the stock solution of 1,500 μmol/L. When in use, the working solution was prepared by diluting the stock solution of 1,500 μmol/L with DW (v:v = 1:9) until the final concentration reaching 150 μmol/L. The concentration of 150 μmol/L was obtained according to previous reports (Li X. et al., 2017).

For experimental treatments, the sterilized seeds were evenly placed on a 9-cm petri dish and then supplemented with 10 ml of DW with and without 150 μmol/L MT. Subsequently, all seeds were incubated for 7 days (Li et al., 2019) in the artificial chambers (12 h PAR, 300 μmol photons m^{-2} s^{-1} light/12 h dark; relative humidity: 65–75%) with the following temperature conditions: (i) 30/30°C (as the control check, CK); (ii) 24/24°C (as LT at 24°C, LT24); (iii) 23/23°C (as LT at 23°C, LT23); (iv) 20/20°C [as LT at 20°C (LT20)]; (v) 16/16°C (as LT at 16°C, LT16); and (vi) 15/15°C (as LT at 15°C, LT15). About 60 seeds derived from the same mother plant were served as one biological replicate, and six biological repeats were employed per time for each treatment. Seed germination was observed and recorded daily within the investigated days, and the evaporated water was added by the weighing method.

Determination of Seed Morphological Index

Germinated seeds were evaluated according to the conditions that (i) the seed coat was broken, (ii) the length of radicle over exceeded the seed length, and (iii) the plumule length at least reached half of the seed length. The germination number of seeds was recorded every day in the germination period. Germinability was counted on the 5th day, whereas the germination percentage was counted on the 7th day. Seedlings were selected from each treatment to measure moisture absorption, healthy seedling rates, radicle length, plumule length, and fresh weight on the 7th day. The germination index and vital index were calculated

[1] http://www.uniprot.org/

[2] http://cbi.hzau.edu.cn/cgi-bin/CRISPR/

according to the method as mentioned in a previous study (Liu and Liu, 2018).

Quantification of the Associated Physiological Attributes

To determine physical efficiency, seedlings on the 7th day were dried at 105°C for 15 min and 85°C for 24 h (Zhu et al., 2012), and the weights were recorded for calculation.

Respiratory rates were measured using the devices of LI-6400XT system (LI-COR Biosciences, Lincoln, NE, United States) (Wang et al., 2005).

Membrane permeability was determined using the conductometry method (Liu and Zhu, 2014). Briefly, the cutup pieces of 0.3-g plumules were fully immersed into DW through a sealed vacuum. After being placed at room temperature for 60 min, the conductance values of S_1 were determined by using the conductometer (DDS-11C). The abovementioned samples were then transferred to a boiling water bath for 10 min, and the conductance values of S_2 were detected after cooling to room temperature. The conductance values of DW (S_0) were used as a blank control. Relative conductance (L) was calculated as follows: $L = (S_1 - S_0)/(S_2 - S_0)$.

Contents of the soluble sugar were analyzed by using the anthrone colorimetric method described by Fairbairn (1953). Briefly, 0.3 g of plumules were immersed into the DW and then boiled for 30 min in a water bath to collect the extracts. After adding sulfuric acid and anthrone, the absorbances of the extracts at 620 nm were determined to calculate the contents by a standard curve.

Contents of the soluble protein were determined with the Coomassie brilliant blue g-250 (G-250) as previously described (Sedmak and Grossberg, 1977). In brief, 0.3 g of plumules were ground in 1 M of pre-cold phosphatic buffer solution (containing 0.1% PVP, 0.1 M ethylenediaminetetraacetic acid (EDTA), 1 M ascorbic acid, pH 7.8), and then centrifuged at 15,000 *g* for 30 min at 4°C. The supernatant was collected and then incubated with G-250 for 2 min. The absorbances at 595 nm were measured to calculate the protein contents by a standard curve.

To determine the free proline contents (Bates et al., 1973; Song et al., 2021), 0.3 g of plumules were homogenized in 3% aqueous sulfosalicylic acid, and the filtered homogenate was collected to incubate in a boiling water bath for 10 min. After cooling to room temperature, the filtrate was centrifuged at 5,000 *g* for 10 min. The supernatants were continuously reacted with 2.5% ninhydrin and glacial acetic acid at 100°C for 60 min. After terminated in an ice bath, the reaction was added with 4 ml toluene. The chromophore phase was collected to measure the absorbance at 520 nm. The proline concentration was determined by using a standard curve.

Phospholipid contents were determined by using the Phospholipid Assay Kit (ab234050, Colorimetric, Abcam, Shanghai, China) according to the instructions of the manufacturer.

Acid values were determined using the method of potassium hydroxide (KOH) titration (Szafrańska, 2015). Briefly, 0.3-g plumules were ground in liquid nitrogen and totally solvent into the mixture of ethanol-diethyl ether. The mixture was then added with a phenolphthalein-alcohol indicator and was subsequently titrated with 0.1 M KOH until the reddish color would not disappear in the time period of 30 s.

Determination of Enzymic Activities

Acid phosphatase (ACP) activity was measured using the method previously described by Olde Venterink (2011). Briefly, 0.1 g of plumules were homogenized in an ice-cold buffer [50 mM acetic acid-sodium acetate, pH 5.8, 50 mM-mercaptoethanol, 12.5% (v/v) glycerol], and the homogenate was then centrifuged at 20,000 *g* for 10 min at 4°C. The supernatant was incubated with 5 mM para-nitrophenylphosphate (p-NPP) at 30°C for 30 min, and then the reaction was terminated with 0.3 M NaOH. The absorption at 405 nm was measured to determine the ACP activity with a standard curve.

To detect the activity of cytochrome oxidase (CCO), 0.1 g of samples were homogenized in an ice-cold buffer [50 mM HEPES-NaOH, pH 7.4, 2 mM $MgCl_2$, 50 mM mercaptoethanol, 12.5% (v/v) glycerol], and the homogenate was then centrifuged at 20, 000 *g* for 10 min at 4°C. The supernatants were used for enzyme activity assays with the Cytochrome C Oxidase Assay Kit (ab239711, Abcam, Shanghai, China) according to the instructions of the manufacturer.

The activity of succinic dehydrogenase (SDH) was determined spectrophotometrically using 2,6-dichlorophenolindophenol (DCPIP) according to the method mentioned in a previous study (Robinson and Lemire, 1995). Briefly, 0.1 g of plumules were homogenized in an ice-cold buffer [50 mM phosphate buffer, pH 7.4, 0.4 M sucrose, 10 mM EDTA, 50 mM-mercaptoethanol, 12.5% (v/v) glycerol], and the homogenate was then centrifuged at 20,000 *g* for 10 min at 4°C. The supernatant was incubated with the reaction medium (containing 20 mM phosphate buffer, pH 7.2, 0.1% Triton X-100, 4 mM sodium azide, 50 mM DCPIP) and 10 mM succinate at 37°C for 10 min. The reduction of DCPIP at 600 nm was used to calculate SDH activity by using the molar absorption coefficient of reduced DCPIP (21.0 mM^{-1} cm^{-1}).

The activity of α-amylase was determined according to the method described in a previous study (Ishimoto et al., 2015). Briefly, 0.1 g of plumules were ground in a mortar with 20 mM sodium phosphate buffer (pH 6.7), and then centrifuged at 15,000 *g* for 10 min at 4°C. The supernatants were incubated with the substrate solution (1% potato starch, 20 mM NaCl, 0.1 mM $CaCl_2$) for 15 min at 30°C. The reaction was then terminated by the addition of 3,5-dinitrosalicylic acid reagent in a boiling water bath for 10 min. The absorbance was measured at 546 nm, and α-amylase activity was expressed as micromoles of maltose liberated per minute.

Determination of Antioxidant Activity

The activities of antioxidant enzymes were performed using the methods mentioned in a few previous studies (Zhou et al., 2014; Jiang et al., 2021) with little modifications. Briefly, the homogenized powder with liquid nitrogen from 0.3 g plumules was suspended with 50 mM pre-cold phosphate buffer (containing 0.2 mM EDTA, 2%

polyvinylpyrrolidone (W/V), pH 7.8), and then immediately centrifuged at 4°C for 10 min (12,000 *g*). Subsequently, the supernatants were collected and used for the determination of the activity assays of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and peroxidase (POD, EC 1.11.1.7).

Quantification of Malondialdehyde and Hydrogen Peroxide

Malondialdehyde (MDA) content was determined according to the methods proposed in a previous study (Heath and Packer, 1968) with 0.1 g of plumules. Quantification of hydrogen peroxide (H_2O_2) concentration was derived according to the methods of Velikova et al. (2000) with 0.1 g of plumules.

Measurement of Endogenous Melatonin, Abscisic Acid, and Gibberellin Contents

Melatonin was extracted with 0.1 g of plumules according to the method mentioned in a previous study (Pape and Lüning, 2006) and was determined using an ELISA kit (Engvall and Perlmann, 1971) (Shanghai Enzyme Biotechnology Co., Ltd., Shanghai, China) following the instructions of the company.

The quantification of both ABA and GA was derived according to the methods mentioned in a previous study (Jahan et al., 2021) with little modifications. For endogenous ABA, 0.1 g of plumules were ground in liquid nitrogen and mixed with pre-cold extraction solution (methanol/water/formic, 16:3:1, v/v/v). After the centrifugation at 12,000 *g* for 15 min at 4°C, the supernatants were resuspended with 80% methanol and then filtered through a Sep-Pak C18 cartridge (Waters, Milford, MA, United States). The filtrates were collected for the measurement of the relative contents of ABA using the ELISA kit (Qingdao Sci-Tech Innovation Quality Testing Co., Ltd., Qingdao, China) according to the protocols of the manufacturer.

For endogenous GA, 0.1 g of plumules were ground in liquid nitrogen and incubated with pre-cold extraction solution (containing 80% methanol, 5% formic, 1 mM butylated hydroxytoluene) for 4 h at 4°C. After the centrifugation at 3,500 *g* for 15 min at 4°C, the supernatant was resuspended with 80% methanol and then filtered through a Sep-Pak C18 cartridge (Waters, Milford, MA, United States). Subsequently, all the filtrates were used for the quantification of the relative GA contents using the ELISA kit (Qingdao Sci-Tech Innovation Quality Testing Co., Ltd., Qingdao, China).

Quantitative Real-Time PCR

Total RNA was extracted with 0.1 g of plumules by using the Qiagen Spin Plant RNA Mini kit (Qiagen, Hilden, Germany). Quantitative real-time PCR (qRT-PCR) analysis was performed according to the method previously proposed by Li R.Q. et al. (2017) and Li et al. (2021). Relative gene expression was calculated in relative to the rice *UBQ5 gene* (Jain et al., 2006) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Gene-specific primers for qRT-PCR were listed in **Supplementary Table 1**.

Electrophoretic Mobility Shift Assay

The full-length coding sequence of *OsABI5* was cloned into the pCZN1 vector to construct the combined OsABI5–pCZN1 vector, which was then transferred to *Escherichia coli* (Arctic-Express, Hilliard, OH, United States) for the production of recombinant protein. Glutathione–Sepharose 4B beads (GE Healthcare, Buckinghamshire, United Kingdom) were employed to collect the purified protein from the lysate cells. In parallel, 25-bp DNA fragments from the *OsCAT2* promoter [harboring the ABA response element (ABRE) motif, − 505th to − 481th distance to transcription start site (TSS)] were biotin-labeled at the 3′-end by using the electrophoretic mobility shift assay (EMSA) Probe Biotin Labeling kit (Promega, Madison, WI, United States). Unlabeled probes were subjected to cold competition experiments. The cold probe concentrations were 100 ×. EMSA was performed using the Light Shift Chemiluminescent EMSA kit (Promega, Madison, WI, United States) according to the instructions of the manufacturer. The EBNA protein was used as a negative control. The biotin signals were imaged using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, United States). EMSAs were repeated three times, and the representative results were shown.

Analysis of Genes for Their Expression

Genes were identified through a homolog search of the Rice Annotation Project (RAP) database[3]: ABA-insensitive 5 (*OsABI5*, Os01g0859300); tryptophan decarboxylase (*OsTDC*, Os08g0140300); tryptamine 5-hydroxylase (*OsT5H*, Os12g026800); *O*-methyltransferase (*OsASMT*, Os09g0344500); serotonin *N*-acetyltransferase (*OsSNAT*, Os05g0481000); abscisic aldehyde oxidase (*OsAAO*, Os07g0281700); cytochrome P450 99A3 (*OsCYP99A3*, Os04g0178400); 9-*cis*-epoxycarotenoid dioxygenase 1 (*OsNCED1*, Os02g0704000); *OsNCED2* (Os12g0435200); *OsGA1* (Os05g0333200); *OsGA2ox1* (GA 2-oxidase1, Os05g0158600); *OsGA2ox2* (Os01g0332200); *OsGA2ox3* (Os01g0757200); *OsGA2ox4* (Os05g0514600); and catalase-2 (*OsCAT2*, Os02g0115700). These genes were subjected to qRT-PCR analysis by using gene-specific primers (**Supplementary Table 1**).

Statistical Analysis

Values were expressed as means ± SEs with six biological replicates. Comparisons of data from different groups were analyzed using the ANOVA test followed by the Tukey's multiple comparison test with $p < 0.05$.

RESULTS

Effects of Melatonin Treatment on the Germination Rate Under Different Low-Temperature Stress Conditions

To determine the effects of MT treatment on the seed germination rates under LT stress, the seeds of WT were treated

[3]https://rapdb.dna.affrc.go.jp/

with MT under different LTs (depicted as LT15, LT16, LT20, LT23, and LT24). Compared with the control, the germination rates were greatly inhibited under all LT conditions (**Figure 1**). However, no significant difference of the germination rates was detected between feeding with and without MT under LT15 and LT24 conditions (**Figure 1**). In addition, more accumulation of H_2O_2 and MDA was detected at LT15 (**Supplementary Figure 2**), making severe effects on the seed germination. Conversely, the feeding of MT greatly improved the germination rates under LT16 and LT23 stress, reaching 2.36 fold and 1.30 fold, respectively, in relative to non-treatment groups on the 7th day (**Figure 1**). Especially, under LT20 conditions, the exogenous MT greatly increased the germination rates within the investigated 3–7 days (**Figure 1**). Thus, the middle one, LT20, within the LT of 16–23°C was selected, and the germinated seeds on the 7th day under LT20 stress were employed for further investigation.

Effects of Melatonin Treatment on Seed Germination Under Low-Temperature Stress

As shown in **Figure 2A**, after LT20 treatment, seed germination was suppressed in WT, *abi5-1*, and *abi5-2*. Nonetheless, the feeding of MT greatly rescued the phenotypical performances of WT, but no significant difference was observed in *abi5-1* and *abi5-2* (**Figure 2A**).

To further explore the effects of *OsABI5* mutation on the MT alleviation under LT20 stress, the germination-associated index was studied (**Figure 2B**, **Supplementary Figures 3A,B**, and **Supplementary Table 2**). Compared with the control, all the investigated indices in WT were significantly reduced after LT20 treatment while the MT addition greatly relieved the damages of LT20 to seed germinability, germination percentage, and germination index (**Figure 2B** and **Supplementary Figures 3A,B**), as well as seedling performance, including vital index, moisture absorption, and healthy seedling rates (**Figure 2B**, **Supplementary Figures 3C,D**, and **Supplementary Table 2**). However, in *abi5-1* and *abi5-2*, the investigated index under LT20 stress were significantly lower than the control. The germination percentage (**Figure 2B**), germinability, and germination index of seed (**Supplementary Figures 3A,B**), as well as healthy seedling rates (**Figure 2B**), vital index, and moisture absorption (**Supplementary Figures 3C,D**), were rarely or never recovered in the conditions of MT incubation in *abi5-1* and *abi5-2*. These results suggested that MT treatment relieved LT20-induced damages to the performance of seed germination, and that *ABI5* mutation greatly affected the alleviation effects of MT on LT20 stress.

Effects of Melatonin Treatment on the Physiological Attributes Associated With Seed Germination

Because seed germination requires the nutritive materials (i.e., soluble sugar and soluble protein) derived from the enzymatic decomposition of the storage materials as well as the

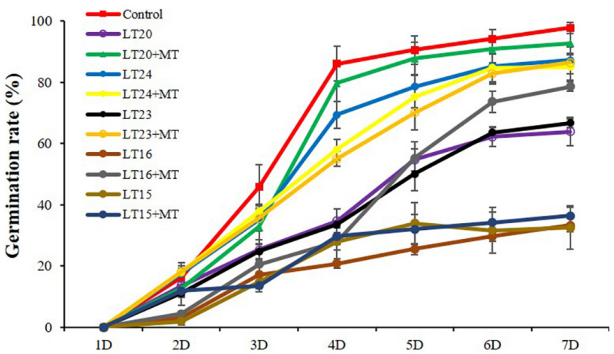

FIGURE 1 | Germination rates of rice seed germination during different low-temperature (LT) stress conditions in the presence or absence of melatonin (MT) treatment. Exogenous applications of MT (150 mM) were employed to provide treatment for rice seeds under different LT stress conditions for 7 days. Control: constant 30°C; LT15: constant 15°C; LT20: constant 20°C; LT24: constant 24°C.

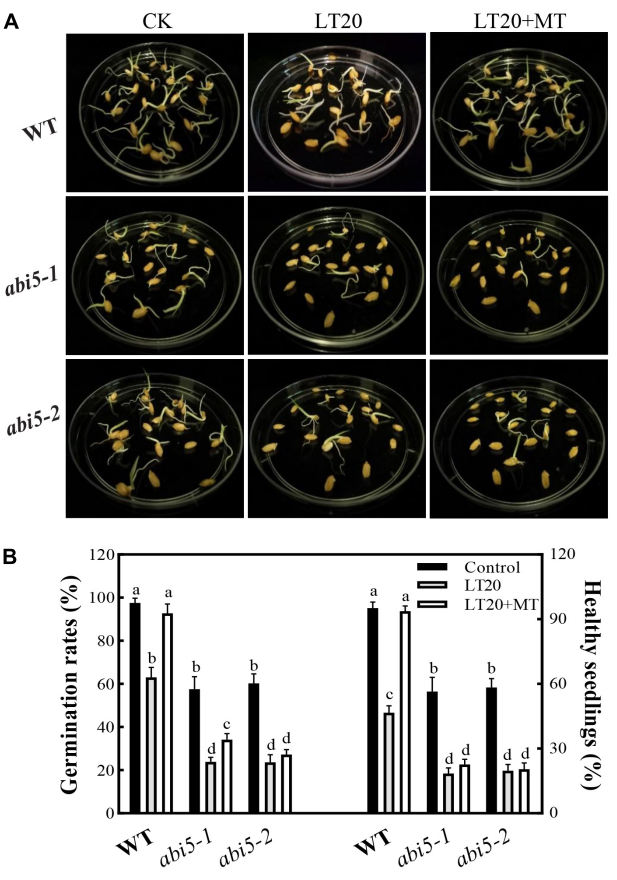

FIGURE 2 | Effects of exogenous MT treatment on the seed germination performance under LT stress. **(A)** Phenotypes, **(B)** germination percentage, and healthy seedling rates under LT stress (constant 20°C for 7 days) without (LT20) or with (LT20 + MT) MT treatment. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's honestly significant difference (HSD) test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

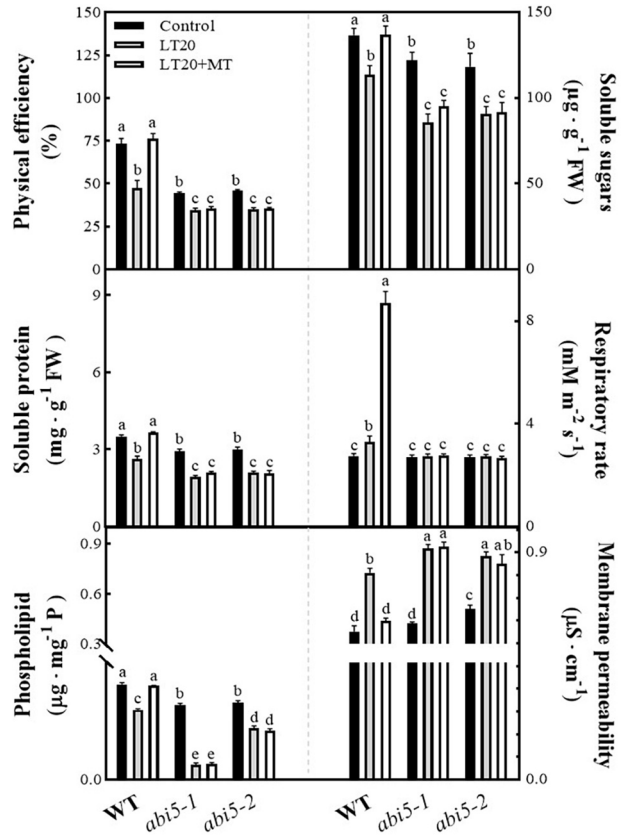

FIGURE 3 | Effects of exogenous MT treatment on seed germination-associated physiological attributes under LT stress. Physical efficiency, soluble sugar contents, soluble protein contents, phospholipid contents, respiratory rate, and membrane permeability under LT stress (constant 20°C for 7 days) without (LT20) or with (LT20 + MT) MT treatment. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

energy provided by respiration, thus the physiological attributes associated with germination, including soluble sugar, soluble protein, physical efficiency, and respiratory rates, were studied to explore the physiological effects of MT on LT20 stress (**Figure 3** and **Supplementary Table 3**). Compared with CK, the physical efficiency and the soluble sugar contents, soluble protein contents, and phospholipid contents were significantly reduced under LT20 stress in WT, whereas the MT feeding greatly mitigated the decreased trends (**Figure 3**). In addition, the respiratory rates were also significantly enhanced after LT20 treatment but reached much higher in the presence of MT feeding (**Figure 3**). In contrast, the acid value (**Supplementary Figure 4**) and membrane permeability (**Figure 3**) were significantly increased under LT20 treatment but were reduced to the control levels in WT once feeding with MT. Consistent with **Figure 2**, these results suggested that exogenous MT alleviated the adverse effects of LT20 stress on seed germination.

However, in *abi5-1* and *abi5-2*, no significant difference was detected in the investigated physiological attributes under LT20 stress irrespective of MT feedings (**Figure 3** and **Supplementary Figure 4**). Besides, except for the respiratory rates (**Figure 3**), the four positive (**Figure 3**) and the two negative (**Figure 3** and **Supplementary Figure 4**) correlated attributes for seed germination showed significant differences in relative to control under LT20 stress. These results suggested that *OsABI5* mutation greatly repressed the alleviating effects of MT on LT20 stress during seed germination.

Effects of Melatonin Treatment on the Enzymic Activities Associated With Germination

Because enzyme catalysis was required for the degradation of storage substances and respiration reaction during seed germination (Bewley, 1997), thus we next detected the effects of MT on the activities of partial enzymes involved in seed germination, including α-amylase and ACP used for storage metabolism as well as SDH and CCO for respiration (**Figure 4** and **Supplementary Table 4**). After LT20 treatment, the activities of α-amylase, ACP, SDH, and CCO were significantly decreased in WT, whereas the incubation of MT greatly enhanced the activities of these four enzymes (**Figures 4A–D**).

However, in *abi5-1* and *abi5-2*, the activities of α-amylase and ACP were significantly decreased after LT20 stress, but no significant difference was detected between feeding with and without MT under LT20 (**Figures 4A,B**). Interestingly, no significant difference in the activities of SDH and CCO was found between CK and LT20 stress in *abi5-1* and *abi5-2* mutants, even in the conditions of MT feedings (**Figures 4C,D**). Thus, these results suggested that *OsABI5* mutation severely affected the alleviation effects of MT on LT20 stress to inhibit enzyme catalysis, which is required for the utilization of storage substances during seed germination.

Effects of Melatonin Treatment on Reactive Oxygen Species Accumulation and Antioxidant Activity

Low-temperature stress leads to the accelerated production of harmful metabolites and simultaneously activates the enzymatic antioxidant system to prevent cells from damage (Fujino et al., 2004; Hussain et al., 2016). Thus, we further detected the accumulation of MDA, H_2O_2, and free proline, as well as an antioxidant system under LT20 stress. As shown in **Figures 5A,B**, under LT20 stress, the concentration of MDA and H_2O_2 was greatly increased in all investigated samples, whereas MDA and H_2O_2 accumulated in *abi5* mutants were more than those accumulated in WT. Nonetheless, the incubation of MT with seeds under LT20 significantly suppressed the production of MDA and H_2O_2 in WT but failed to inhibit the accelerated accumulation of harmful metabolites in *abi5-1* and *abi5-2* (**Figures 5A,B**). In addition, in WT, the contents of free proline under LT20 stress were significantly increased in relative to CK but were restored to CK levels after MT treatment (**Figure 5C**). Similarly, in *abi5-1* and *abi5-2*, free proline detected under LT20 was more than that detected under CK, but MT treatment made

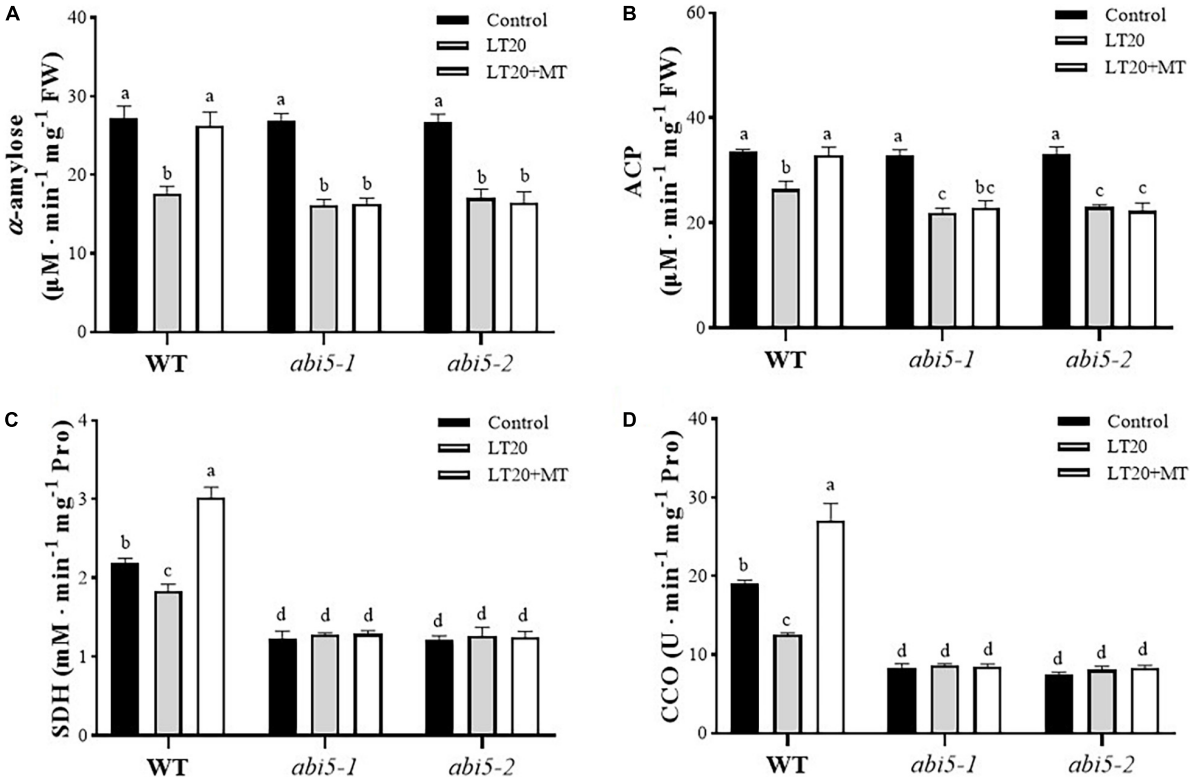

FIGURE 4 | Effects of exogenous MT treatment on seed germination associated with enzymes under LT stress. Activities of **(A)** α-amylase, **(B)** acid phosphatase (ACP), **(C)** succinic dehydrogenase (SDH), and **(D)** cytochrome oxidase (CCO) under LT stress (constant 20°C for 7 days) without (LT20) or with MT (LT20 + MT) treatment. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

no significant changes, indicating that MT failed to relieve LT stress in *abi5* mutants (**Figure 5C**).

Correspondingly, the activities of CAT, SOD, and POD showed antagonistic correlations with the accumulation of MDA and H_2O_2 under LT20 stress (**Figure 5** and **Supplementary Table 5**). The activities of CAT, SOD, and POD in WT were significantly enhanced under LT20 as compared to CK, especially in the conditions of MT treatment (**Figures 5D–F**). However, in *abi5-1* and *abi5-2*, no significant difference was detected under LT20 stress compared with CK, irrespective of MT treatment, suggesting that *OsABI5* mutation suppressed the activities of an antioxidant system (**Figures 5D–F**). These results indicated that *OsABI5* mutation has negative effects on the MT functions and enables the promotion of LT20-induced production of harmful substances during seed germination.

Effects of Exogenous Melatonin Treatment on the Biosynthesis of Endogenous Melatonin

To explore the effects of *OsABI5* mutation on the biosynthesis of endogenous MT, the contents of endogenous MT and the expression levels of the genes involved in MT biosynthesis were further detected (**Figure 6** and **Supplementary Table 6**). Under LT20 stress, the contents of endogenous MT in WT were increased by 105.6% and 55.6% in the presence and absence of MT, respectively, compared to CK (**Figure 6A**). However, in *abi5-1* and *abi5-2*, compared with CK, no significant difference in the contents of endogenous MT was detected under LT20 stress irrespective of MT treatment, suggesting that *OsABI5* mutation negatively affected the biosynthesis of endogenous MT (**Figure 6A**).

Moreover, the expression levels of *OsTDC*, *OsT5H*, *OsSNAT*, and *OsASMT* in WT were also increased by 2–2.9 folds under LT20 stress but significantly enhanced up to fourfold to 4.8 fold once MT feeding (**Figures 6B–E**). However, in *abi5-1* and *abi5-2*, there were no significant differences in transcriptional abundance presented under LT20 stress with or without MT (**Figures 6B–E**). All these results indicated that *OsABI5* mutation affects the expression of genes for the biosynthesis of endogenous MT under LT20 stress even in the conditions of exogenous MT treatment.

Effects of Exogenous Melatonin Treatment on the Biosynthesis of Endogenous Abscisic Acid

Seed germination requires the cooperation of different phytohormones, such as MT, ABA, and GA under various stress conditions (Ahmad et al., 2012), and it is well known that ABA serves as a positive regulator of ABI5 signals during seed

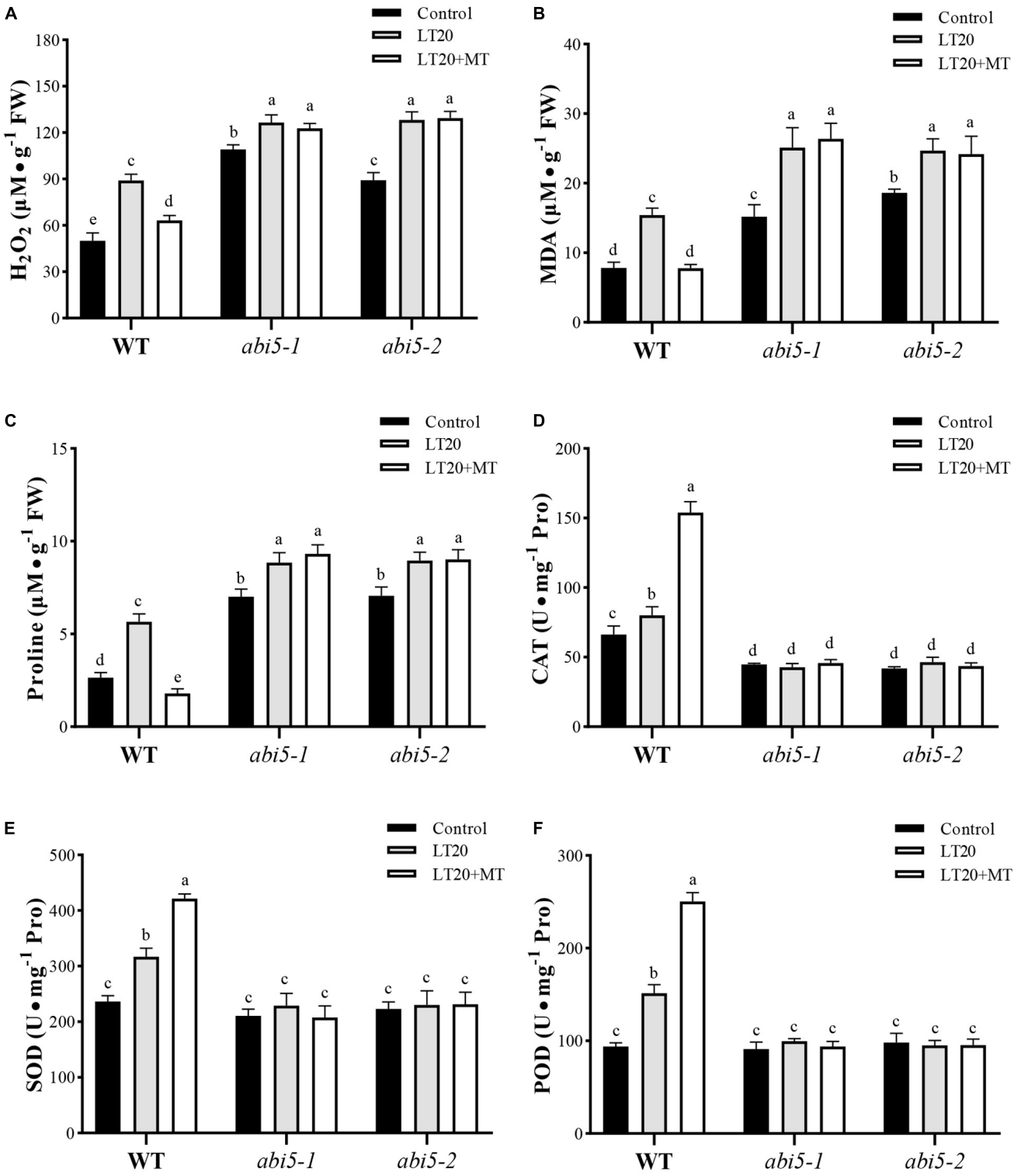

FIGURE 5 | Effects of exogenous MT treatment on an antioxidant system under LT. **(A)** Hydrogen peroxide (H_2O_2) content, **(B)** malondialdehyde (MDA) content, **(C)** proline (Pro) content, **(D)** catalase (CAT) activity, **(E)** superoxide dismutase (SOD) activity, and **(F)** peroxidase (POD) activity under LT stress (constant 20°C for 7 days) without (LT20) or with (LT20 + MT) MT treatment in wild type (WT) and *abi5* mutants. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

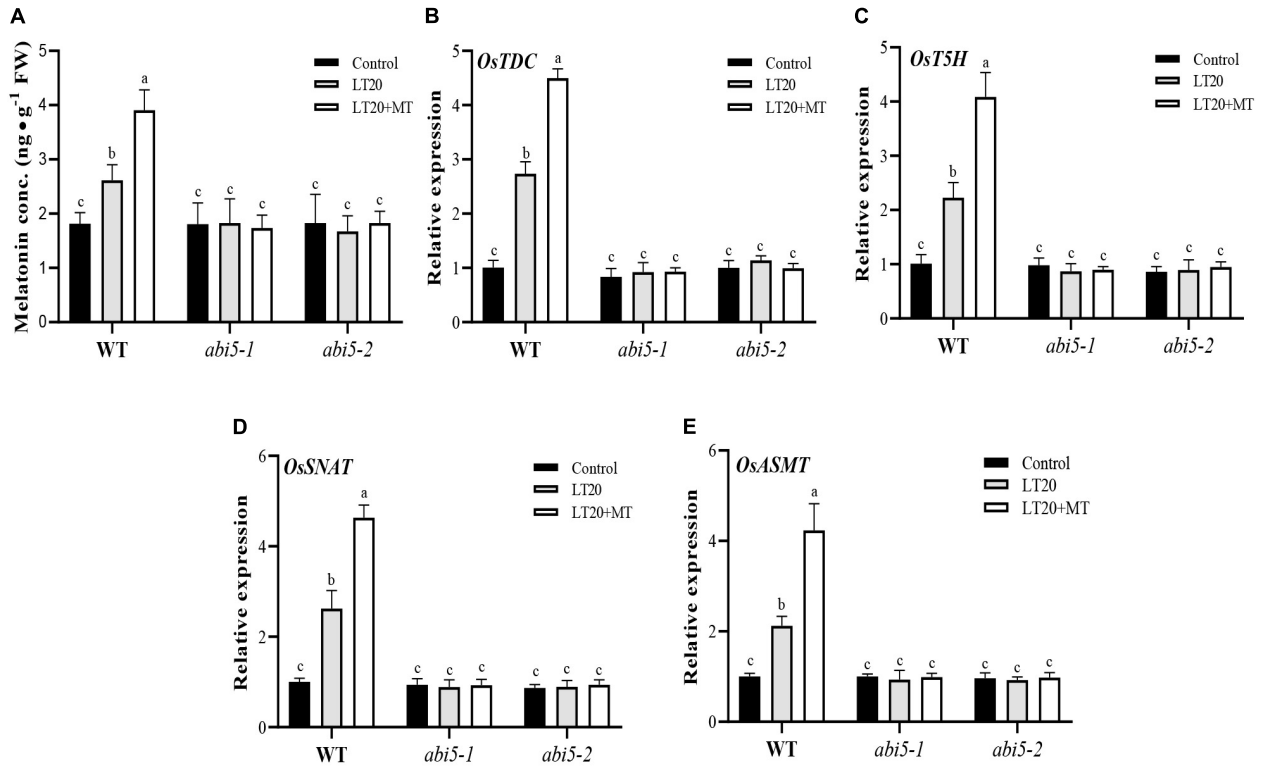

FIGURE 6 | Effects of exogenous MT treatment on the biosynthesis of endogenous MT under LT. **(A)** Endogenous MT content and **(B–E)** transcript abundance of MT biosynthesis genes (*TDC*, *T5H*, *SNAT*, and *ASMT*) under LT stress (constant 20°C for 7 days) without (LT20) or with (LT20 + MT) MT treatment in WT and *abi5* mutants. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

germination (Skubacz et al., 2016; Bi et al., 2017). Thus, the contents of endogenous ABA and GA as well as the expression levels of genes involved in ABA and GA metabolism were further detected in the conditions with or without exogenous MT under LT20 stress (**Figure 7** and **Supplementary Tables 7, 8**).

Under LT20 stress, the contents of ABA, and the expression levels of genes for ABA biosynthesis were significantly upregulated in WT, *abi5-1*, and *abi5-2* (**Figures 7A,B** and **Supplementary Figures 5A,B**). By contrast, under LT20 stress, the treatment of MT significantly decreased the ABA concentration and the expression of *OsNCED1*, *OsNCED2*, and *OsAAO* in WT while *abi5-1* and *abi5-2* remained to be in the same level as the single LT20 treatment irrespective of MT feedings (**Figures 7A,B** and **Supplementary Figures 5A,B**).

However, as a catabolic gene for ABA, *OsCYP99A3* showed contrast expression with the genes of ABA synthesis (**Figure 7**). LT20 treatment enhanced expression of *OsABI5* and reduced expression of *OsCYP99A3*, but the feeding of MT significantly induced the transcriptional levels of *OsABI5* and promoted the expression of *OsCYP99A3* (**Figures 7C,D**). Nonetheless, in *abi5-1* and *abi5-2*, no significant difference of transcriptional abundance of *OsABI5* was detected between LT20 stress and the control, even under MT treatment (**Figure 7C**). Moreover, in *abi5-1* and *abi5-2*, although the expression of *OsCYB99A3* was downregulated after LT20 treatment, no significant difference of the *OsCYB99A3* expression was detected under LT20 stress despite MT treatment (**Figure 7D**). All these results suggested that MT had antagonism effects on ABA under LT20 stress, which was weakened or failed due to *OsABI5* mutation.

Effects of Exogenous Melatonin Treatment on the Biosynthesis of Endogenous Gibberellin

Compared with CK, GA contents under LT20 stress were significantly decreased by 35.3% in WT, whereas the feeding of MT significantly increased the GA contents to CK levels (**Figure 7E**). However, no change of GA concentration was detected under LT20 stress in *abi5-1* and *abi5-2*, even in the presence of MT (**Figure 7E**), suggesting that *OsABI5* mutation negatively affected the effects of MT in promoting GA synthesis.

Furthermore, the expression levels of *OsGA1* (**Figure 7F**) and the genes involved in GA catabolism (**Supplementary Figures 6A–D**) under LT20 stress were significantly enhanced in WT, but MT treatment led to a higher expression of *OsGA1* and decreased expression of *OsGA2ox1, OsGA2ox2, OsGA2ox3*, and *OsGA2ox4* (**Figure 7F** and **Supplementary Figures 6A–D**). Nonetheless, in *abi5-1* and *abi5-2*, no significant difference of transcriptional abundance of *OsGA1*, *OsGA2ox1*, *OsGA2ox2*, *OsGA2ox3*, and *OsGA2ox4* was detected under

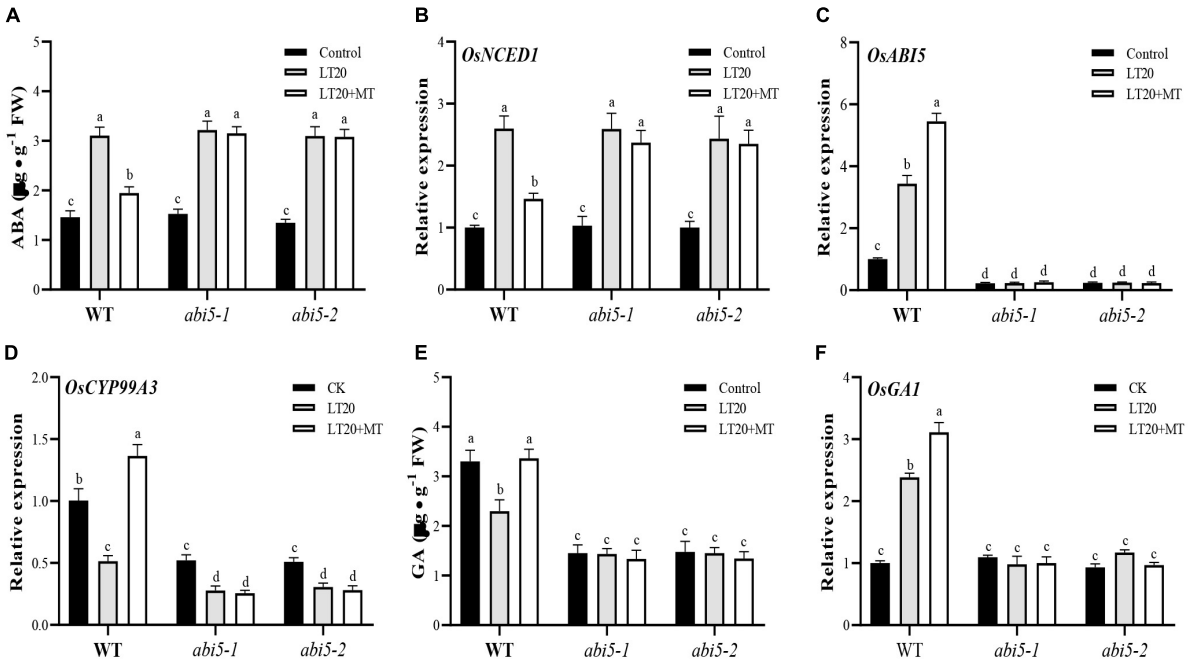

FIGURE 7 | Effects of exogenous MT treatment on the endogenous abscisic acid (ABA) and gibberellin (GA) biosynthesis under LT. **(A)** Endogenous ABA content, **(B)** transcript abundance of ABA biosynthesis gene (*OsNCED1*), **(C)** ABA signaling gene [ABA INSENSITIVE 5 (*OsABI5*)], **(D)** ABA of catabolic gene (*OsCYP99A3*), **(E)** endogenous GA content, and **(F)** transcript abundance of GA signaling genes (*OsGA1*) under LT stress (constant 20°C for 7 days) without (LT20) or with (LT20 + MT) MT treatment in WT and the *abi5* mutants. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

LT20 stress, irrespective of MT treatment (**Figure 7F** and **Supplementary Figures 6A–D**). These results suggested that MT had a synergistic interaction on GA biosynthesis, which was inhibited in *OsABI5* mutants.

Regulation of OsABI5 on *OsCAT2* Expression

The expression of *OsCAT2*, one-gene encoding catalysis, is closely associated with seed germination and dormancy (Bailly et al., 2008; Ishibashi et al., 2017). The expression of *OsCAT2* in WT was induced by LT treatment, whereas higher transcription abundance was detected once feeding with MT (**Figure 8A**). However, in *abi5-1* and *abi5-2*, no significant difference of *OsCAT2* expression was observed under LT20 stress with or without MT (**Figure 8A**). EMSA assay demonstrated that OsABI5 was directly bound to the promoter areas of *OsCAT2* (**Figure 8B**). The results suggested that the regulation of OsABI5 on the expression of *OsCAT2* was affected by MT under LT20 stress.

DISCUSSION

Melatonin Alleviates Low-Temperature Stress During Seed Germination

Due to its origin from tropical and subtropical areas, rice is susceptible to temperature, especially for LT, which would greatly limit growth and yield (Hussain et al., 2016; Li and Yang, 2020). The optimal temperature for rice s germination is in the range of 25–35°C as the temperature of less than 15°C would greatly inhibit the germination rates (Fujino et al., 2004). Li and Yang (2020) found that the seed germination of two rice genotypes was greatly inhibited at an LT of 15°C. Currently, we also found that the germination rates of rice seeds were greatly decreased under different LT stress conditions, especially in the temperature below 20°C (**Figure 1**). For example, under LT15 stress, the germination of seeds was severely suppressed, with the disrupted cell metabolism and accumulated harmful substances (**Figure 1** and **Supplementary Figure 2**). However, the feeding of MT did not greatly improve the germination rates at both 15°C and 24°C (**Figure 1**). Because LT stress (15°C) would severely disrupt the activity of enzymes being essential for seed germination, and cause the accumulation of oxidative products, i.e., MDA and H_2O_2 (**Supplementary Figure 2**), and thus the feeding of MT also could not restore the germination rates. Meanwhile, the temperature of 24°C was very close to the optimal temperature for rice seed germination, thus little effects were shown on the seed germination (**Figure 1**). Nevertheless, the germination rates were greatly inhibited under the LT of 16°C and 23°C, whereas they were greatly restored after feeding with exogenous MT, reaching 2.36 and 1.30 folds in relative to non-treatment groups (**Figure 1**). In addition, under LT20 stress, the middle temperature in the range of 16–23°C, the seed germination was also greatly improved after feeding with exogenous MT (**Figures 1**, **2**). Therefore, MT can alleviate LT

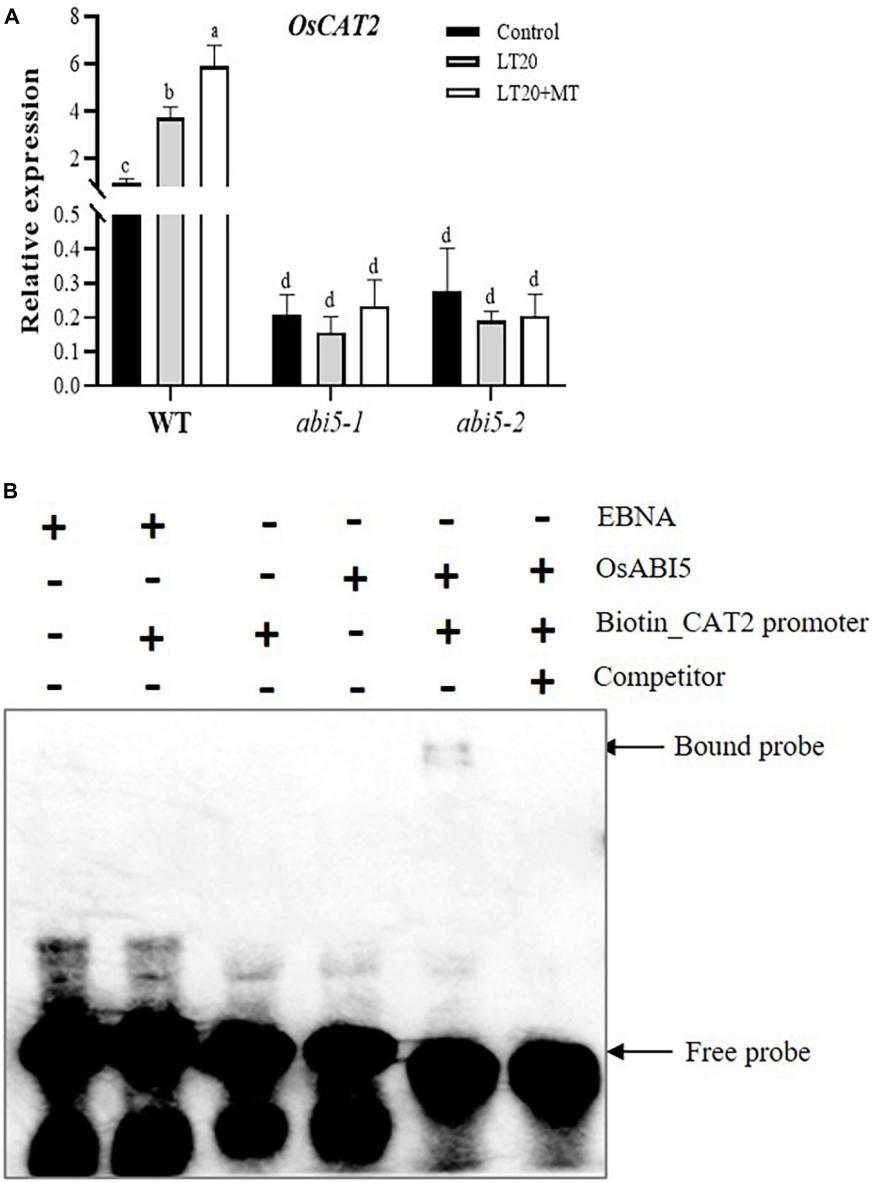

FIGURE 8 | Expression of *CATALYSE 2* (*OsCAT2*) under LT stress treated with exogenous MT and electrophoretic mobility shift assay (EMSA) assays for a direct interaction between OsABI5 and the *OsCAT2* promoter. **(A)** Transcript abundance of *OsCAT2* under LT stress (constant 20°C for 7 days) without (LT20) or with MT (LT20 + MT) treatment in WT and *abi5-1* and *abi5-2* mutants. **(B)** An EMSA validated the interaction of OsABI5 with the promoters of *OsCAT2*. The purified OsABI5 protein obtained using prokaryotic expression was used for the *in vitro* assay, and the EBNA protein was used as a negative control. Unlabeled probes (competitor) were subjected to cold competition experiments (100×). Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates. "+" and "–" indicate the presence or absence, respectively, of proteins and probes in the loading mixture. About 4% of the polyacrylamide gel was used here.

stress in the range of 16–23°C, and shows potential application values in production.

Seed dormancy or delayed germination is an adaptative response to adverse environments, and in turn, germination will be triggered when the conditions are favorable for seedling recruitments under natural environments (Clauss and Venable, 2000; Gutterman, 2002). This was greatly related to the counteraction of various bioactive phytohormones, such as GA and ABA (Miransari and Smith, 2014). Therefore, it was feasible to improve seed germination by using phytohormones, i.e., MT, in agricultural productions. Indeed, MT has been widely and effectively applied to alleviate the stress-induced delays of seed germination in various plants (Posmyk et al., 2008; Zhang et al., 2013, 2014; Hernández et al., 2015; Chen et al., 2020; Simlat et al., 2020). In this research, we found that the applications of exogenous MT significantly improved the germination rates (**Figures 1**, **2A,B** and **Supplementary Figures 3A,B**) and seedling performance (**Supplementary Figures 3C,D**), which were largely

related to the recovery of water absorption (**Supplementary Figure 3D**) and membrane permeability (**Figure 3**). However, under LT20 stress, the metabolism of storage substances and respiratory rate were also inhibited (**Figure 3**), but the acid value (**Supplementary Figure 4**) and harmful substances (**Figures 5A,B**) were greatly accumulated. On the contrary, the MT applications efficiently enhanced the supplies of nutrients (**Figure 3**), such as soluble sugars and proteins, as well as sufficient water (**Supplementary Figure 3D**) for seed germination under LT20 stress. Therefore, these results suggested that MT alleviated the LT -stress-induced inhibition of seed germination.

In addition, despite the support of moderate ROS to seed germination, over the accumulation of ROS and MDA would prevent or delay germination, especially under adverse conditions (Bailly et al., 2008; Wojtyla et al., 2016). Here, LT stress promoted the concentrations of H_2O_2 and MDA during germination while the treatment of MT greatly inhibited the production of LT stress-induced ROS (**Figures 5A–C**), which in turn enabled the promotion of germination rates (**Figure 2B** and **Supplementary Figures 3A,B**). Thus, the effects of MT on the LT stress germination were dependent on the effective functioning of an antioxidant system. Moreover, the interaction between H_2O_2 and ABA was involved in the regulation of seed germination and dormancy in barley (Ishibashi et al., 2017). In this research, the concentration of ABA showed consistent changes with the accumulation of H_2O_2 under LT stress (**Figures 5A**, **7A**). In addition, the *OsCAT2* expression, directly regulated by OsABI5, was responsible for the CAT activity (**Figure 8**). However, the *OsCAT2* expression was even higher in the conditions of upregulated *OsABI5* under MT treatment (**Figures 7E**, **8**). Interestingly, Ishibashi et al. (2017) demonstrated that ABA-induced HvABI5 enabled the promotion of CAT2, a H_2O_2 scavenging enzyme, to suppress the ROS signals for GA biosynthesis in dormant seeds. This seemed to be consistent with our results. Moreover, Mittler and Blumwald (2015) proposed that stress-induced ABA could synergistically enhance the ROS produced by NADPH oxidase in guard cells, which led to the accumulation of ABA in the positive feedback loop model. In addition, the regulation of OsABI5 on seed germination may be involved in other regulatory mechanisms, such as in the recently reported OsABI5/OsKEAP1 system (Liu et al., 2021). ABA contents between dormant seeds and LT-stressed seeds showed obvious differences, which might be served as other reasonable attributes.

All in all, LT stress greatly inhibited the seed germination while MT treatment could alleviate LT-induced damages to seeds *via* systemic acquired acclimation, i.e., enhanced antioxidant activity.

Melatonin Alleviates Different Low-Temperature Stress Conditions *via* Cross Talk With Abscisic Acid During Seed Germination

The regulation of MT on abiotic-stressed seed germination at least involves two ways, by cross talk with other plant hormones (Zhang et al., 2014; Cao et al., 2019; Xiao et al., 2019; Chen et al., 2021) and by small molecular signals (e.g., ROS). For

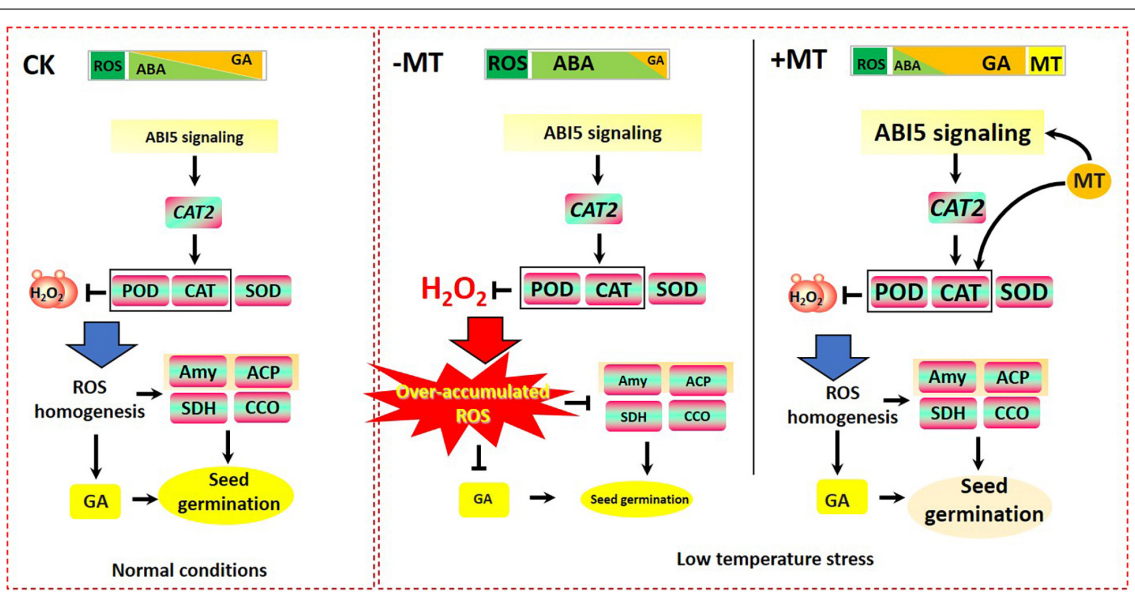

FIGURE 9 | Models for explaining the alleviated effects of MT on LT-stressed seed germination in rice. During seed germination, LT stress caused the accumulation of reactive oxygen species (ROS) and ABA, which was helpful to maintain seed dormancy. However, the disruption of redox homeostasis caused by LT stress-induced ROS accumulation greatly suppressed the biosynthesis of GA and the efficient applications of storage materials (i.e., soluble sugar), to eventually prevent seed germination. However, MT treatment greatly activated OsABI5 to regulate *OsCAT2* and promoted the antioxidant systems (i.e., SOD, POD, and CAT) to scavenge ROS, which would consequently restore the steady states of ROS homeostasis in the germinating seeds; the homeostasis of ROS, in turn, enabled the promotion of the catabolism of ABA, which finally relieved the inhibitions for seed germination and simultaneously contributed to the biosynthesis of GA as well as the effective functioning of enzymes involved in seed germination (i.e., Amy, ACP, SDH, and CCO), and thereby promoting seed germination.

example, MT is involved in the regulation of ABA and GA to promote seed germination under salt stress (Zhang et al., 2014; Chen et al., 2021). It is well known that GAs and ABA play vital roles in seed germination and ABA acts as a key molecule in dormancy (Finkelstein et al., 2008; Rodríguez et al., 2015; Shu et al., 2016) while GAs prefer to promote seed germination (Jacobsen et al., 2002; Tuttle et al., 2015). Such an antagonistic interaction of GA and ABA was consistent with our results. In this study, LT treatment led to the biosynthesis of ABA, which in turn enabled the inhibition of the GA, thus inhibiting seed germination (**Figures 2**, **7** and **Supplementary Figure 3**). This was also concluded from the gene expression levels for ABA. For example, as the catabolic gene for ABA, *OsCYP99A3* was induced in the presence of moderate ROS contents (i.e., H_2O_2) during seed germination while over-accumulated ROS would greatly repress its expression, which had eventually helped to accumulate more ABA to inhibit seed germination (**Figures 2**, **5**, **7**). However, the feeding of exogenous MT not only increased the expression of *OsCYP99A3* to promote the catabolism of ABA but also greatly rescued the biosynthesis of GA and MT, which was beneficial to seed germination (**Figures 2**, **6**, **7**). On the other hand, LT stress also induced the accumulation of harmful substances, especially H_2O_2 (**Figure 5A**), which eventually broke the redox homeostasis that was essential for seed germination. MT has been proved to be an effective antioxidant for scavenging the excess ROS (Galano et al., 2011). In this case, the treatment of MT indeed repressed the production of H_2O_2 under LT conditions (**Figure 5A**). All these results suggested that MT was involved in the regulation of seed germination under LT stress, possibly *via* its antagonism with ABA. Nonetheless, MT showed a synergetic correlation with ABA to regulate seed germination under non-stress conditions (Wei et al., 2018; Lv et al., 2021), which was largely due to the different applied doses of MT.

Apart from various hormones, more and more studies have demonstrated that small molecules also played a function in the regulation of seed germination (Lv et al., 2021). For example, ABA acting as the major stress phytohormone is involved in the response of the seed to adverse environments during germination (Skubacz et al., 2016) while ABI5, a key module of the core ABA signaling, mediated seed germination partially through the ROS homeostasis (Bi et al., 2017). Moreover, as an effective antioxidant, phytomelatonin was supposed to regulate seed germination through ABI5-mediated ROS signals. There were two reasons to explain this point. Firstly, MT treatment has antagonistic effects on the LT stress-induced accumulation of ABA while *abi5* abolished the effects of MT on the biosynthesis of ABA (**Figures 7A,B** and **Supplementary Figure 5**). Secondly, under LT stress, MT greatly enabled the promotion of the CAT activity (**Figure 5D**), and the expression of its encoded gene, *OsCAT2*, which was directly regulated by OsABI5 (**Figure 8A**). This was also concluded from the failure of *OsCAT2* in response to LT stress in *abi5* mutants, even in the presence of MT feedings (**Figure 8A**).

Therefore, in the context of seed germination, LT stress was found to result in the accumulation of ROS and ABA (**Figures 5**, **9**), which was helpful to maintain seed dormancy, while the disruption of redox homeostasis caused by LT stress-induced ROS accumulation greatly suppressed the biosynthesis of GA (**Figure 7E**) and the efficient applications of storage materials (i.e., soluble sugar; **Figures 3**, **4** and **Supplementary Figure 4**), which eventually prevented seed germination (**Figures 1**, **2** and **Supplementary Figure 3**). However, MT treatment greatly activated OsABI5 to regulate *OsCAT2* and enabled the promotion of the antioxidant systems (i.e., SOD, POD, and CAT; **Figures 5**, **9**) to scavenge ROS, which consequently restored the steady states of ROS homeostasis in the germinating seeds; the homeostasis of ROS, in turn, enabled the promotion of the catabolism of ABA (**Figure 7**), which finally relieved the inhibitions for seed germination and simultaneously contributed to the biosynthesis of GA (**Figure 7**) as well as the effective functioning of enzymes (**Figures 4**, **7**), and thereby promoting seed germination (**Figure 9**).

CONCLUSION

We now demonstrate that MT synergistically acts with an ABI5-mediated signal to regulate rice seed germination under LT stress, possibly through a direct regulator of *OsCAT2*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The authors declare that the experiments were performed in compliance with the current laws of China.

AUTHOR CONTRIBUTIONS

RL and MJ conceived the study. RL, MJ, and YS carried out the experiments and performed data analysis. RL, MJ, and HZ finished the first draft. RL and HZ finished the final version. All authors gave their consent for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.727596/full#supplementary-material

Supplementary Figure 1 | A schematic diagram of an ABA-INSENSITIVE 5 (*OsABI5*) and a single-guide RNA (sgRNA) target site for clustered regularly interspaced short palindromic repeats-associated protein 9- (CRISPR/Cas9-) mediated mutagenesis. **(A)** Exons, introns, and untranslated regions (UTRs) are indicated by solid boxes, lines, and blank boxes, respectively. cN2-F and cN2-R are the primers for genotyping mutation, and its position is indicated by arrowheads. The mutation is identified within the target site of *OsABI5* generated through CRISPR/Cas9-mediated genome editing in rice. The PAM sequences (NGG) are boxed and the 20-nt target sequences are underlined. Mutations are shown in red letters for insertion or in "–" for deletion. **(B)** The three-dimensional structures of OsABI5 were analyzed on the SWISS-MODEL (https://www.swissmodel.expasy.org/).

Supplementary Figure 2 | Effects of exogenous melatonin (MT) treatment on the concentration of **(A)** hydrogen peroxide (H_2O_2) and **(B)** malondialdehyde (MDA) at 15°C. The concentration of H_2O_2 and MDA was measured under low-temperature (LT) stress (constant 15°C for 7 days) without (LT15) or with (LT15 + MT) MT treatment in wild type (WT) and *abi5* mutants. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's honestly significant difference (HSD) test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

Supplementary Figure 3 | Effects of exogenous MT treatment on seed germination performance under LT stress. **(A)** Germinability, **(B)** germination index, **(C)** vital index, and **(D)** moisture absorption under LT stress (constant 20°C for 7 days) without [LT of constant 20°C (LT20)] or with (LT20 + MT) MT treatment. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

Supplementary Figure 4 | Effects of exogenous MT treatment on the acid value during seed germination under LT stress. The acid value during LT stress (constant 20°C for 7 days) without (LT20) or with MT (LT20 + MT) treatment. Different letters denote significant variations between treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

Supplementary Figure 5 | Effects of exogenous MT treatment on endogenous abscisic acid (ABA) biosynthesis. Transcript abundance of ABA biosynthesis genes, **(A)** *OsNCED2* and **(B)** *OsAAO* under LT stress (constant 20°C for 7 days) without (LT20) or with MT (LT20 + MT) treatment in WT and *abi5* mutants. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

Supplementary Figure 6 | Effects of exogenous MT treatment on endogenous gibberellin (GA) biosynthesis. **(A–D)** Transcript abundance of GA of catabolic genes (*OsGA2ox1*, *OsGA2ox2*, *OsGA2ox3*, and *OsGA2ox4*) during LT stress (constant 20°C for 7 days) without (LT20) or with (LT20 + MT) MT treatment in WT and *abi5* mutants. Different letters denote significant variations between the treatments, and the average values were measured by Tukey's HSD test at $p < 0.05$. Data were represented as mean ± SE of six biological replicates.

Supplementary Table 1 | Primers used in this study.

Supplementary Table 2 | Two-way ANOVA test for seed germination performance under low-temperature (LT) stress.

Supplementary Table 3 | Two-way ANOVA test for seed germination-associated physiological attributes during LT stress.

Supplementary Table 4 | Two-way ANOVA test for seed germination associated with enzymic activities under LT stress.

Supplementary Table 5 | Two-way ANOVA test for an antioxidant system under LT.

Supplementary Table 6 | Two-way ANOVA test for the biosynthesis of endogenous melatonin (MT).

Supplementary Table 7 | Two-way ANOVA test for endogenous abscisic acid (ABA) biosynthesis.

Supplementary Table 8 | Two-way ANOVA test for endogenous gibberellin (GA) biosynthesis.

Supplementary Table 9 | Two-way ANOVA test for the *OsCAT5* expression level.

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Molecular Mechanisms Underlying the Biosynthesis of Melatonin and Its Isomer in Mulberry

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Mulberry (*Morus alba* L.) leaves and fruit are traditional Chinese medicinal materials with anti-inflammatory, immune regulatory, antiviral and anti-diabetic properties. Melatonin performs important roles in the regulation of circadian rhythms and immune activities. We detected, identified and quantitatively analyzed the melatonin contents in leaves and mature fruit from different mulberry varieties. Melatonin and three novel isoforms were found in the *Morus* plants. Therefore, we conducted an expression analysis of melatonin and its isomer biosynthetic genes and *in vitro* enzymatic synthesis of melatonin and its isomer to clarify their biosynthetic pathway in mulberry leaves. *MaASMT4* and *MaASMT20*, belonging to class II of the *ASMT* gene family, were expressed selectively in mulberry leaves, and two recombinant proteins that they expressed catalyzed the conversion of *N*-acetylserotonin to melatonin and one of three isomers *in vitro*. Unlike the *ASMT*s of *Arabidopsis* and rice, members of the three *ASMT* gene families in mulberry can catalyze the conversion of *N*-acetylserotonin to melatonin. This study provides new insights into the molecular mechanisms underlying melatonin and its isomers biosynthesis and expands our knowledge of melatonin isomer biosynthesis.

Keywords: mulberry, melatonin, melatonin isomers, biosynthesis, *N*-acetylserotonin methyltransferase (ASMT)

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INTRODUCTION

Melatonin, a kind of biogenic indolamine, is a pineal secretory product and was first discovered in the bovine pineal gland in 1958 (Lerner et al., 1958). Since then, melatonin has been detected in other species, such as bacteria, algae, fungi, animals, and plants (Pöggeler et al., 1991; Hattori et al., 1995; Manchester et al., 1995; Sprenger et al., 1999). Melatonin performs important roles in the regulation of many physiological processes, such as circadian rhythms, sleep, mood, body temperature, appetite, retina physiology and immune system (Lieberman et al., 1984; Lewy et al., 1992; Dollins et al., 1994). Additionally, melatonin is a free radical scavenger and acts as a wide-spectrum antioxidant to scavenge hydroxyl radicals and hydrogen peroxide (Reiter, 1998; Stasica et al., 1998; Reiter et al., 2013).

Melatonin was first reported in plants in 1995, and is contained in almost all tested plants, at concentrations ranging from pico- to nano-grams per gram of tissue (Garcia-Parrilla et al., 2009; Gomez et al., 2013). Although differing melatonin concentrations have been reported

in some plants and fermentation products, there is limited information on melatonin isomers (Rodrigueznaranjo et al., 2011; Kocadagli et al., 2014). Melatonin isomers were first reported in wine using HPLC coupled with mass/mass spectrometry (MS/MS), and the presence of melatonin isomers has been confirmed (Rodrigueznaranjo et al., 2011; Gomez et al., 2012, 2013; Vitalini et al., 2013). The distributions of melatonin and its isomers vary widely in wine products (Gomez et al., 2013). Melatonin isomers have been identified in other fermentation and plant products, such as bread, beer, and orange juice (Fernández-Pachón et al., 2013; Kocadagli et al., 2014). Recently, a melatonin isomer was found in sesame extract (Vitalini et al., 2020). Based on the data of Diamantini et al. (1998), isomer structures in wine were tentatively identified as 1-(2-alkanamidoethyl)-6-methoxyindole and *N*-acetyl-3-(2-aminoethyl)-6-methoxyindole (Tan et al., 2012).

The melatonin biosynthetic pathway was characterized earlier in animals. The source of melatonin is L-tryptophan, and it is produced when tryptophan hydroxylase (TPH) catalyzes the production of 5-hydroxytryptophan by tryptophan (Fitzpatrick, 1999). Then, 5-hydroxytryptophan is catalyzed by aromatic amino acid decarboxylase (AADC) to serotonin, acetyltransferase catalyzes the formation of serotonin to *N*-acetyl-5-hydroxytryptamine, and finally *N*-acetyl-5-hydroxytryptamine is catalyzed by *O*-methyltransferase (OMT) to melatonin (Arnao and Hernández-Ruiz, 2014; Huang et al., 2017). The melatonin synthetic pathways in plants and animals differ. For example, in plants, in the initial step, tryptophan is decarboxylated to form tryptamine instead of being hydroxylated to 5-hydroxytryptophan, which is then hydroxylated to form serotonin (Back et al., 2016). Thereafter, the other melatonin biosynthetic processes are similar to those in animals. These steps involve serotonin *N*-acetyltransferase (SNAT) and *N*-acetylserotonin methyltransferase (ASMT) (Kang et al., 2011, 2013). However, the biosynthesis of melatonin isomers is still completely unclear (Gomez et al., 2013).

Mulberry (*Morus alba* L.) is an important medicinal herb with multiple functions, such as antioxidant, anti-cancer and hypoglycemic, and is involved in regulating immunity and sleep (Chen et al., 2003; He et al., 2013; Sánchez-Salcedo et al., 2015). According to the literature, mulberry leaves and fruit contained higher melatonin content than other tissues (Chen et al., 2003; Pothinuch and Tongchitpakdee, 2011; Wang et al., 2016). In addition, the melatonin contents among different varieties of the same species of plant could be significantly different by several 100-fold (Vitalini et al., 2013; Wang et al., 2016). We detected, identified and quantitatively analyzed the melatonin contents in leaves and mature fruit of different mulberry varieties. We found that melatonin isomers were present from all the tested mulberry varieties. The complete sequence of the mulberry genome is available and provides an opportunity for the characterization of biosynthetic genes involved in melatonin and its isomers (He et al., 2013; Jiao et al., 2020). Thus, we identified 37 putative melatonin and its isomer biosynthetic genes and analyzed their expression patterns and enzyme functions to clarify the biosynthetic pathway of melatonin and its isomer in mulberry (Zheng et al., 2021). The results provided insights into the molecular mechanism underlying melatonin and its isomer biosynthesis in mulberry and expanded our knowledge of melatonin isomer biosynthesis.

MATERIALS AND METHODS

Plant Materials

The leaves were collected from different varieties of mulberry trees. All the mulberry trees were grown without chemical pesticides, and without of wild silkworms (**Table 1** and **Supplementary Table 1**). Mulberry (*Morus* spp.) plants were grown at the Mulberry Germplasm Nursery in Southwest University, China. The mature mulberry leaves of 50 mulberry varieties were harvested in May, 2015. The mature mulberry leaves were harvested at 10: 00 AM on April 28th, June 28th, and August 28th, 2016. Leaves at different maturation stages (represented by the 1st, 5th, 10th, 15th, and 20th leaf positions) from three varieties were harvested per variety in July, 2016. The 1st leaves were selected from positions of 1∼3 from the top of each branch, the 5th leaves were from positions 4 and 5, the 10th leaves were from positions 9 and 10, the 15th leaves were from positions 14 and 15, and the 20th leaves were from positions 19 and 20. These mulberry leaves were oven-dried at 50°C to a constant mass and then pulverized. The powders were passed through a 100-mesh sieve and stored at −40°C until used.

Mulberry varieties "Dashi," "Baiyuhuang," "Jialing NO. 30," and "Zhongsang 5801" are the same materials used to detect melatonin and isomers and to clarify the molecular mechanisms of their biosynthesis. These mulberry varieties grown in the mulberry field of the Chongqing Sericulture Science and Technology Research Institute, China and were harvested in April 2017 (**Supplementary Table 1**). Mulberry varieties "Dashi," "Baiyuhuang," "Jialing NO. 30," and "Zhongsang 5801" grown in natural conditions. Parts of the mature mulberry leaves and fruit were picked, lyophilized and ground into powders. The fruits were collected at 33–37 days after full-bloom (Liu et al., 2015). They were then stored in a −40°C refrigerator for the detection of melatonin and its isomers. Other parts of the samples were immediately frozen in liquid nitrogen and stored at −80°C for molecular biology experiments.

Reagent and Chemicals

All the experimental event sequences were controlled using UNIFI software (Waters). Melatonin (CAS NO.73-31-4), *N*-acetylserotonin (CAS NO.1210-83-9) and tryptophan ethyl ester standards were purchased from Sigma-Aldrich Chemical (Sigma, St Louis, MO, United States), and methanol was purchased from Merck (Merck, Darmstadt, Germany). The *N*-acetyl-6-methoxytryptamine standard was purchased from FUJIFILK Wako Pure Chemical Corporation (FUJIFILK Wako, Tokyo, Japan).

Detection, Identification and Quantitative Analyses of Melatonin and Its Isomers

Melatonin was extracted according to the modified method of Vitalini et al. and others (Chen et al., 2003; Vitalini et al., 2013;

TABLE 1 | Contents of melatonin and its isomers in the mature mulberry leaves from 50 varieties harvested in May, 2015.

Name of mulberry variety	Specie name of mulberry variety	Name of sample*	Mel (ng/g) (DW)	SD	MI-1 (ng/g) (DW)	SD	SUM (ng/g) (DW)
Naxi	*Morus sp.*	S1-L	nd	0	0.23	0.03	0.23
Chuansang	*Morus notabilis* C. K.	S2-L	0.83	0.01	0	0.01	0.83
Zhichuisang	*Morus multicaulis* Perr.	S3-L	nd	0	0.99	0.08	0.99
Jialing-NO.30	*Morus multicaulis* Perr.	S4-L	0.36	0.02	0.63	0.06	1
Zhenzhubai (2X)	*Morus alba* Linn	S5-L	nd	0	1.03	0.04	1.03
Hongguo-NO.2	*Morus atropurpurea* Roxb.	S6-L	nd	0	1.2	0.13	1.2
Jialing-NO.40	*Morus multicaulis* Perr.	S7-L	0.37	0.05	0.85	0.08	1.22
Jialing-NO.20	*Morus multicaulis* Perr.	S8-L	0.43	0.01	0.82	0.16	1.26
Xiaoguansang	*Morus multicaulis* Perr.	S9-L	nd	0	1.27	0.05	1.27
Zhongsang5801	*Morus multicaulis* Perr	S10-L	nd	0	1.36	0.09	1.36
Zhenzhubai (4X)	*Morus alba* Linn.	S11-L	nd	0	1.44	0.15	1.44
Dahuayepisang	*Morus cathayana* Hemsl.	S12-L	nd	0	1.66	0.06	1.66
Hongguo-NO.1	*Morus multicaulis* Perr.	S13-L	nd	0	1.75	0.05	1.75
Sri Lanka2	*Morus bombycis* koidz.	S14-L	nd	0	1.77	0.1	1.77
Yuanyepisang	*Morus alba* Linn.	S15-L	nd	0	1.9	0.05	1.9
Guofeng	*Morus sp.*	S16-L	nd	0	2.01	1	2.01
Sri Lanka1	*Morus bombycis* koidz.	S17-L	nd	0	2.56	0.21	2.56
Jianchi	*Morus bombycis* koidz.	S18-L	nd	0	2.62	0.69	2.62
Yanbian	*Morus alba* Linn.	S19-L	nd	0	2.68	0.34	2.68
Georgia mulberry	*Morus multicaulis* Perr.	S20-L	nd	0	2.76	0.23	2.76
Xinyizhilai (2X)	*Morus alba* Linn.	S21-L	nd	0	2.97	0.39	2.97
GM31	*Morus sp.*	S22-L	nd	0	3.11	0.05	3.11
Hongyousang	*Morus alba* Linn.	S23-L	nd	0	3.58	0.02	3.58
Ya'an-NO.7	*Morus sp.*	S24-L	nd	0	4.06	0.32	4.06
Baiyuwang	*Morus multicaulis* Perr.	S25-L	nd	0	4.09	0.4	4.09
Xinong6071	*Morus bombycis* koidz.	S26-L	0.58	0.02	3.51	0.21	4.09
Yidachimu	*Morus bombycis* koidz.	S27-L	nd	0	4.55	0.09	4.55
Xiqing-NO.3	*Morus bombycis* koidz.	S28-L	nd	0	5.15	0.18	5.15
Ya'an-NO.1	*Morus sp.*	S29-L	nd	0	5.81	0.42	5.81
Cambodia mulberry	*Morus sp.*	S30-L	nd	0	5.93	0.18	5.93
Xiqing-NO.5	*Morus multicaulis* Perr.	S31-L	nd	0	6.13	0.52	6.13
Qiangbing	*Morus sp.*	S32-L	nd	0	6.18	0.28	6.18
Jialing-NO.16	*Morus alba* Linn.	S33-L	0.46	0.01	5.89	0.25	6.35
Laos mulberry	*Morus sp.*	S34-L	nd	0	6.79	0.2	6.79
Shan305	*Morus alba* Linn.	S35-L	nd	0	7.09	0.57	7.09
Huosang-NO.1	*Morus mizuho* Hotta.	S36-L	nd	0	7.26	0.33	7.26
Yunnanchangguosang	*Morus laevigata* Wall.	S37-L	nd	0	7.96	0.33	7.96
Guangyuan-NO.5	*Morus sp.*	S38-L	nd	0	8.22	0.79	8.22
Tongxiangqing	*Morus multicaulis* Perr.	S39-L	nd	0	8.45	1.01	8.45
Xiqing-NO.1	*Morus alba* Linn.	S40-L	nd	0	8.65	0.54	8.65
Zijin	*Morus alba* Linn.	S41-L	nd	0	8.8	0.63	8.8
Xinongxinyizhilai (4X)	*Morus alba* Linn.	S42-L	nd	0	9.09	0.14	9.09
Lunjiao40	*Morus atropurpurea* Roxb.	S43-L	0.26	0.02	9.84	0.44	10.1
Xiqing-NO.4	*Morus multicaulis* Perr.	S44-L	nd	0	10.28	0.55	10.28
Xiqing-NO.2	*Morus bombycis koidz.*	S45-L	nd	0	10.3	1.79	10.3
Ganluo-NO.6	*Morus sp.*	S46-L	nd	0	10.64	0.12	10.64
India mulberry	*Morus indica L.*	S47-L	nd	0	12.67	0.58	12.67
Xiaohuayepisang	*Morus cathayana* Hemsl.	S48-L	nd	0	14.39	0.26	14.39
Shenlongjiachangsuisang	*Morus wittiorum Hand.*-Mazz.	S49-L	0.51	0.01	18.99	0.17	19.5
Cesha	*Morus alba* Linn.	S50-L	nd	0	20.58	0.76	20.58
	Average						5.49 ng/g

**The nomenclature of Name of sample is S for Sample, Arabic numerals for the number of mulberry variety, and the last capital letter for tissue of mulberry. For example, S1-L is S for Sample, 1 for the number of mulberry variety "Naxi," and L for leaves of mulberry variety "Naxi." "Mel" is melatonin. "MI-1" is melatonin isomer-1. "nd" is no detected. "sp." indicate that no species name has been determined. Data are means ± SDs (n = 3).*

Wang et al., 2016). Briefly, 5 g of oven-dried or freeze-dried sample was accurately weighed and transferred to a 50-mL centrifuge tube. A 10-mL aliquot of methanol was added to each sample tube and vortexed for 2 min. Ultrasonication in an ultrasonic water bath (200 W, 20°C) followed by 30 min on ice was used to assist and accelerate the extraction of melatonin. After centrifugation at 12,000 × *g* for 5 min at 4°C, the supernatants were collected and filtered through a 0.22-μm syringe filter and stored in amber vials suitable for subsequent UPLC-MS/MS analyses (Pothinuch and Tongchitpakdee, 2011; Wang et al., 2016). All the samples were analyzed using an Agilent 1290-6495 UPLC-MS/MS (Agilent, Waldbronn, Germany). Each sample was tested on a C18 column (2.1 cm × 5.0 cm, 1.8 μm) using the following parameters: ion source, AJS-ESI +; acquisition mode, MRM mode; dry gas temperature, 250°C; dry gas flow rate, 14 L/min; nitrogen pressure, 30 psi; sheath gas temperature, 375°C; sheath gas flow rate, 12 L/min; capillary voltage, 4,000 V; and nozzle voltage, 500 V. The mobile phases were 0.1% formic-acid in water (A) and MeOH (B). The gradient elution program was set as follows: 0–2 min, 10% (B); 2–4 min, 10–90% (B); 4–6 min, 90–98% (B); 6–8 min, 98–10% (B). The flow rate was set at 0.15 mL/min and the injection volume was set at 3.0 μL. The melatonin and isomers contents of each sample were determined in triplicate.

An UHPLC-Q-TOF-MS system, performed on a Waters Xevo G2-XS QTOF system equipped with a heated ESI mode and coupled to a Waters I-Class UHPLC system (Waters, Milford, CT, United States) was used for the analysis of melatonin isomers, melatonin, tryptophan-ethyl ester and samples. Each sample was tested on a BEH C18 column (2.1 cm × 5.0 cm, 1.7 μm) (Waters). The mobile phases were 0.1% formic-acid in water (A) and MeOH (B). The gradient elution program was set as follows: 0–2 min, 10–15% (B); 2–4 min, 15–30% (B); 4–8 min, 30–40% (B); 8–10 min, 40–60% (B); 10–12 min, 60–85% (B). The flow rate was set at 0.4 mL/min and the injection volume was set at 1.0 μL. ESI was operated in the positive electrospray ionization modes. The electrospray capillary voltage was 2.5 kV, the capillary skimmer was set to 40 V, a countercurrent flow of nitrogen gas (120°C) was employed for the desolvation, and argon was used to improve the fragmentation. The scan range was from *m/z* 80–400 at a resolution of 60.000.

Quantitative Real-Time PCR Analysis

The total RNAs isolated from four mulberry leaves and fruit ("Dashi," "Baiyuhuang," "Jialing NO. 30," and "Zhongsang 5801") were extracted, independently, using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's instructions. The RNA quality and concentration were measured using a ND-1000 UV spectrophotometer (Thermo, Madison, WI, United States). First-strand cDNA was synthesized using 3 μg of total RNA with M-MLV reverse transcriptase (Promega, Madison, WI, United States) in a 25 μL reaction system. The amino acid sequences encoded by 37 potential genes involved in the biosynthesis of melatonin and its isomers were downloaded from the *M. notabilis* database[1]. The primers (**Supplementary Table 2**) used for qRT-PCR were designed based on the gene sequences obtained from *Morus* genome. Each qRT-PCR was performed using SYBR Premix EXTaq II (TaKaRa) and StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions. The diluted cDNA (2 μL) was used as a template. The mulberry *Actin3* gene (GenBank accession NO. HQ163775.1) was used as a control to normalize target gene expression data. **Supplementary Table 2** contains a list of gene-specific primers for qRT-PCR. The process of detecting the expression levels of mulberry genes by qRT-PCR was as follows: 10 μL SYBR Premix ExTaq II, 0.4 μL primer-F (10 μM), 0.4 μL primer-R (10 μM), 0.4 μL ROX reference dye, 2 μL template, 6.8 μL ddH_2O, for a 20-μL total. The reaction was as follows: 40 cycles of pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s and extension at 60°C for 30 s.

Cloning and Recombinant Overexpression of Key Genes Involved in Melatonin Isomer Biosynthesis

Based on the *M. notabilis* database (see text footnote 1), the sequences of the *ASMT* genes in mulberry were established. All the primer pairs (**Supplementary Table 3**) were designed using Premier 5.0 (Biosoft International Palo Alto, United States) to clone *MaASMT4*, *MaASMT9*, *MaASMT19* and *MaASMT20* from "DaShi" using PCR. Total RNA extraction and cDNA synthesis were performed as previously described. The PCR products were cloned into the pMD19-T cloning vector, and the inserts were verified and sequenced.

Functional and *in vitro* Activity Analyses of the Key Enzymes Involved in Melatonin Isomer Biosynthesis

The ASMT cDNA from pMD19-*MaASMT4,* pMD19-*MaASMT9*, pMD19-*MaASMT19* and pMD19-*MaASMT20* were first subcloned into the pCold TF vector (TaKaRa, Beijing, China;[2], Code No. 3365) by LR recombination to obtain the respective expression vectors pCold TF-*MaASMT4*, pCold TF-*MaASMT9*, pCold TF-*MaASMT19* and pCold TF-*MaASMT20* according to the instruction of *pEASY*®-Basic Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China;[3], Order NO. CU201). Next, these expression vectors were independently transformed into *Escherichia coli* BL21 (DE3). Each of the obtained positive *E. coli* strains was inoculated into a sterilized test tube, and positive *E. coli* strains and fresh medium containing 100 mg/L ampicillin were mixed at a1:100 ratio and then incubated at 37°C until an OD_{600} of 0.6. After the addition of 1 mM isopropyl-b-D-thiogalactopyranoside (+ IPTG), the culture was grown at 28°C and shaken at 250 rpm for 8 h. Cell culture and purification steps were performed using Ni-NTA chromatography according to the manufacturer's instructions (Sangon Biotech, Shanghai, China;[4], Order NO. C600033). The protein concentration was determined by the Bradford method using a protein assay dye (Beyotime,

[1]http://morus.swu.edu.cn/morusdb/

[2]https://www.takarabiomed.com.cn/

[3]https://www.transgen.com.cn/

[4]https://www.sangon.com/

Shanghai, China). Purified recombinant MaASMT4, MaASMT9, MaASMT19 and MaASMT20 were dissolved in 30% glycerol and stored at −80°C or analyzed further (Byeon et al., 2014).

Samples of the purified recombinant proteins (2.0 μg) were incubated in a total volume of 100 μL of 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM *N*-acetylserotonin and 0.5 mM *S*-adenosyl-L-methionine at 28°C for 30 min, and reactions were terminated by the addition of 300 μL of MeOH. Then, 10 μL aliquots were analyzed using UPLC-MS/MS as described above (Kang et al., 2011; Sangkyu et al., 2013). Non-enzymatic reaction products, which were generated in the absence of any enzyme, were subtracted. The analyses were performed in triplicate.

Statistical Analyses

All the data were expressed as the means ± standard deviations analyzed with SPSS statistical software (SPSS Inc., Chicago, IL, United States).

RESULTS

Detection and Identification of Melatonin and Its Isomers in Mulberry Leaves

UPLC-MS/MS was used to determine whether the retention time and the collision-induced ion fragments of mulberry samples in positive mode were consistent with those of the melatonin standard (**Figure 1**). The retention time of the melatonin standard was 2.05 min (**Figure 1A**). The main collision-induced ion fragments derived from the melatonin standard were 233.0, 216.2, 174.1, and 159.2. In melatonin collision-induced ion fragments, the relative abundance of 174.1 was greatest, which indicated that the most stable abundant fragment of melatonin was 174.1 (**Figure 1A**). Therefore, the collision-induced ion fragment of 174.1 was used for the quantification of melatonin in accordance with a previous report (Rodrigueznaranjo et al., 2011). We detected three compounds with the same *m/z* as melatonin using UPLC-MS/MS (**Figures 1B–D**). The three compounds were named as Mel, MI-1 and MI-2, respectively. The Mel were detected at 2.05 min and the same *m/z* value (233.0) as that of the melatonin standard. These data indicated that Mel was melatonin (**Figure 1C**). In addition, Compared to the melatonin standard, the two natural compounds (MI-1 and MI-2) have the same *m/z* value (233.0) and different retention times (1.27 and 1.85 min), and the most abundant fragment of the MI-1 in mulberry leaves occurred at 159.2 (**Figures 1B–D**). Diamantini et al. (1998) previously reported the characterization of metastable collision-induced ion fragments of melatonin isomers. The data showed that the different positions of the substituents on the indole ring influence the fragmentation patterns and the relative abundances of the generated ions (Tan et al., 2012). Therefore, MI-1 and MI-2 were thought to be melatonin isomers, and the collision-induced ion fragment of 159.2 was used for the quantification of MI-1 (Gomez et al., 2012). MI-2 was only present in four mulberry varieties, and the low content made it impossible to quantify. The structure of Mel and the predicted structures of MI-1 and MI-2 are shown in **Figures 1E–G** and based on the different collision-induced ion fragment patterns (Diamantini et al., 1998; Gomez et al., 2012).

Analysis of Melatonin and Its Isomers in Different Mulberry Varieties

The contents of melatonin and its isomers from the 50 mulberry varieties are shown in **Table 1**, and 42 varieties only contained the novel natural melatonin isomer in their leaves. One mulberry variety only contained melatonin and the remaining seven mulberry varieties contained both melatonin and its isomers, with their total contents ranging from 0.23 to 20.58 ng/g (DW). The highest melatonin content was detected in the sample from S2-L (0.83 ng/g DW) and the highest melatonin isomer content was detected in the sample from S50-L (20.58 ng/g DW) (**Table 1**). The content of the melatonin isomers ranged from 0.23 to 20.58 ng/g (DW). The average content of melatonin isomers in mulberry leaves was 5.49 ng/g (DW) (**Table 1**).

Spatio-Temporal Distributions of Melatonin and Its Isomers in Mulberry

To uncover the effects of daily environmental conditions (season) on the production of melatonin and its isomers in mulberry leaves, leaves were collected every 2 months at harvest day. The changes in the content of melatonin and its isomer in three mulberry varieties were different in different months (**Supplementary Figure 1**). The melatonin isomer contents in mulberry leaves at the same developmental stage from different varieties increased from April 28th to June 28th, but showed the opposite trend from June 28th to August 28th. The average melatonin isomer content in S44-L was higher than that in S4-L and S26-L at different harvest time points.

To measure melatonin and its isomer in the leaves of S4-L, S26-L and S44-L at different maturation stages, leaves at different positions (1st, 5th, 10th, 15th, and 20th) were collected. In the three mulberry varieties, the MI-1 content was higher at the 20th leaf position than at the other leaf positions (**Supplementary Figure 2**). The melatonin isomer contents in leaves at different maturation stages were significantly different in S4-L, S26-L and S44-L ($P < 0.05$). Thus, the melatonin isomer contents in the leaves of three different mulberry varieties increased with leaf position. However, the levels of melatonin and its isomer were below the detection limits in the 1st leaves of the three mulberry varieties. In general, the melatonin isomer contents increased with leaf position. To our knowledge, this is the first report of melatonin isomers being naturally present in the tissues of a woody plant. In addition, although melatonin isomers do exist in fermentation products and herbaceous plants, the biosynthesis of melatonin isomer was still completely unclear (Gomez et al., 2013). Therefore, we conducted a series of experiments using the same materials to clarify the molecular mechanism underlying the biosynthesis of melatonin and its isomers in mulberry. Melatonin and isomers from different tissues (leaves, fruit) of four different mulberry varieties were detected using UPLC-MS/MS (**Figure 2** and **Supplementary Table 1**). As shown in **Figure 2**, the retention time of the melatonin standard was 2.15 min and contained mainly three ion fragments, 216.2, 174.1,

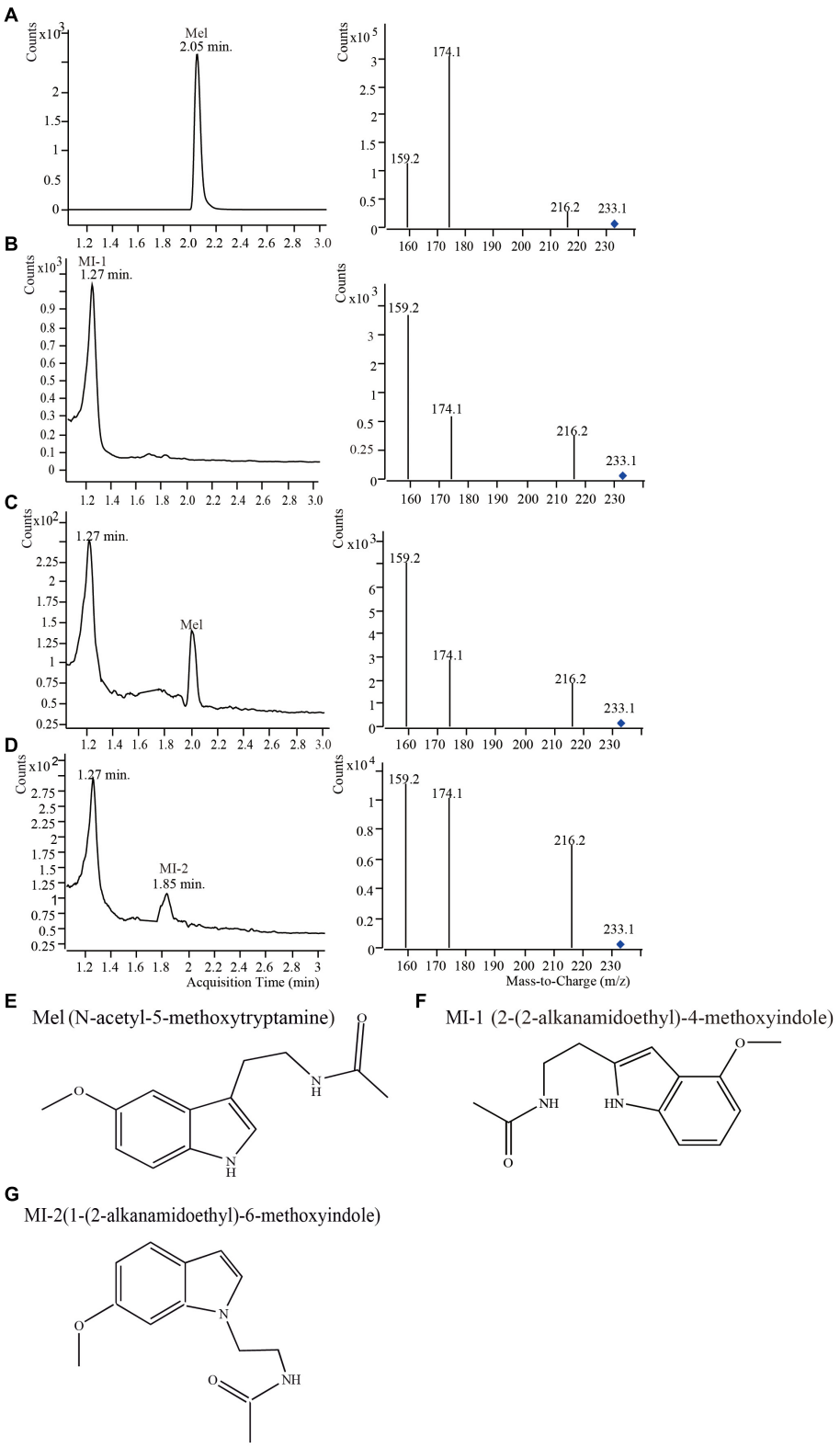

FIGURE 1 | Mass spectra of mulberry leaf samples from different varieties harvested in May, 2015 detected using UPLC-MS/MS, and the structural formulae of melatonin and its isomers. **(A)** Melatonin standard; **(B)** Sample S-36-L; **(C)** Sample S-4-L; **(D)** sample S-12-L; **(E)** melatonin; **(F)** 2-(2-alkanamidoethyl)-4-methoxyindole; **(G)** 1-(2-alkanamidoethyl)-6-methoxyindole.

and 159.2. The relative abundance of the main ion fragments of melatonin standard was highest at 174.1 (**Figure 2A**). Three compounds having *m/z* 233.0, the same *m/z* as the melatonin standard, were detected at retention times of 1.85, 2.15 and 2.19 min (**Figures 2B,C**). The three compounds were named as Mel, MI-2 and MI-3 in accordance with a previous method. Mel showed the same *m/z* value (233.0) and retention time as the melatonin standard (**Figure 2B**). The relative abundances of the main fragment ions of the samples (216.2 or 159.2) were inconsistent with those of the melatonin standard (174.1). Thus, MI-2 and MI-3 were identified as melatonin isomers (**Figures 2B,C**). As an authentic marker is not available to identify the current isomer detected by UPLC-MS/MS, a tentative identification was performed by comparing relative abundances of minority fragment ions. Previous studies have demonstrated that 216.2 is important as the primary ion, and the relative abundances of secondary ions can indicate the positions of the methoxy and amide groups (Gomez et al., 2012). The predicted structure of MI-2 is 1-(2-alkanamidoethyl)-6-methoxyindole. In accordance with previous reports, MI-3 is presumed to be *N*-acetyl-6-methoxytryptamine (**Figure 2D**; Diamantini et al., 1998; Gomez et al., 2013). Four different mulberry varieties contained melatonin and two melatonin isomers (MI-2 and MI-3) in their leaves. However, four different mulberry varieties fruits only contained melatonin and one melatonin isomer (MI-2). We found that MI-3 was only present in the leaves and not in the fruit of the four different mulberry varieties (**Supplementary Table 1**). Furthermore, the total contents of melatonin and melatonin isomers in leaves and fruits of "Dashi" were the highest among the four mulberry cultivars (**Supplementary Table 1**).

Tissue-Specific Expression of Genes Involved in the Biosynthesis of Melatonin and Its Isomers

The complete sequence of the mulberry genome is now available (He et al., 2013; Jiao et al., 2020). It provides the opportunity to elucidate the molecular mechanisms underlying the biosynthesis of melatonin and its isomers. Based on the analysis of the mulberry genome database, 37 potential genes, one *TDC*, seven *T5Hs*, six *SNAT*s, three *COMT*s, and 20 *ASMT*s, were identified as being associated with the biosynthesis of melatonin (Zheng et al., 2021). The qRT-PCR was used to detect the expression levels of genes involved in the biosynthesis of melatonin in leaves and fruit of mulberry varieties "Dashi" and "Baiyuhuang" (**Figures 3A,B**). All the genes involved in the biosynthesis of melatonin, except *MaASMT4* and *MaASMT20*, had different expression patterns in both the leaves and fruit of the two varieties (**Figure 3B**). *MaTDC* was expressed in various tissues, and the gene expression in fruit was significantly higher than that in leaves (**Figure 3B**). *MaT5H2* was expressed highest in these two tissues compared with the expression levels of six other genes (**Figure 3B**). The expression levels of the six *MaSNAT* genes differed in two tissues of two varieties, but their expression patterns were consistent (**Figure 3B**). Among the three *MaCOMT* genes, the expression level of *MaCOMT1* was the highest (**Figure 3B**). The expression levels of *ASMT* gene family members were different in the two varieties (**Figure 3B**). The *MaASMT4* gene was abundantly expressed in the leaves of "Dashi" and "Baiyuhuang," but was almost not expressed in the fruit of the two mulberry varieties. The expression level of the *MaASMT20* gene in the leaves of the two mulberry varieties was over 25-fold greater than in their fruit (**Figure 3B**). A similar result was confirmed in the other two mulberry varieties ("Jialing NO. 30" and "Zhongsang 5801") (**Supplementary Figure 3**). These results suggested that *MaASMT4* and *MaASMT20* may be involved in the synthesis of melatonin isomer (MI-3), which was only detected in mulberry leaves.

Gene Cloning and Analysis of the *MaASMT4* and *MaASMT20*

We successfully cloned *MaASMT4* and *MaASMT20* from "Dashi." The cloned *MaASMT4* was 1,077 bp in length, encoding 358 amino acids, while the cloned *MaASMT20* was 1,098 bp in length, encoding 365 amino acids (GenBank accession NOs. MN937267 and MN937270). The amino acid sequences of MaASMT4 and MaASMT20 were aligned with AtASMT, MdASMT and OsASMT1 (**Figure 4**). The conserved motif for *S*-adenosyl-L-methionine binding sites in the MaASMT4 and MaASMT20 sequences were identical to those of the AtASMT, MdASMT and OsASMT sequences. The conserved motif for the putative substrate binding sites of MaASMT4 and MaASMT20 were also identical to those of AtASMT, MdASMT and OsASMT, but the catalytic domains were not conserved.

Function and Activity Analyses of *MaASMT4* and *MaASMT20*

While the animal ASMT enzyme has been characterized in detail at the biochemical level, there have been limited studies of plant ASMT enzymes. *MaASMT4* and *MaASMT20* full-length cDNAs were independently cloned into pCold TF vectors for the expression of recombinant proteins in *E. coli* (**Figure 5A**). We purified recombinant proteins as described in the Materials and Methods. The obtained MaASMT4 and MaASMT20 proteins showed the predicted molecular weights of 39 and 41 kDa, respectively, in the SDS-PAGE analysis (**Figure 5B**). Then, we co-incubated *S*-adenosyl-L-methionine and *N*-acetylserotonin separately with MaASMT4 and MaASMT20 in the reaction buffer and analyzed the products by UPLC-MS/MS (**Figures 5C–F**). The retention time for the melatonin standard was 2.81 min and that of the MI-3 standard was 2.91 min (**Figures 5C,D**). The target compound in both the MaASMT4 and MaASMT20 reaction samples showed the same *m/z* value (233.0) and the same retention time (2.91 min) as that of MI-3 standards standard (**Figures 5C–F**). These data indicated that MaASMT4 and MaASMT20 were able to convert *N*-acetylserotonin to melatonin and MI-3.

DISCUSSION

In this study, the contents of melatonin and its isomers were analyzed among 50 mulberry varieties and they differed significantly by up to 90-fold. The measured melatonin content

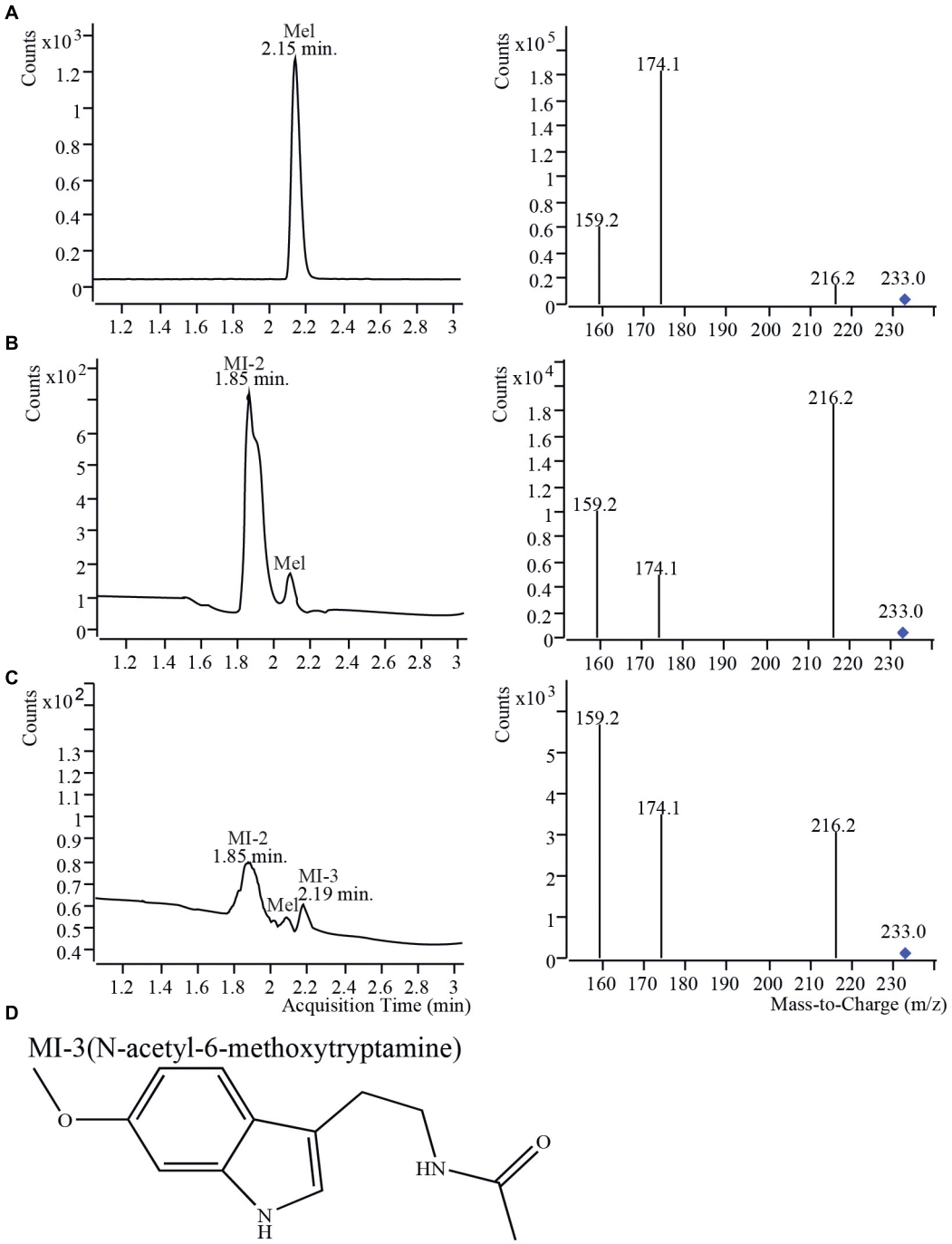

FIGURE 2 | Mass spectra of mulberry leaf and fruit samples from "Dashi" harvested in 2017 detected using UPLC-MS/MS. **(A)** Melatonin standard; **(B)** Mulberry fruit; **(C)** Mulberry leaves; **(D)** The structure of a melatonin isomer. MI-3, *N*-acetyl-6-methoxytryptamine.

is affected by genetic and non-genetic factors (Wang et al., 2016). The non-genetic factors include growth conditions and analytical methods, such as LC-ECD-UV, LC-MS, and LC-MS/MS (Zhao et al., 2013; Mukherjee et al., 2014; Wang et al., 2016). The variation in the contents of melatonin and its isomers in mulberry leaves of different varieties may mainly depend on genetic traits, because samples at the same maturity stage were collected from mulberry trees that were grown under the same environmental conditions and melatonin was measured using the same analytical methods. The melatonin contents of mulberry leaves from the three other reports differed greatly, which may be attributed to the selectivity and sensitivity of different analytical

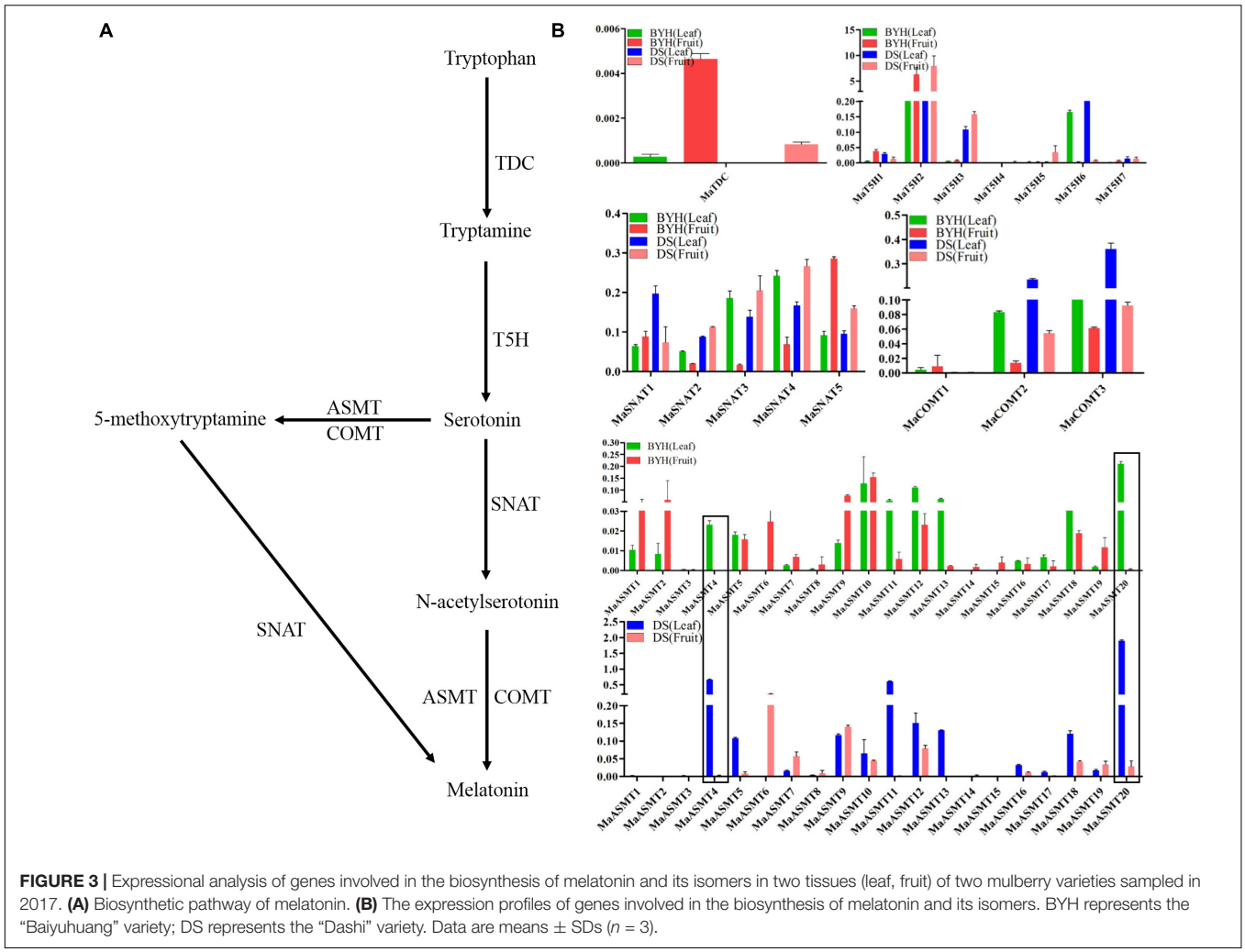

FIGURE 3 | Expressional analysis of genes involved in the biosynthesis of melatonin and its isomers in two tissues (leaf, fruit) of two mulberry varieties sampled in 2017. **(A)** Biosynthetic pathway of melatonin. **(B)** The expression profiles of genes involved in the biosynthesis of melatonin and its isomers. BYH represents the "Baiyuhuang" variety; DS represents the "Dashi" variety. Data are means ± SDs (n = 3).

methods for the identification and quantification of melatonin (Chen et al., 2003; Pothinuch and Tongchitpakdee, 2011; Wang et al., 2016). The total content of melatonin and melatonin isomers in this study was similar to the results reported by Wang et al. (2016). Our measurement of melatonin in mulberry may be more reliable because a more precise analytical method, UPLC-MS/MS, was used.

Although some studies have used MS to detect melatonin in plants, little attention has been paid to the detection and characterization of melatonin isomers (Iriti et al., 2006; Feng et al., 2014; Vitalini et al., 2020). Here, natural compounds (MI-1, MI-2, and MI-3) having the same m/z as melatonin, were found in mulberry using UPLC-MS/MS. MI-1, MI-2 and MI-3 were thought to be melatonin isomers. Melatonin isomers have been detected in wine, and one of these isomers has been identified as tryptophan-ethyl ester, a compound with the same m/z as melatonin standard (Gardana et al., 2014; Iriti and Vigentini, 2015). However, UHPLC-Q-TOF-MS was carried out to confirm that in mulberry none of these compounds is tryptophan-ethyl ester but were other melatonin isomers. Tryptophan-ethyl ester has the same m/z as melatonin standard but its retention time was inconsistent with those of the two natural compounds (**Supplementary Table 4**). Therefore, the novel natural compounds in mulberry leaves were not tryptophan-ethyl ester. To our knowledge, this is the first identification of novel melatonin isomers in a woody plant, and this lays the foundation for the further clarification of the biosynthetic pathway of melatonin and its isomers in plants.

To our knowledge, the physiological functions of melatonin isomers in plants are completely unknown. However, Tarzia et al. (1997) tested the biological activity and the ability of a synthetic melatonin isomer (A1/M6) to bind to melatonin membrane receptors. Spadoni et al. (2001) reported that changing the positions of either the methoxy or N-acetylaminoethyl side chains in the indole ring results in marked alterations in their antioxidant and cytoprotective capacities (Tarzia et al., 1999). For example, if the methoxy side chain is located in position 4 of the indole ring, then this melatonin isomer possesses the strongest antioxidant capacity, but if the N-acetylaminoethyl side chain is in position 3, then the isomer is most effective as a cytoprotective agent (Spadoni et al., 2001). Therefore, we initially speculated

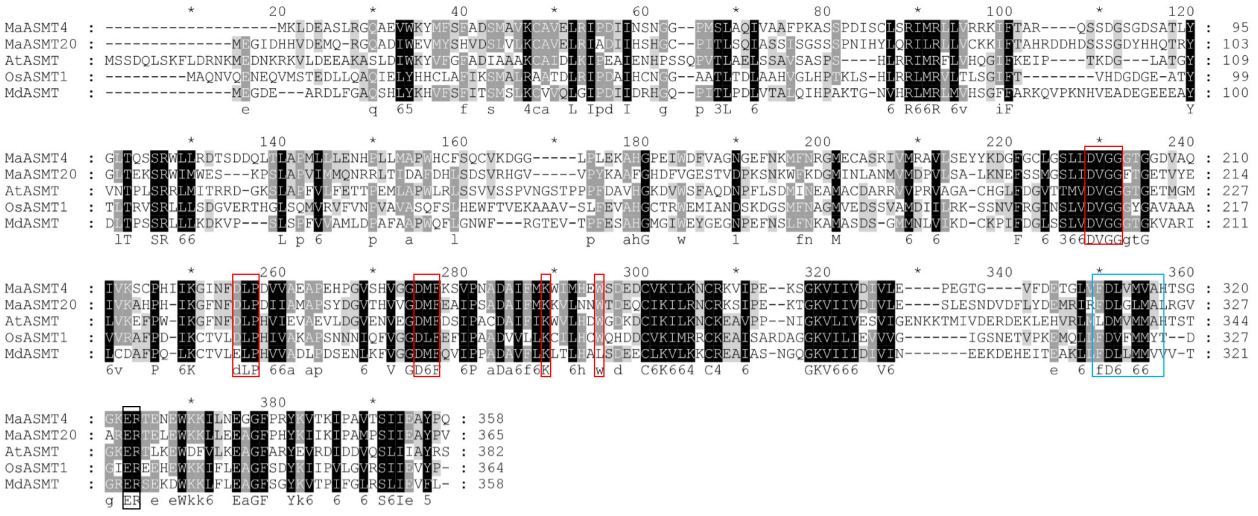

FIGURE 4 | Multiple sequences alignment of MaASMTs with the ASMTs from other plant species. The positions of the different conserved domains are represented by different colored boxes. The conserved motif for the *S*-adenosine-L-methionine binding is boxed in red, putative substrate-binding residues are boxed in black, and catalytic residues are boxed in blue.

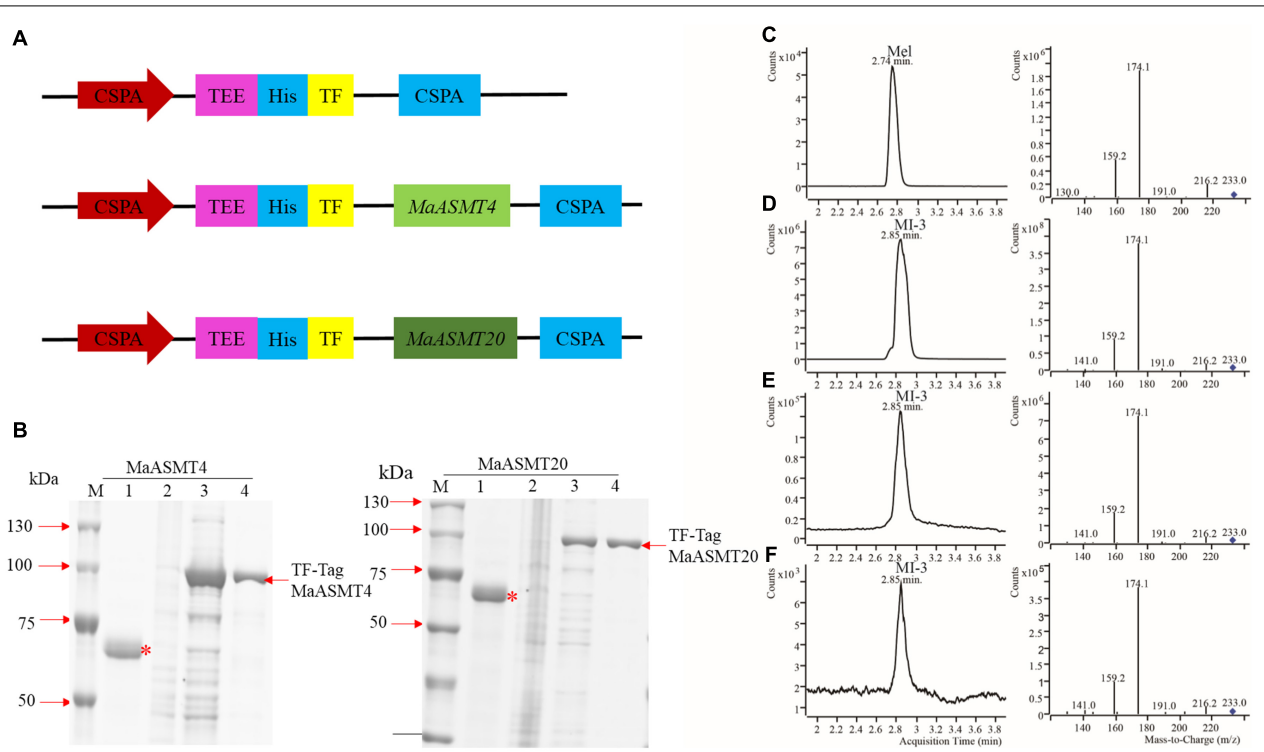

FIGURE 5 | Expression and activity analyses of MaASMT4 and MaASMT20 proteins. **(A)** Schematic diagram of the *E. coli* expression vector in pCold TF harboring *MaASMT4* and *MaASMT20*. **(B)** Purification of N-terminal His × 6-tagged MaASMT4 and MaASMT20 proteins. *E. coli* BL21 (DE3) cells harboring either pCold TF-*MaASMT4* or pCold TF-*MaASMT20* were incubated with IPTG for 8 h at 28°C. Lane 1: protein marker; lane 2: pCold TF (+ IPTG); lane 3: recombinant (–IPTG) lane 4: recombinant (+ IPTG); lane 5: purified protein. "TF" is Trigger Factor, a prokaryotic ribosome-related molecular chaperone that facilitates the translation and folding of peptides. "*" indicates the Trigger factor protein. "–IPTG" indicates no addition of 1 mM IPTG to the *E. coli* BL21 (DE3) culture, "+IPTG" indicates addition of 1 mM IPTG to the *E. coli* BL21 (DE3) culture. Products of *in vitro* enzymatic reactions detected by UPLC-MS/MS. **(C)** Melatonin standard; **(D)** *N*-acetyl-6-methoxytryptamine standard; **(E)** Chromatogram of *N*-acetylserotonin catalyzed by MaASMT4; **(F)** Chromatogram of *N*-acetylserotonin catalyzed by MaASMT20.

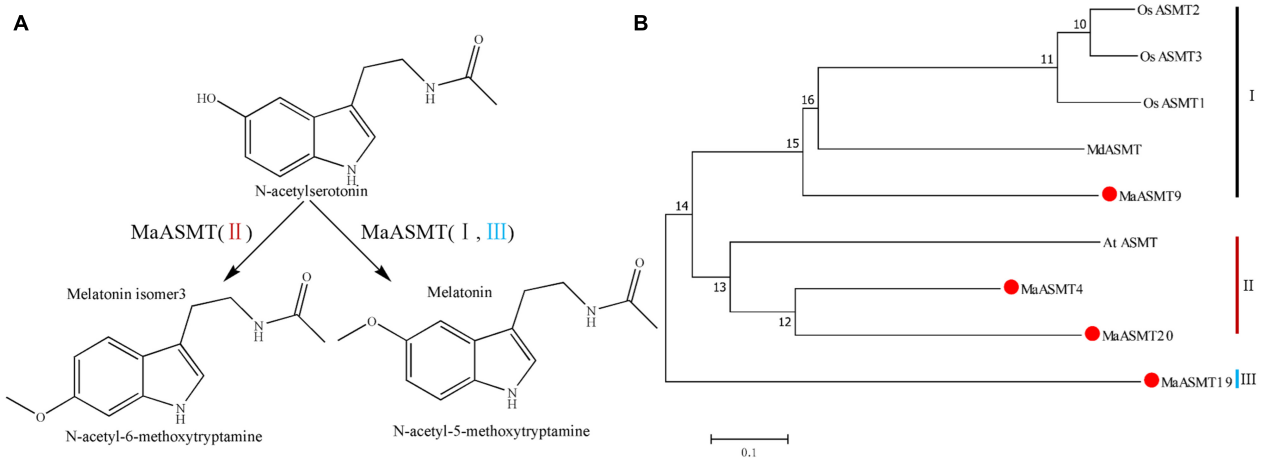

FIGURE 6 | The final step of the biosynthetic pathways of melatonin and its isomer and a phylogenetic tree of the ASMT family. **(A)** The final step of the biosynthetic pathways of melatonin and its isomer in "Dashi." ASMT: *N*-acetylserotonin methyltransferase. **(B)** Phylogenetic tree of amino acid sequences of ASMT in "Dashi" and other plants. The phylogenetic tree was generated with the neighbor-joining method using MEGA5 software. ASMTs were separated into three groups: class I (black), II (red) and III (blue). The function of ASMT genes has been characterized. Information of ASMT genes was listed in **Supplementary Table 5**.

that melatonin and its isomers in mulberry play similar functions during plant growth and development.

In plants, several isoforms of SNAT and ASMT have been identified in apple and rice (Kang et al., 2011; Bixiao et al., 2015; Tan and Reiter, 2020). The activity levels of some of these isoforms do not always correlate with the production of melatonin. Whether some of the SNAT and ASMT isoforms are actually enzymes for the synthesis of melatonin isoforms is unknown (Yeong et al., 2013). ASMT has three groups, classes I, II and III (Sangkyu et al., 2013; Liu et al., 2017; Pan et al., 2019). Our result suggested that *MaASMT4* and *MaASMT20*, belonging to the class II *ASMT* gene family, were the key genes for the biosynthesis of melatonin isomer, MI-3, which only occurs in mulberry leaves (**Figures 5**, **6A**). *ASMT* genes of rice and apple belong to class I, and *ASMT* gene of *Arabidopsis* belong to class II (**Figure 6B**). The proteins encoded by these genes only catalyze the conversion of *N*-acetylserotonin to melatonin (Sangkyu et al., 2013; Byeon et al., 2016). Therefore, this indicated that the functions of the *MaASMT4* and *MaASMT20* genes were different from those of other *ASMT* genes. In addition, the other two *MaASMTs* could be used in *in vitro* enzyme activity experiments. They were class I member MaASMT9 and class III member MaASMT19 (**Figure 6B**). Thus, MaASMT9 (GenBank accession NO. MN937268) and MaASMT19 (GenBank accession NO. MN937269) were also cloned from "Dashi." MaASMT9 and MaASMT19 full-length cDNAs were independently cloned into pCold TF vectors for the expression of recombinant proteins in *E. coli* BL21 (DE3) (**Supplementary Figure 4A**). The MaASMT9 and MaASMT19 proteins were purified according to description in the Materials and Methods, and the obtained proteins were with the predicted molecular weights of 41 kDa and 40 kDa, respectively (**Supplementary Figure 4B**). We co-incubated *S*-adenosyl-L-methionine and *N*-acetylserotonin separately with MaASMT9 and MaASMT19 in the reaction buffer and analyzed the products by UPLC-MS/MS. The recombinant proteins of the two MaASMTs (classes I and III) were only able to convert *N*-acetylserotonin to melatonin (**Supplementary Figures 4C–F**).

Why the ASMT proteins perform distinct functions? We analyzed sequences of ASMTs that contained conserved OMT domains in plants, including *S*-adenosyl-L-methionine binding sites, substrate binding sites and catalytic sites (**Supplementary Figure 5**). The conserved motif for the *S*-adenosyl-L-methionine binding sites and substrate binding sites were well conserved among MaASMTs, AtASMT, MdASMT, and OsASMT1. However, the conserved motif in the catalytic domains of the seven ASMT proteins was not conserved. The variation among the catalytic domains may cause changes in the functions of the MaASMT enzymes belonging to class II of the ASMT family. The molecular mechanisms involved in their functional changes need to be further studied.

In conclusion, we were the first to discover three naturally occurring melatonin isomers in mulberry. In addition, MI-3, one of the three melatonin isomers was only found in mulberry leaves, was synthesized by two enzymes, MaASMT4 and MaASMT20, belonging to the class II ASMT protein family. The present study provides some new insights into the functions of MaASMT4 and MaASMT20 from the class II subgroup of the ASMT family in mulberry plants and lays a foundation for the further understanding of the biosynthesis of melatonin and its isomers. Unfortunately, the biosynthetic mechanism responsible for the higher levels of MI-1 and MI-2 in mulberry have not yet been clarified. Therefore, the analysis of the biosynthesis mechanism of MI-1 and MI-2 needs be conducted in further research.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

ShaZ and AZ designed the research and wrote the manuscript. CL, MY, SW, and WF helped in the preparation for experiments materials. YZ and ShuZ conducted experiments and analyzed the data. AZ and ZX provided the financial aid for the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.708752/full#supplementary-material

Supplementary Figure 1 | Contents of melatonin and its isomers in the mature mulberry leaves from three varieties harvested during different months in 2016. Melatonin and its isomers were measured using UPLC-MS/MS. 1, April 28th; 2, June 28th; 3, August 28th. Significant differences ($P < 0.05$) are marked with different letters above the bars.

Supplementary Figure 2 | Contents of melatonin and its isomers in mulberry leaves at different maturity stages from three varieties harvested during July, 2016. Melatonin and its isomers were measured using UPLC-MS/MS. 1, 1st leaves; 2, 5th leaves; 3, 10th leaves; 4, 15th leaves; 5, 20th leaves. Significant differences ($P < 0.05$) are marked with different letters above the bars.

Supplementary Figure 3 | Expressional analysis of *ASMT* genes in two tissues (leaf and fruit) of two mulberry varieties sampled in 2017. **(A)** The expression profiles of the *MaASMT*s in two tissues of two mulberry varieties. Sample names are shown above the heat maps. Color scale indicates the degree of expression: green, low expression; red, high expression. **(B)** Expressional analysis of *MaASMT4* and *MaASMT20*. JL-NO30 represents the "Jialing NO. 30" variety; ZS-5801 represents the "Zhongsang 5801" variety. Significant differences ($P < 0.01$) are marked with different asterisks above the bars.

Supplementary Figure 4 | Expression and activity analyses of MaASMT9 and MaASMT19 proteins. **(A)** Schematic diagram of the *E. coli* expression vector in pCold TF harboring genes of interest. **(B)** Purification of N-terminal His × 6-tagged MaASMT9 and MaASMT19 proteins. *E. coli* BL21 (DE3) cells harboring either pCold TF-*MaASMT9* or pCold TF-*MaASMT19* were incubated with IPTG for 8 h at 28°C. Lane 1: protein marker; lane 2: pCold TF (+ IPTG); lane 3: recombinant (−IPTG) lane 4: recombinant (+ IPTG); lane 5: purified protein. Products of *in vitro* enzymatic reactions were detected by UPLC-MS/MS. "TF" is Trigger Factor, a prokaryotic ribosome-related molecular chaperone that facilitates the translation and folding of peptides. "*" indicates the Trigger Factor. "−IPTG" indicates no addition of 1 mM IPTG to the *E. coli* BL21 (DE3) culture, "+IPTG" indicates the addition of 1 mM IPTG to the *E. coli* BL21 (DE3) culture. Sequence data have been deposited with GeneBank (Accession NOs are MN937268 for MaASMT9 and MN937269 for MaASMT19). **(C)** melatonin standard; **(D)** melatonin isomer standard (MI-3); **(E)** Chromatogram of *N*-acetylserotonin catalyzed by MaASMT9; **(F)** Chromatogram of *N*-acetylserotonin catalyzed by MaASMT19.

Supplementary Figure 5 | Sequence analysis of ASMT proteins in plants. The alignment was performed using GeneDoc and the positions of the different conserved domains are represented by different colored boxes. The conserved motif for the *S*-adenosine-L-methionine binding are boxed in red, putative substrate-binding residues are boxed in black, and catalytic residues is boxed in blue.

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Melatonin Enhanced the Tolerance of *Arabidopsis thaliana* to High Light Through Improving Anti-oxidative System and Photosynthesis

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Land plants live in a crisis-filled environment and the fluctuation of sunlight intensity often causes damage to photosynthetic apparatus. Phyto-melatonin is an effective bioactive molecule that helps plants to resist various biotic and abiotic stresses. In order to explore the role of melatonin under high light stress, we investigated the effects of melatonin on anti-oxidative system and photosynthesis of *Arabidopsis thaliana* under high light. Results showed that exogenous melatonin increased photosynthetic rate and protected photosynthetic proteins under high light. This was mainly owing to the fact that exogenous melatonin effectively decreased the accumulation of reactive oxygen species and protected integrity of membrane and photosynthetic pigments, and reduced cell death. Taken together, our study promoted more comprehensive understanding in the protective effects of exogenous melatonin under high light.

Keywords: *Arabidopsis*, melatonin, high light, ROS, photosynthetic protein

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HIGHLIGHTS

- Melatonin inhibited the burst of reactive oxygen species by regulating enzymatic and non-enzymatic antioxidant systems under high light.
- Melatonin improved the photosynthesis under high light through maintaining the integrity of photosynthetic apparatus.
- Exogenous melatonin functions partially through improving the accumulation of endogenous melatonin, but it could not completely compensate for the deficiency of endogenous melatonin.

INTRODUCTION

Plants depend on sunlight absolutely as an overall energy source so that they develop multiple protein complexes to accomplish photosynthesis. These protein complexes include Photosystem II (PSII), Photosystem I (PSI), cytochrome b_6f complex, and so on (Jarvis and Lopez-Juez, 2013). When light energy is insufficient, plants capture more light energy through changing

the location of chloroplast (Salgado-Luarte and Gianoli, 2011). When the absorbed light energy of plants exceeds their demand, the photosynthetic complexes will be injured, leading to the decrease of photosynthetic efficiency (Demmig-adams and Adams, 1992). At the same time, reactive oxygen species (ROS) bursts (Jarvis and Lopez-Juez, 2013), and the resultant ROS is toxic to plants (Nishiyama et al., 2001). Although the damage mechanism of photosynthetic apparatus caused by high light is still controversial, it is indisputable that the high light finally leads to injure D1 subunit of PSII (Allakhverdiev and Murata, 2004). The photodamage of D1 occurs at all light intensities, but the photoinhibition occurs only when the balance between the photodamage and repair of D1 is broken (Allakhverdiev and Murata, 2004). Fortunately, plants had established an elaborate protective mechanism, including chloroplast avoidance movement which could minimize light exposure, ROS scavenging systems that are composed of SOD, POD, APX, etc. (Apel and Hirt, 2004), and PSII repair cycle (Kirchhoff, 2014). Although this multi-level photoprotective mechanism helps plants to minimize the injury on the photosynthetic machinery, the damage is unavoidable. Even the damage would affect plant growth and development, resulting in yield reduction and death.

Melatonin (N-acetyl-5-methoxytryptamine), a kind of indoleamine which widely exists in organism, was discovered in plants in 1995 and numerous studies proved that melatonin has involved in multiple processes in plants, including the development of flower (Lee et al., 2019), the architecture of root (Yang et al., 2021), the ripening of fruit (Wang et al., 2020), the senescence of leaf (Wang et al., 2013), the regulation of circadian rhythms, and the protective effect on chlorophyll and photosynthesis (Arnao and Hernandez-Ruiz, 2015). Melatonin alleviated oxidative damage through effectively scavenging ROS and reactive nitrogen species (RNS) (Arnao and Hernandez-Ruiz, 2015). And its metabolites, such as 2-hydroxylmelatonin and N1-acetyl-N2-formyl-5-methoxykynuramine, could also directly and efficiently scavenge ROS (Tan et al., 2007). Besides, melatonin also inspired antioxidant activity by stimulating antioxidant enzymes and could augment the ascorbate-glutathione (AsA-GSH) cycle to scavenge excess ROS (Li et al., 2015). And melatonin helped plants to defend against multiple abiotic stresses, such as cold, heavy metals, salt, drought, and so on (Arnao and Hernandez-Ruiz, 2015). Exogenous melatonin relieved the photoinhibition of tomato seedlings by improving non-photochemical quenching under cold stress (Ding et al., 2017). Similarly, the accumulation of melatonin in water hyacinth under sunlight was significantly higher than that under artificial low-light (Tan et al., 2007). This implies that melatonin can be induced by high light. Supporting these results, the expression of the melatonin-synthesis-related gene *ASMT* in apple had been up-regulated by high light, leading to the accumulation of melatonin (Zheng et al., 2017). In addition, melatonin enhanced the tolerance to high light in *Arabidopsis thaliana* (Lee and Back, 2018). However, the underlying physiological and molecular mechanism of the elevated tolerance to high light by melatonin remains unclear in plants.

Plants need light for photosynthesis and thus gain energy for their growth, but excessively high light does harm to photosynthetic apparatus. There were many researches on the high light stress in plants, but the role of melatonin under high light had been less explored. Lee and Back (2018) found that high light led to the brust of ROS, and the synthesis of melatonin was induced by chloroplastidic singlet oxygen and promoted the accumulation of melatonin. At the same time, melatonin increased the activity of antioxidant enzymes, thus enhancing the tolerance of plants to high light. In addition, Yao et al. (2020) reported the synthesis of melatonin was induced by UV-B. The wavelength of light spectrum also affected the synthesis of melatonin. Afreen et al. (2006) reported that the melatonin concentrations were highest in red-light-exposed plants and followed the blue light and white light. A lot of study showed that high light inhibited photosynthesis, but the role of melatonin in this physiological process is still unclosed.

Based on the reported relationship between melatonin and light intensity, we suggested that melatonin decreased the level of ROS by regulating antioxidant system to protect the photosynthesis under high light. To test this hypothesis, we measured ROS accumulation, membrane lipid peroxide, photosynthetic parameters, antioxidant enzyme, and PSII protein after the melatonin pretreatment under high light. The results demonstrated that melatonin provided effective ROS scavenging ability for plants and preserved the integrity of the photosynthetic protein, and then enhanced the tolerance to high light.

MATERIALS AND METHODS

Plant Materials and Treatments

Arabidopsis thaliana, including wild-type (Col-0) and mutants, were grown in pots filled with the mixture of humus, perlite, and vermiculite at the ratio of 1:1:1 with 60% relative humidity and illumination of 120 μmol m^{-2} s^{-1} for a 16 h (22°C)/8 h (20°C) day/night photoperiod. SALK_032239 (*SNAT-1*) and SALK_020577 (*SNAT-2*) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, United States). Arabidopsis leaves were sprayed 100 μmol/L melatonin (with 0.02% Tween-20) on the 26th day, and sprayed again after 24 h. Then, the seedlings were exposed to high light (1,000 μmol m^{-2} s^{-1}) for 3 h. All experiments were performed in triplicate.

Determination of Chlorophyll and Carotenoid Content

Chlorophyll (Chl) and carotenoid was determined by the previously described method (Lichtenthaler and Wellburn, 1983). Fresh leaves (0.1 g) were cut and homogenized with 5 ml of 80% (v/v) acetone, then centrifuged at 8,000 r min^{-1} for 10 min. The absorbance of the supernatant was recorded with a

Abbreviations: PSII, photosystem II; PSI, photosystem I; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; O_2^-, superoxide anion radicals; EL, electrolyte leakage; MDA, malondialdehyde; SOD, superoxide dismutase; POD, peroxidase; APX, ascorbate peroxidase; GPX, glutathione peroxidase.

spectrophotometer (UV-1750, Shimadzu, Japan) at 663, 646 and 470 nm.

Melatonin Measurement

Extraction of melatonin from *Arabidopsis* was performed as described by Han et al. (2017). The quantification of melatonin was performed with liquid a chromatography (HPLC) system (1290 LC, Agilent, United States) couple to a mass spectrum (MS) system (6470 LC-MS/MS, Agilent, United States) according to Han et al. (2017). Separations were carried out on a 150 × 2.1 mm, 1.8 μm, Eldath RS-C18 column. Solvent A was methanol, and solvent B was methanol with 0.1% formic acid, v/v. The injection volume was 1 μL, and solvent A was from 20 to 80% at a flow rate of 0.3 mL min^{-1}. Mass spectrum parameters were as follows: positive ion mode; turbo 1 speed, 100%; turbo 2 speed, 100%; sheath gas temperature, 300°C; sheath gas flow, 11.0 L min^{-1}; capillary current, 59 nA; capillary, 3,368 V; MS 1 heater, 100°C; MS 2 heater, 100°C; rough vac, 9.91E-1 Torr; high vac, 3.60E-5 Torr; and m/z, 159.0.

Measurement of Photosynthetic Characteristics and Chlorophyll Fluorescence

The photosynthetic rate (P_n) and stomatal conductance (g_s) of leaves was measured with a potable photosynthesis system (GSF-3000, Heinz-Walz Instruments, Effeltrich, Germany). Intact leaves were measured at a temperature of 22°C, the light intensity of 120 μmol m^{-2} s^{-1} and 1,000 μmol m^{-2} s^{-1}, photosynthetically active radiation (PAR) of 750 μmol m^{-2} s^{-1}, the relative humidity of 65% (Huang et al., 2019).

Chlorophyll fluorescence was imaged with a modulated imaging fluorometer (the Imaging PAM M-Series Chlorophyll Fluorescence System, Heinz Walz Instruments, Effeltrich, Germany). The maximum efficiency of PSII photochemistry (Fv/Fm) and non-photochemical quenching (NPQ) was imaged and calculated after adaption in the dark for 30 min (Huang et al., 2019).

Determination of H_2O_2 and O_2^-

Histochemical detection of ROS was conducted as described by Han et al. (2017) Briefly, hydrogen peroxide (H_2O_2) and superoxide anion radicals (O_2^-) were visually detected with 0.5 mg/ml 3,3′-diaminobenzidine (DAB) and 1 mg/ml nitro blue tetrazolium (NBT), respectively. Then, the tissues were decolorized for 2 h in boiling ethanol (85%). The quantification of H_2O_2 and O_2^- was determined as described by Han et al. (2017).

Determination of EL and MDA

Electrolyte leakage (EL) of leaves was measured with a conductivity meter (DDS-309+, Chengdu, China) as described by Han et al. (2017) The relative EL was obtained according to the ratio of the initial conductivity to the absolute conductivity. The degree of membrane lipid peroxidation in leaves was estimated by malondialdehyde (MDA) content. MDA was evaluated using thiobarbituric acid assay (Han et al., 2017).

Trypan Blue Staining

The method of trypan blue dyeing according to Liang et al. (2015). Leaves were detached and stained with lactophenol-trypan blue solution (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 10 mg of trypan blue, dissolved in 10 ml of distilled water) at 70°C for 1 h and then boiled for approximately 5 min and left staining overnight. After destaining in chloral hydrate solution (2.5 g of chloral hydrate dissolved in 1 ml of distilled water) for 3 days to reduce background staining, samples were equilibrated with 70% glycerol for scanning.

Assay of Antioxidant Enzymes and Non-enzymatic Antioxidant

For determination of SOD, POD, APX and GPX activities, the sample (0.5 g) was homogenized in 5 ml pre-cooled extract solution (50 mm potassium phosphate buffer, pH 7.8). The homogenate was centrifuged for 20 min at 12,000 r min^{-1} at 4°C, and the supernatant was used for further analysis.

The supernatant was used for assays of specific enzymatic activities. The activity of SOD (EC 1.15.1.1) was assessed according to Han et al. (2017) by measuring its ability to inhibit the photochemical reduction of NBT. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of NBT reduction. The activities of antioxidant enzymes, namely peroxidase (POD, EC 1.11.1.7), glutathione peroxidase (GPX, EC 1.11.1.9) and ascorbate peroxidase (APX, EC 1.11.1.11), were assayed following the methods of Huang et al. (2019).

The antioxidants including reduced ascorbic acid (AsA), dehydroascorbate (DHA), reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined with the enzymatic cycling assay method (Han et al., 2017). For GSH, 0.5 g sample was extracted in an ice bath with 5 ml 100 mm potassium phosphate buffer (pH 7.5) containing 5 mm EDTA. After centrifugation, 2 ml supernatant was mixed with 1 ml 100 mm phosphate buffer (pH 7.5) and 0.5 ml 4 mm DTNB (5,5′-dithio-bisnitrobenzoic acid). The reaction mixture was incubated at 25°C for 10 min, and the absorbance at 412 nm was measured. For the GSSG assay, the GSH in the supernatant was cleared first, and GSSG content was quantified as described by Han et al. (2017). The GSH and GSSG content was calculated according to their standard curves and expressed as μmol g^{-1}(FW).

Thylakoid Protein Analysis

Thylakoid membrane protein was isolated as described by Fristedt et al. (2010). Western blotting was performed according to Chen et al. (2009). The first antibody was PSII D1, D2, CP43, PsbS, Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6, and PSI PsaD, Lhca1, Lhca2, Lhca3 polyclonal antibody (Agrisera, Umea, Sweden), and the second antibody was goat anti-rabbit-HR (horseradish peroxidase; Agrisera, Umea, Sweden).

Data Analysis

All experiments were repeated at least three times, and all data are presented as mean ± standard deviation. Statistical analysis was done with IBM SPSS Statistics 20.0 software (IBM Corp., Armonk, NY, United States). Asterisks indicate significantly different values at $^*p < 0.05$.

RESULT

Application of Exogenous Melatonin Enhanced the Accumulation of Melatonin in Leaf Tissue

After melatonin pretreatment, the level of melatonin in wild type increased 111.25%, and that in *snat-1* and *snat-2* increased 59.32 and 57.75%, respectively (**Figure 1**). Exogenous melatonin increased the content of melatonin in wild-type chloroplasts, but had no significant effect on the mutants (*snat-1*, *snat-2*). In addition, high light increased the level of melatonin in the wild type, but not in *snat-1* and *snat-2*. The application of melatonin further increase the level of melatonin in leaf tissue and chloroplast under high light. The above results suggested that exogenous melatonin could increase the content of melatonin by absorption and transport, and also might promote the synthesis of melatonin. Furthermore, high light could promote the synthesis of melatonin.

Melatonin Protected Photosynthetic Pigments Under High Light

High light caused *Arabidopsis* leaves curling and chlorosis while melatonin pretreatment alleviated this symptom to a certain extent (**Supplementary Figure S1A**). But the application of melatonin had no effect on the fresh weight and dry weight of *seedlings* (**Supplementary Figures S1B,C**). The level of chlorophyll and carotenoid significantly decreased under high light, but this situation was significantly ameliorated after melatonin pretreatment (**Figure 2**). But the level of chlorophyll and carotenoid in *snat-1* and *snat-2* was still lower than that in the wild type after melatonin pretreatment under high light. This indicated that the lack of endogenous melatonin could influence the rescue of chlorophyll and carotenoid by exogenous melatonin under high light.

Melatonin Protected Photosynthesis Under High Light

Under growth light, exogenous melatonin had little influence on photosynthesis. The Fv/Fm of *snat-1* and *snat-2* decreased more than WT under high light, but they all recovered after melatonin pretreatment (**Figure 3A**). NPQ significantly increased after 3 h high light, and the NPQ of the *snat-1* and *snat-2* were higher than WT (**Figure 3B**). High light significantly decreased P_n and g_s in both WT and mutants, and mutants showed a larger drop (**Figures 3C,D**). Exogenous melatonin increased P_n under high light, but showed no effects on g_s. These results showed that high light could cause obvious damage to the photosynthesis and reduce photosynthetic efficiency, but melatonin could reverse this trend.

Melatonin Decreased the Level of ROS and Reduced the Damage to Cell

H_2O_2 and O_2^- are two major ROS which produced in chloroplasts under high light and caused oxidative damage to the photosystem. The histochemical staining and quantitative analysis showed high light promoted the production of H_2O_2 and O_2^-, and exogenous melatonin decreased their accumulation (**Figure 4**). The content of ROS in the chloroplast showed the same trend as that in leaves (**Supplementary Figure S2**). These results showed that high light caused the brust of ROS but exogenous melatonin relieved this dilemma.

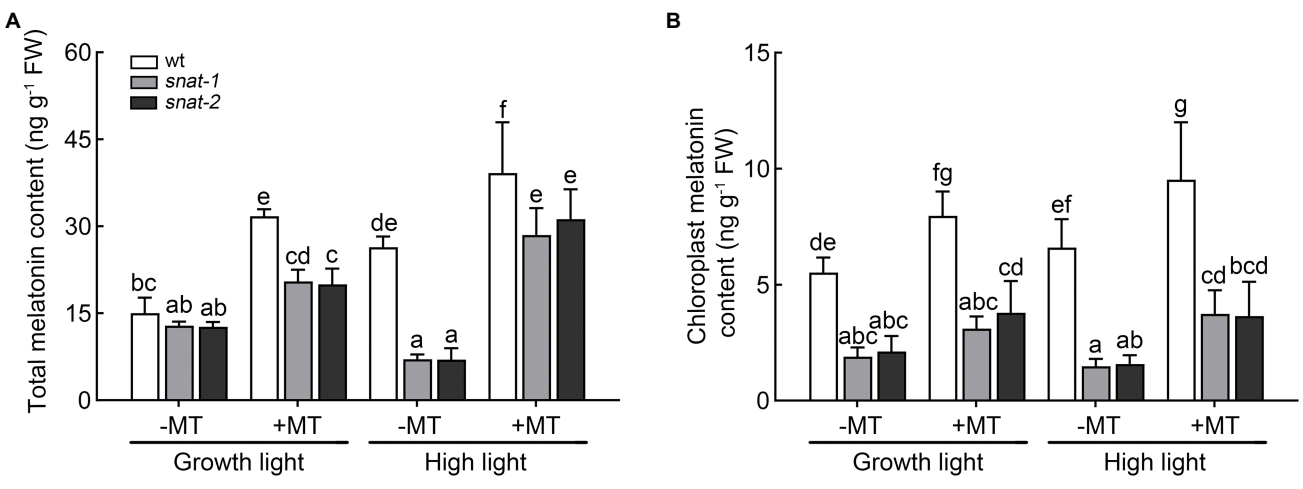

FIGURE 1 | Effects of exogenous melatonin on the content of melatonin. **(A)** The melatonin content in leaves. **(B)** The melatonin content in chloroplasts. Data represent means ± SDs of three replicate samples. Different letters denote significant differences (Tukey's HSD *post hoc* test; $p < 0.05$).

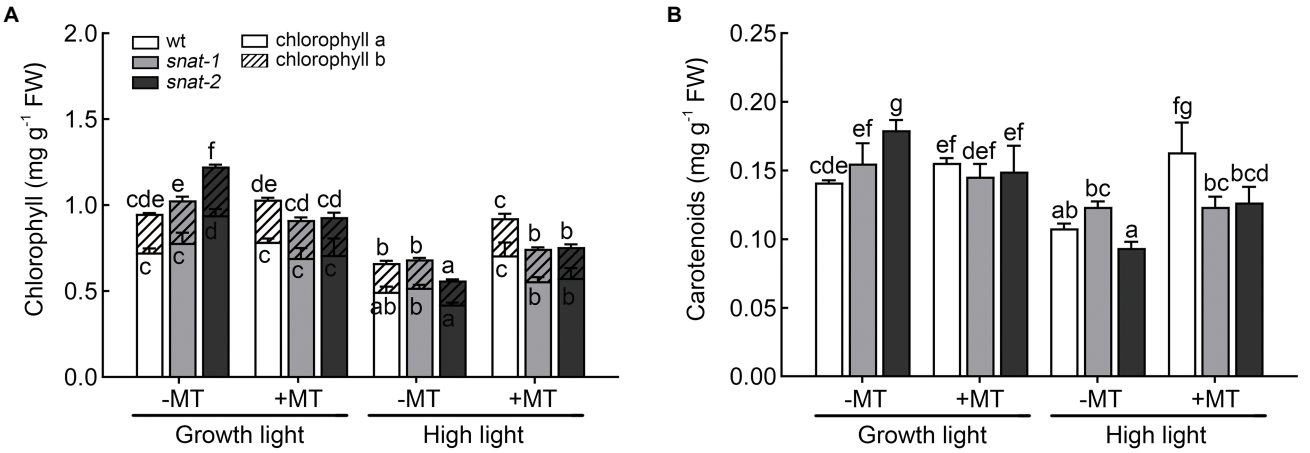

FIGURE 2 | Effects of exogenous melatonin on photosynthetic pigments. **(A)** The chlorophyll level. **(B)** The carotenoid level. Others are the same as in **Figure 1**.

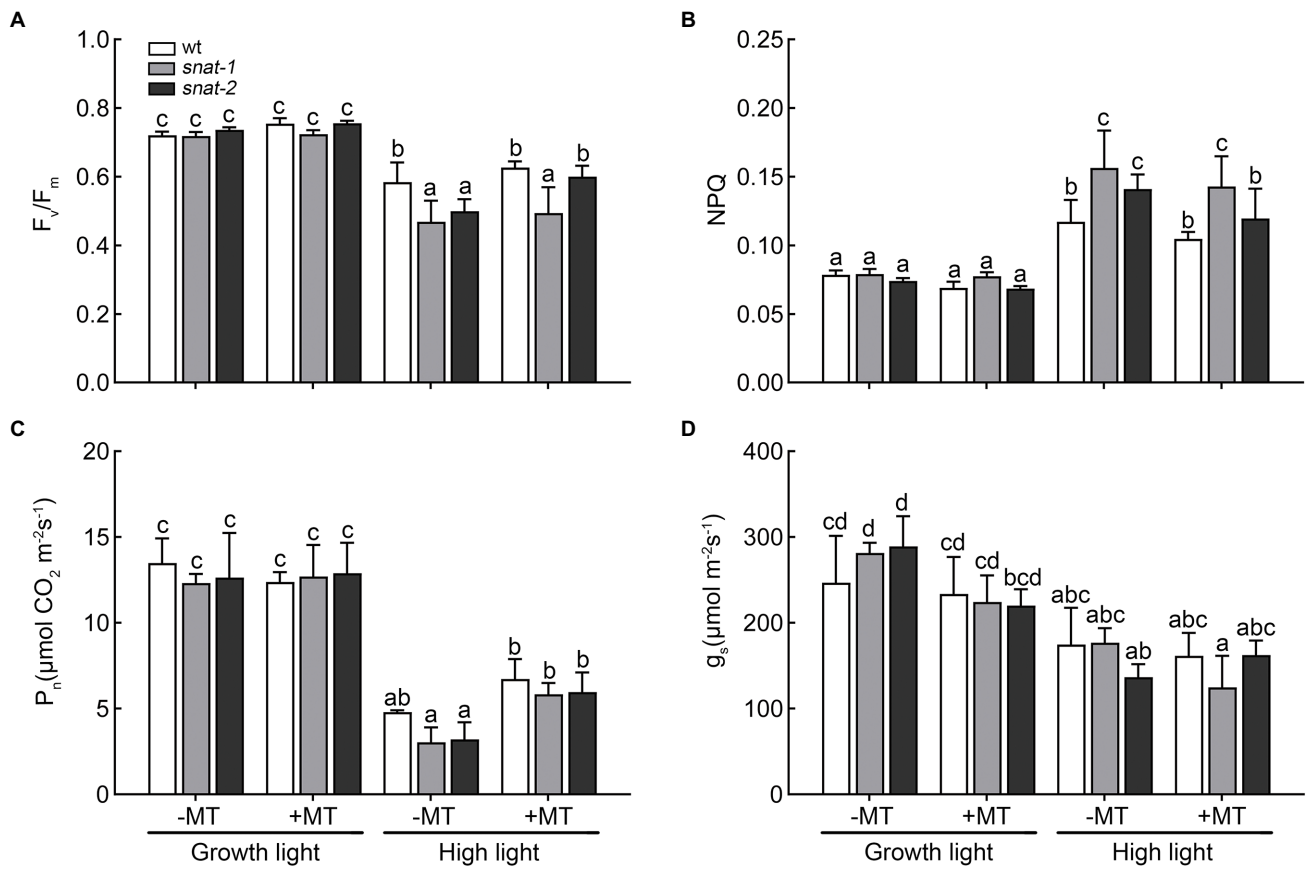

FIGURE 3 | Effects of exogenous melatonin on photosynthesis. **(A)** Maximum PSII quantum yield (F_v/F_m). **(B)** Non-photochemical quenching (NPQ). **(C)** Net photosynthetic rate (P_n). **(D)** Stomatal conductance (g_s). Others are the same as in **Figure 1**.

The levels of EL and MDA increased significantly under high light. Exogenous melatonin lessened the increase of EL and MDA, and the alleviation role in *snat-1* and *snat-2* was weaker than that in WT (**Figures 5A,B**).

Cell death enhanced under high light, and it was even worse in *snat-1* and *snat-2*. Melatonin pretreatment reduced the level of cell death under high light, but it was still more serious in *snat-1* and *snat-2* (**Figure 6**).

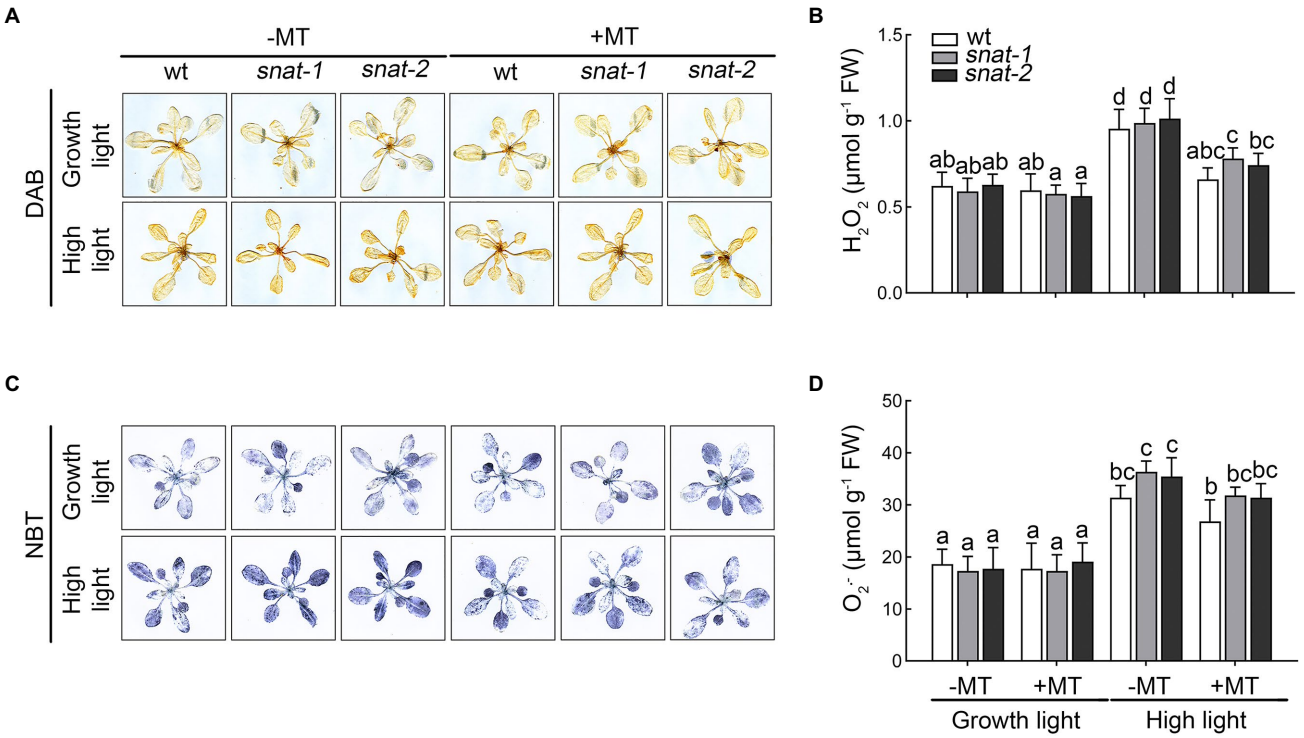

FIGURE 4 | Effects of exogenous melatonin on reactive oxygen species. **(A)** Accumulation of hydrogen peroxide (H_2O_2) was visualized by 3,3′-diaminobenzidine (DAB) staining. **(B)** The content of hydrogen peroxide (H_2O_2). **(C)** Accumulation of superoxide anion radicals ($O_2^{\cdot-}$) was visualized by nitrotetrazolium blue (NBT) staining. **(D)** The content of superoxide anion radicals ($O_2^{\cdot-}$).

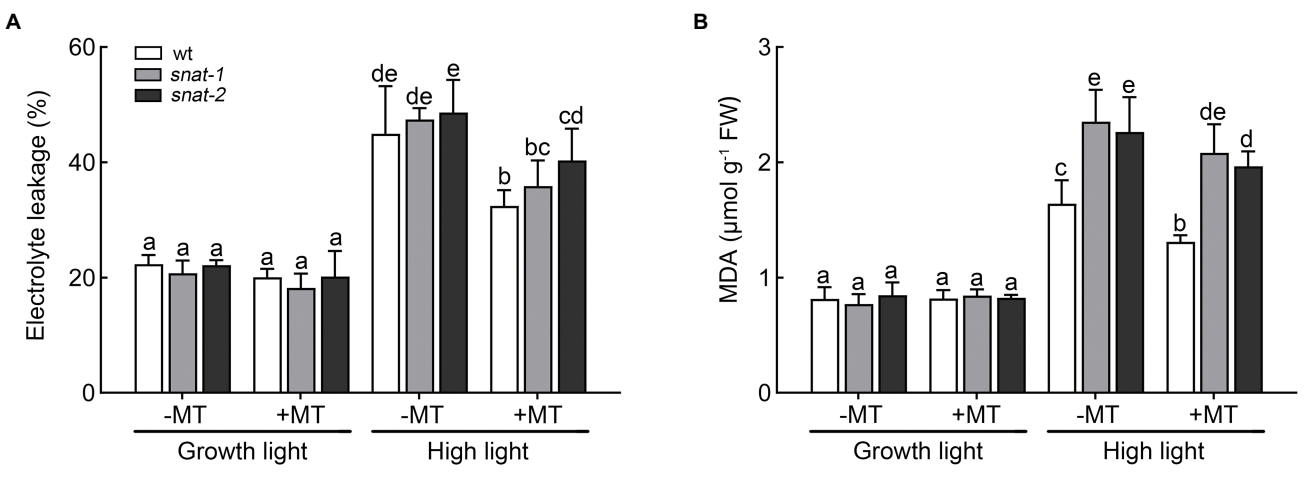

FIGURE 5 | Effects of exogenous melatonin on the damage of cellular membranes. **(A)** Electrolyte leakage (EL). **(B)** Malondialdehyde (MDA). Others are the same as in **Figure 1**.

These results showed that exogenous melatonin could alleviate the damage of cell membrane and inhibit cell death under high light. It is worth noting that exogenous melatonin could not completely compensate for the deficiency of endogenous melatonin.

Melatonin Promoted the Antioxidant Ability in Plant Under High Light

The content of soluble sugar and proline increased under high light, and exogenous melatonin could downregulate the level of soluble sugar and proline (**Supplementary Figure S3**).

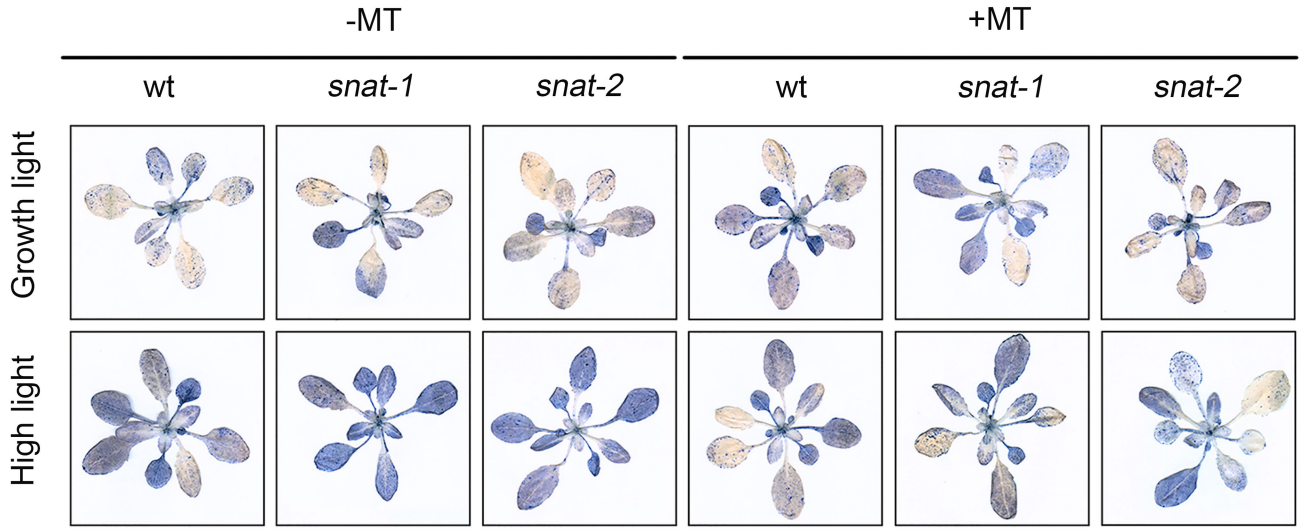

FIGURE 6 | Effects of exogenous melatonin on cell death. Trypan-blue staining of the Col-0 and mutants (*snat-1*, *snat-2*) under the growth light and high light.

High light increased the content of AsA and GSH in WT, but there was no obvious effect on that of mutants (**Table 1**). Exogenous melatonin increased the content of AsA and GSH in *snat-1* and *snat-2*, but not in WT under high light. High light decreased the ratio of AsA/DHA, and the ratio in WT was higher than that of *snat-1* and *snat-2*. Exogenous melatonin reversed this trend.

Different antioxidant enzyme showed different response to high light and melatonin (**Figure 7**). The activities of POD, APX and GPX increased but SOD activity decreased under high light. Exogenous melatonin enhanced the activities of SOD, APX, and GPX but it decreased POD activity under high light.

On the one hand, melatonin directly removed ROS as a scavenger. On the other hand, it also regulated the level of non-enzymatic antioxidant and the activity of antioxidant enzymes. Therefore, melatonin works as a key regulator between antioxidants and ROS and contributes to the homeostasis of them.

Exogenous Melatonin Protected Photosystem Protein Under High Light

Under growth light, the content of PSII proteins except Lhcb1 in *snat-1* and *snat-2* was lower than that in WT (**Figure 8; Supplementary Figures 4, 5**). Exogenous melatonin decreased the content of PSII proteins in WT and the content of PSII core proteins and Lhcb1 in *snat-1* and *snat-2*. High light decreased the content of PSII proteins in WT, and the content of PSII proteins except Lhcb2, Lhcb3, Lhcb4 in *snat-1* and *snat-2* also reduced. However, exogenous melatonin increased the content of PSII proteins except Lhcb6 in WT and the level of PSII proteins except Lhcb1 in *snat-1* and *snat-2* under high light.

Under growth light, the content of PSI proteins in *snat-1* and *snat-2* was lower than that of WT (**Figure 8; Supplementary Figure 6**). Exogenous melatonin decreased the level of PsaD, Lhca2 and Lhca3 in WT, and lowered the content of PsaD in *snat-1* and *snat-2*. High light decreased the content of PSI proteins in WT and *snat-1*. Exogenous melatonin improved the level of Lhca1, Lhca2 and Lhca3 under high light.

These results showed that melatonin reduced the level of photosystem proteins under growth light but it could alleviate the damage of photosystem proteins caused by high light.

DISCUSSION

Light is a source of energy and signal for plant growth. Plant has to go through a dark - low light - high light - low light - dark cycle every day. High light often causes destruction of the photosynthetic system and even cell death. Melatonin is a multitasking biomolecule, and it is involved in numerous physiological processes in plants, including redox reactions, biosynthesis, circadian clock, and stress defenses (Arnao and Hernandez-Ruiz, 2015). According to recent research, high light seriously destroyed the photosynthetic structure of chloroplasts and weakened its photosynthesis, and finally inhibited the growth of plants (Ding et al., 2017). In the present study, high light caused ROS burst and the reduction of photosynthesis. However, the application of exogenous melatonin significantly alleviated the damage caused by high light. Also, stress triggered endogenous melatonin response. Melatonin is effective in striving against stress, but the reception of stress signal, the activation of endogenous melatonin biosynthesis and the action process of melatonin were all restricted by many factors. At the cellular level, a stress signal is firstly received by the cell membrane, and then transferred to the nucleus. These starts to activate the melatonin biosynthesis pathway in mitochondria and chloroplasts by upregulating the melatonin-biosynthesis genes (Moustafa-Farag et al., 2020). Melatonin is effective in striving against stress,

TABLE 1 | Effects of exogenous melatonin on non-enzymatic antioxidant.

		Growth light		High light	
		−MT	+MT	−MT	+MT
AsA	Col-0	2.130 ± 0.059^{ab}	2.145 ± 0.057^{ab}	2.469 ± 0.069^{c}	2.451 ± 0.115^{c}
	snat-1	2.234 ± 0.246^{abc}	2.101 ± 0.094^{a}	2.134 ± 0.069^{ab}	2.445 ± 0.171^{c}
	snat-2	2.263 ± 0.162^{abc}	2.081 ± 0.060^{a}	2.105 ± 0.062^{a}	2.371 ± 0.222^{bc}
DHA	Col-0	0.680 ± 0.062^{a}	0.661 ± 0.071^{a}	0.971 ± 0.033^{bc}	0.917 ± 0.036^{c}
	snat-1	0.736 ± 0.071^{a}	0.663 ± 0.071^{a}	1.101 ± 0.051^{bc}	0.980 ± 0.116^{bc}
	snat-2	0.685 ± 0.151^{a}	0.647 ± 0.060^{a}	0.969 ± 0.041^{b}	1.000 ± 0.047^{bc}
GSH	Col-0	0.454 ± 0.029^{a}	0.458 ± 0.025^{a}	0.556 ± 0.023^{b}	0.564 ± 0.029^{b}
	snat-1	0.429 ± 0.025^{a}	0.455 ± 0.010^{a}	0.460 ± 0.017^{a}	0.521 ± 0.031^{b}
	snat-2	0.453 ± 0.028^{a}	0.450 ± 0.019^{a}	0.463 ± 0.019^{a}	0.551 ± 0.043^{b}
GSSG	Col-0	0.146 ± 0.014^{a}	0.144 ± 0.017^{a}	0.245 ± 0.091^{c}	0.184 ± 0.056^{abc}
	snat-1	0.162 ± 0.017^{ab}	0.161 ± 0.028^{ab}	0.228 ± 0.036^{bc}	0.240 ± 0.052^{c}
	snat-2	0.154 ± 0.013^{ab}	0.158 ± 0.040^{ab}	0.218 ± 0.024^{abc}	0.196 ± 0.017^{abc}

Different letters denote significant differences (Tukey's HSD post hoc test; $p < 0.05$).

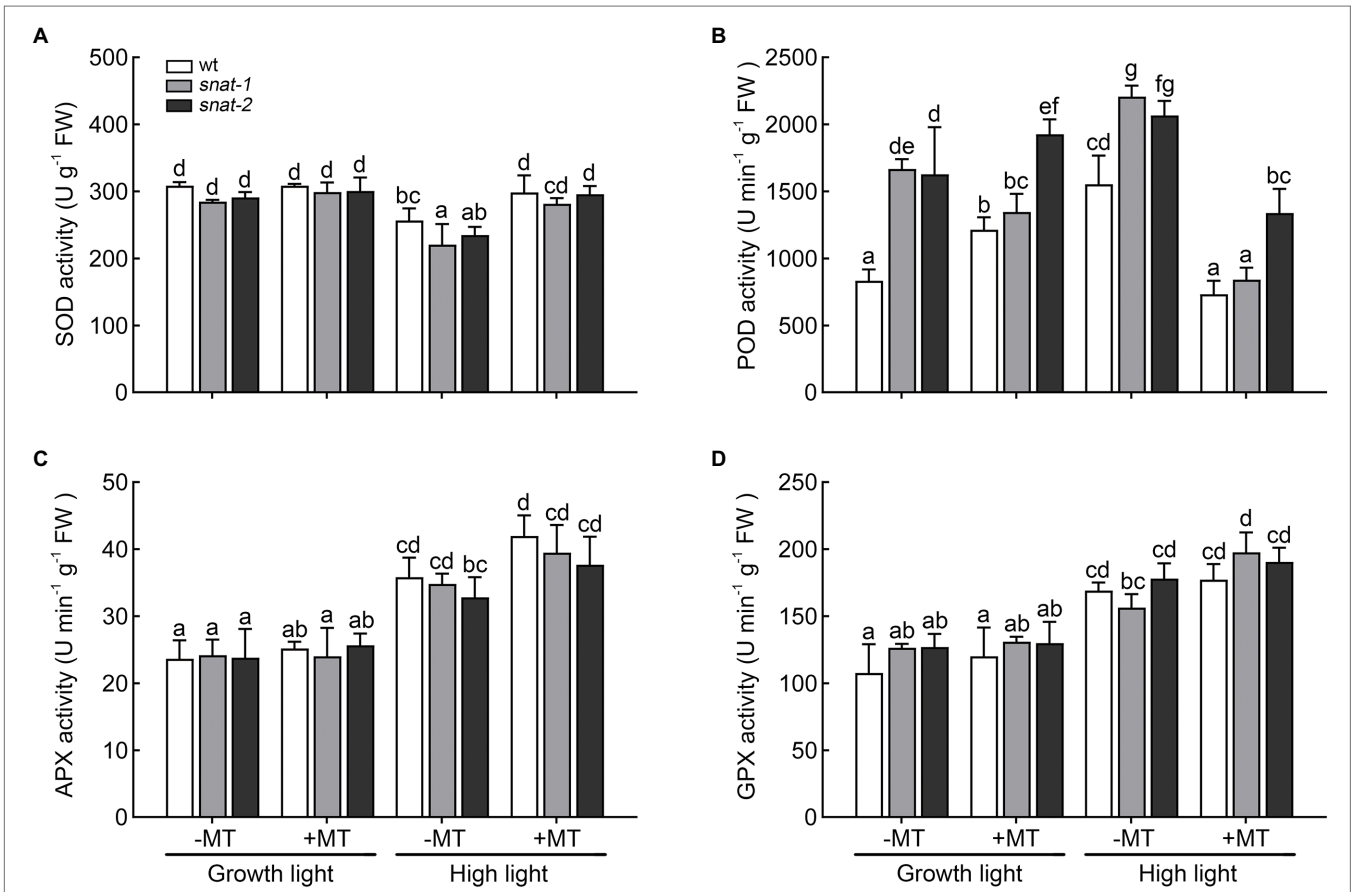

FIGURE 7 | Effects of exogenous melatonin on the activity of antioxidant enzyme. **(A)** Superoxide dismutase (SOD) activity. **(B)** Peroxidase (POD) activity. **(C)** Ascorbate peroxidase (APX) activity. **(D)** Glutathione peroxidase (GPX) activity. Others are the same as in **Figure 1**.

but the reception of stress signal, the activation of endogenous melatonin biosynthesis and the action process of melatonin were all restricted by many factors. In addition to ROS brust caused by high light to promote endogenous melatonin response, it seems that there are other pathways to promote the synthesis of melatonin. Transcription factors like MYB, bHLH, bZIP, ERF, NAC, and WRKY are major players in stress signaling and some constitute major hubs in the signaling webs (Tripathi et al., 2014). Hu et al. (2019) reported that high light up-regulated constantly the expression of 29 transcriptional factors, which

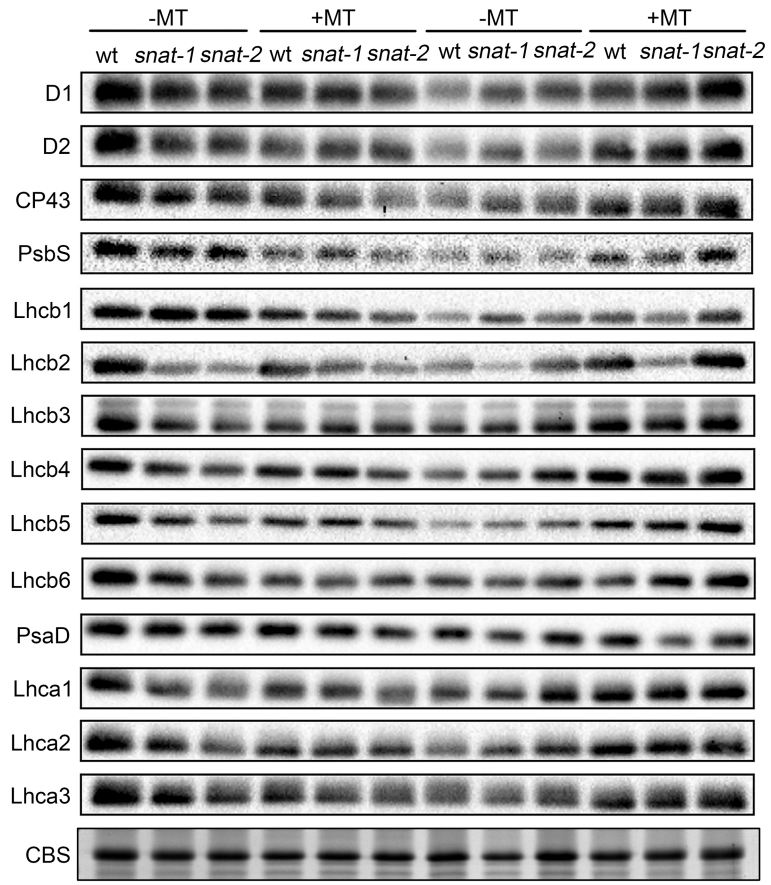

FIGURE 8 | Effects of exogenous melatonin on thylakoid membrane proteins. Immunoblotting was performed with antibodies against D1, D2, CP43, PsbS, Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6, PsaD, Lhca1, Lhca2 and Lhca3. SDS-PAGE of thylakoid proteins stained with coomassie brilliant blue (CBS) worked as loading control.

could regulate the expression of genes associated with photosynthesis and ROS scavenging-related genes. Zhao et al. (2010) found that UV-B radiation induced *OsWRKY89* to participate in light responses. Transcription factor regulatory networks are also involved in the regulation of melatonin synthesis. Wei et al. (2017) reported that MeWRKY79 and MeHsf20 of cassava could act with W-box and thermal stress element HSEs (Heat-stress elements) in the promoter of *MeASMT* to induce its expression. And previous research found one cysteine2/histidine2-type zinc finger transcription factor, ZAT6, was involved in melatonin-mediated stress response in *Arabidopsis* (Shi and Chan, 2014). Maybe melatonin participated in light response through some of these transcription factors. In addition, transcription factor could directly affect the expression of melatonin synthesis gene, and also indirectly affect the effect on antioxidant system.

Reactive oxygen species is the byproducts of photosynthesis, respiration, and other normal metabolism, and it plays an important role in the resistance and tolerance to stress (Chen et al., 2016). Excessive ROS was toxic to cells and organisms, and involved in the programmed response to abiotic stress (Manchester et al., 2015). Yuan et al. (2020) found that high light triggered the accumulation of ROS. Han et al. (2017) found that exogenous melatonin decreased the level of ROS under cold stress. In the present study, H_2O_2 and O_2^- increased significantly under high light, and they decreased with melatonin pretreatment.

Excessive ROS could stimulate membrane lipid peroxidation, and then led to the damage of cell membrane, loss of cellular integrity, and cell death (Chen et al., 2018). MDA and EL are regarded as important indicators of oxidative damage and previous study indicated that melatonin decreased the high level of EL and MDA caused by water stress (Zhang et al., 2013). Consistently, our research suggested that exogenous melatonin decreased the level of EL and MDA and reduced cell death under high light. It is worthwhile to note that the situation of *snat-1* and *snat-2* was worse than WT under high light. Exogenous melatonin application maintained a relatively low level of ROS and reduced the degree of cell damage, further conferring plant resistance to high light.

Enzymatic antioxidant system and non-enzymatic antioxidant system were evolved in response to oxidative stress in plants (Apel and Hirt, 2004). Melatonin alleviated oxidative damage caused by salinity, drought and cold perhaps by directly

enhancing antioxidative enzyme activities, like SOD, POD and APX (Apel and Hirt, 2004). Chen et al. (2018) also found that the application of melatonin increased the activities of antioxidant enzymes in maize seedlings under salt stress. In the present research, melatonin increased the activity of SOD under high light. Exogenous melatonin downregulated the activity of POD and upregulated the activity of APX and GPX. They are all the converter for H_2O_2 but work in different ways. Melatonin inhibited the pathway of POD but promoted the pathway of APX and GPX to scavenge H_2O_2. Previous works showed that exogenous melatonin resulted in higher content of AsA and GSH under salt stress (Chen et al., 2018). But our study suggested that exogenous melatonin had little effect on them and the lack of endogenous melatonin weakened their levels. AsA-GSH cycle is a vital antioxidant system against oxidative stress in plants (Zhang et al., 2015). APX and GPX are the key enzymes of the glutathione ascorbic acid cycle, and melatonin effectively increased their activity. The glutathione-ascorbic acid cycle might play a key role in alleviating the high light stress. Melatonin did not only remove ROS as a scavenger but also regulated the activity of antioxidants in plants. Our results showed that melatonin reduced the accumulation of ROS but decreased the activity of POD. So melatonin was not overkill to ROS. In addition, Li et al. (2020) found that low concentration of melatonin induced the production of ROS and ROS worked as a key signal in many physiological processes. Maybe melatonin not only improves the defense capabilities of plant, but also ensures the role of ROS as a message transmitter in stress depending on its regulation role to the antioxidant system.

Photosynthetic pigments are susceptible to environmental stress. Melatonin effectively alleviated the degradation of chlorophyll and carotenoid under stress and made it with a certain level. Wu et al. (2021) found that melatonin suppressed the activities of chlorophyll catabolic enzymes such as chlorophyllase (CLH), pheophytinase (PPH), pheophorbide a oxygenase (PAO) and down-regulated the expressions of *BoNYC1*, *BoNOL*, *BoCLH*, *BoPPH*, *BoPAO*, *BoRCCR* and *BoSGR1* which involved in chlorophyll catabolism. In addition, Jahan et al. (2020) found melatonin upregulated the expression of chlorophyll synthesis genes, i.e., POR, CAO, CHL G.

The decrease of photosynthesis efficiency in plants after being exposed to adverse environmental might be a key reason for the reduction of crop. The previous studies showed abiotic stress induced irreversible damage to PSII in tomato, oat seeds, *Ligustrum vicaryi* and maize seedlings thereby decreasing photosynthetic rate (Ding et al., 2017; Chen et al., 2018; Alyammahi and Gururani, 2020; Kanwar et al., 2020; Yuan et al., 2020). The decrease of stomatal conductance could result in a declined P_n and reduced assimilation products, thus causing an inhibited growth and a lower yield (Rao and Chaitanya, 2016). In this research, the P_n and g_s reduced under high light, and exogenous melatonin increased P_n. These findings were in line with the report of maize under salt stress (Chen et al., 2018). Hu et al. (2021) suggested that the reduction of Chl a may be one of the reasons for the decrease of P_n in acid rain stressed barley plants. And this was similar with our results (**Figures 2A, 3C**). The increase of P_n by melatonin under high light might be due to its protective effect on chlorophyll. The g_s was mainly controlled by guard cell through regulating the opening and closing of stomata (Assmann, 1999; Vavasseur and Raghavendra, 2005). Erland et al. (2019) employed a novel technique, quantum dot nanoparticles, to visualize the location of melatonin and found melatonin-QD aggregated in guard cells. It is possible that melatonin exerted an effect on g_s through this pathway.

In nature, as soon as there is light, it will cause photooxidative damage to photosynthetic apparatus and then photoinhibition is unavoidable (Allakhverdiev and Murata, 2004). The extent of photoinhibition depends on the balance between photodamage and the repairing cycle (Allakhverdiev and Murata, 2004). Melatonin had been found to protect PSII proteins from oxidative injuries (Han et al., 2017; Huang et al., 2019). In previous work, the protective role of melatonin was confirmed on photosynthetic proteins in maize and tomato under drought and high light stress (Ding et al., 2017; Huang et al., 2019). Among ROS, H_2O_2 in chloroplast is an important inhibitor of the Calvin cycle. It might inhibit the activities of enzymes possessing sulfhydryl groups and reduced the photosynthetic CO_2 assimilation (Hancock et al., 2005). In addition, the photooxidative damage products (especially H_2O_2) firstly stimulated the apparent photoinhibition of PSII by inhibiting the repair of PSII instead of accelerating photodamage to PSII (Nishiyama et al., 2001). In present study, we found that the change in the content of H_2O_2 in the chloroplast showed that the photosynthetic system was suffering from huge oxidative pressure, the proteins of the photosystem were destroyed under high light, and this situation was relieved by exogenous melatonin. Therefore, these results suggested that melatonin significantly inhibited ROS burst under high light. Numerous works had indicated that D1 protein is the key target under environmental stress and the D1 protein remained a relatively high level with melatonin pretreatment in our study.

Taken together, our research evaluated the effect and mechanism of melatonin on *Arabidopsis* under high light. Melatonin effectively protected photosynthesis in response to high light. Melatonin mainly worked through two aspects. On the one hand, melatonin was involved in cellular REDOX regulation. Melatonin directly removed ROS as antioxidants (Zhao et al., 2021). At the same time, melatonin regulated the activity of antioxidant enzyme as a signal molecule (Zhao et al., 2021). Therefore, melatonin protected photosynthetic pigments and proteins through redox homeostasis, and contributed to photosynthesis. On the other hand, melatonin gathered in guard cells (Erland et al., 2019), and might participate in stomatal movement. Simultaneously, the role of endogenous melatonin in plants was indispensable for the responses of plants to stress.

Our findings provided the evidence for melatonin to relieve high light stress, and extended new uses for melatonin as a plant growth regulator. At the same time, endogenous melatonin played an important role to against stress, and its potential mechanism needs further study. Our results and other reports

suggested that melatonin might also be involved in stomatal movement (Assmann, 1999; Vavasseur and Raghavendra, 2005; Erland et al., 2019), but the mechanism is still unclear. Given the key role of melatonin in tolerance against various abiotic stresses, it is of interest to explore the mechanism of melatonin in plant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MY designed the experiments. S-JY, BH, Y-QZ, DH, TC, and C-BD performed the experiments and data analysis. S-JY and MY wrote the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.752584/full#supplementary-material

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Functional Characterization of Serotonin N-Acetyltransferase Genes (*SNAT1/2*) in Melatonin Biosynthesis of *Hypericum perforatum*

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Hypericum perforatum is a traditional medicinal plant that contains various secondary metabolites. As an active component in *H. perforatum*, melatonin plays important role in plant antioxidation, growth, and photoperiod regulation. Serotonin N-acetyltransferase (SNAT) is the key enzyme involved in the last or penultimate step of phytomelatonin biosynthesis. A total of 48 members of SNAT family were screened and analyzed based on the whole genome data of *H. perforatum*, and two *SNAT* genes (*HpSNAT1* and *HpSNAT2*) were functionally verified to be involved in the biosynthesis of melatonin. It was found that *HpSNAT1* and *HpSNAT2* were highly expressed in the leaves and showed obvious responses to high salt and drought treatment. Subcellular localization analysis indicated that these two proteins were both localized in the chloroplasts by the *Arabidopsis* protoplasts transient transfection. Overexpression of *HpSNAT1* and *HpSNAT2* in *Arabidopsis* (SNAT) and *H. perforatum* (wild-type) resulted in melatonin content 1.9–2.2-fold and 2.5–4.2-fold higher than that in control groups, respectively. Meanwhile, *SNAT*-overexpressing *Arabidopsis* plants showed a stronger ability of root growth and scavenging endogenous reactive oxygen species. In this study, the complete transgenic plants of *H. perforatum* were obtained through *Agrobacterium*-mediated genetic transformation for the first time, which laid a significant foundation for further research on the function of key genes in *H. perforatum*.

Keywords: ***Hypericum perforatum***, ***Arabidopsis thaliana***, **melatonin, SNAT gene family, serotonin N-acetyltransferase, hairy root**

INTRODUCTION

Hypericum perforatum, commonly known as St. John's wort (SJW), is an important medicinal plant used for centuries. Extracts of *H. perforatum* are widely used to treat anxiety, depression, sciatica, or even wounds because of their high efficiency, safety, and non-toxic side effects (Barnes et al., 2001). As an important bioactive component in *H. perforatum*, melatonin (N-acetyl-5-methoxytryptamine) is a ubiquitous multifunctional substance acting as a free radical scavenger,

circadian rhythm regulator, and immune regulatory molecule (Galano et al., 2011; Manchester et al., 2015; Ma et al., 2020). It can induce antidepressant effects (Detanico et al., 2009), regulate some nerve regeneration processes (Atik et al., 2011), and protect organisms from environmental and internal oxidative stress (Galano and Reiter, 2018). Since discovering of melatonin in plants in 1995, many pharmacological studies have been carried out to explore its functional and physiological significance (Hattori et al., 1995; Tan et al., 2012; Hardeland, 2016). It has been reported that melatonin can not only regulate the whole growth and development stage of plants, from promoting seed germination to delaying leaf senescence; but also enhance the tolerance of plants to abiotic and biotic stresses, such as drought and salt stress, osmotic stress, extreme temperature, senescence, and pathogen attacks (Lee et al., 2014; Li et al., 2015; Liang et al., 2015; Han et al., 2017).

Melatonin is an indole heterocyclic compound synthesized from tryptophan which was confirmed by ^{14}C-tryptophan isotope tracing experiment in *H. perforatum* (Murch et al., 2000). The biosynthesis of melatonin needs at least six enzymes after four consecutive enzymatic reactions, such as tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), tryptophan hydroxylase (TPH), serotonin N-acetyltransferase (SNAT), N-acetylserotonin O-methyltransferase (ASMT), and caffeic acid O-methyltransferase (COMT), among which SNAT is the penultimate enzyme or the last step enzyme involved in the melatonin biosynthesis. SNAT catalyzes 5-hydroxytryptamine (serotonin, 5-HT) to N-acetyl-5-hydroxytryptamine (NAS) or 5-methoxytryptamine (5-MT) to melatonin, and the subcellular localization analyses of SNAT in different plants was almost located in chloroplasts (Lee et al., 2015a; Byeon et al., 2016). Since the first plant SNAT gene was identified and cloned in rice, it was found that the genes belong to GCN5 related N-acetyltransferase (GNAT) superfamily playing a critical role in regulating the accumulation of melatonin and were located in chloroplasts (Byeon et al., 2016; Lee and Back, 2017). Subsequently, Lee et al. (2015b) cloned *AtSNAT* from *Arabidopsis* and the results showed that the melatonin content of the gene knockout mutant decreased significantly and was more susceptible to *Pseudomonas syringae* infection (Byeon et al., 2015a). Wang et al. overexpressed apple *MzSNAT5* in *Arabidopsis* wild-type plants, which increased the content of melatonin in *Arabidopsis* mitochondria by about three times, thus enhancing the drought resistance of transgenic plants (Lin et al., 2017). Park et al. reported that *PtSNAT* cloned from *Pinus taeda* was also located in chloroplasts (Sangkyu et al., 2014). Byeon et al. revealed that the melatonin content in the *OsSNAT* (AK059369) RNAi line was significantly lower than that in the wild-type plants, and the seedlings grew slowly and had weak tolerance to $CdCl_2$ stress (Byeon and Back, 2016). Wang et al. (2020) found that compared with wild-type plants, the expression of *SlSNAT* (Solyc10g074910) in overexpressed lines increased by about 3–5 times, and the content of melatonin increased by about 3–4 times. The homologous *SNAT* genes in other species such as cyanobacteria (Byeon et al., 2013b), *Vitis vinifera* (Yu et al., 2019), and *Pyropia yezoensis* (Byeon et al., 2015b) have also been identified and characterized by enzymology.

Regulating the expression of enzyme genes or transcription factors through metabolic engineering can effectively improve the biosynthesis of therapeutic compounds in medicinal plants (Rathinasabapathi, 2000; Capell and Christou, 2004). Although DNA recombinant technology has been widely used, genetic improvement in plants is still challenging without robust transformation methods. In recent years, it has been found that the resistance of *H. perforatum* to *Agrobacterium tumefaciens*-mediated leaf disk transformation is largely due to the induction of plant defense response (Hou et al., 2016). Through co-culture with *Agrobacterium rhizogenes*, some research groups obtained positive *H. perforatum* transgenic hairy roots (HR) (Koperdáková et al., 2009; Tusevski et al., 2012; Montazeri et al., 2019). However, *H. perforatum*, the whole grass is used as medicine. If complete transgenic plants are not obtained, the expression detection of many target genes will be limited. Therefore, *A. rhizogenes* was used to infect *H. perforatum* tissue in this study. By optimizing the conditions of *Agrobacterium* infection and explant growth, HRs, and completely transformed plants were successfully induced. The genetic transformation system of *A. rhizogenes* was established.

In this study, based on the whole-genome sequence of *H. perforatum*, the HpSNAT gene family was screened. The key genes *HpSNAT1* and *HpSNAT2* were identified and functionally validated in *Arabidopsis* and *H. perforatum*. Second, the regeneration of mature transgenic plants from transformed *H. perforatum* root cultures was successfully achieved.

MATERIALS AND METHODS

Identification and Sequence Analysis

To identify the *HpSNAT* candidates, the hidden Markov model profile of the SNAT conserved DNA binding domain (PF13508) was used as a query to search the genomic databases of *H. perforatum via* the PFAM databases.[1] In addition, only those genes with complete GNAT domain can be used as members of the SNAT gene family by using InterProScan[2] (Zdobnov and Rolf, 2001). The isoelectric point (PI) and molecular weight (MW) of the HpSNAT proteins were predicted by Compute PI/MW tool on the ExPASy[3] (Gasteiger, 2005).

Isolation and Bioinformatics Analysis of *HpSNAT1* and *HpSNAT2*

Four *SNAT* genes (rice *OsSNAT1* and *OsSNAT2*, *Arabidopsis AtSNAT1* and *AtSNAT2*) were confirmed to have SNAT activity, identified as bait proteins. A neighbor-joining phylogenetic tree of the candidate HpSNATs and four bait protein sequences was constructed using the software MEGA 9.0 with 1000 replicates bootstrap. The protein sequence of HpSNATs was used to predict the conserved motifs by using the program MEME[4] (Bailey et al., 2009) with the following parameters: a maximum

[1]http://pfam.janelia.org

[2]http://www.ebi.ac.uk/interpro/search/sequence-search

[3]http://web.expasy.org/compute_pi/

[4]http://memesuite.org/tools/meme

number of motifs sets at 5, optimum motif width from 70 to 300 bp, and with an *e*-value less than 1e-10. The amino acid sequence of the conserved GNAT domain of HpSNAT proteins was obtained by SMART,[5] and then multiple sequence alignment analysis was carried out by using Geneious v10.22. The upstream 2 kb genomic DNA sequences of *HpSNAT1/2* were uploaded to the PlantCARE database to search for the *cis*-acting regulatory elements in the promoter region (Lescot, 2002). The transcriptome data of different tissues (root, stem, leaf, and flower) were used to display the tissue-specific expression profile in the form of a heat map by using Origin v10.5.

Plant Material and Stress Treatment

Arabidopsis homozygous mutant *snat* (SALK_033944C) and wild-type Columbia-0 (Col-0) seeds were obtained from the European *Arabidopsis* Stock Centre (NASC). Three primers (LP, RP, and BP) were used to screen the homozygous mutants (**Supplementary Figure 1** and **Supplementary Table 1**). The seeds of *H. perforatum* were preserved in our laboratory. The sowing, sterilization, and growth conditions of aseptic tissue culture seedlings of *Arabidopsis* and *H. perforatum* can be referred to in the previous detailed description (Zhou et al., 2021). Three-month-old aseptic seedlings of *H. perforatum* were transferred into liquid Murashige and Skoog (MS; Solarbio) medium containing 250 mM D-Mannitol (Alfa Aesar) and 200 mM NaCl (Solarbio) for drought and high salt stress treatment. One-week-old aseptic seedlings of *Arabidopsis* were transferred to one-half strength MS agar medium with 200 mM D-mannitol and 150 mM NaCl for 7 days to observe their phenotypes, while 4-week-old seedlings in soil were treated with drought stress without watering for 12 days.

Gene Cloning and Vector Construction

Scaf 151.204 (*HpSNAT1*) and Scaf 64.495 (*HpSNAT2*) were cloned from *H. perforatum* transcriptome cDNA (Zhou et al., 2021) using gene-specific primers (**Supplementary Table 1**). The full-length cDNAs mentioned above were inserted into PBI221-GFP to produce the *35S::SNAT-GFP* constructs as subcellular localization vectors. The *HpSNAT* genes were cloned with the Gateway™ Recombination Cloning Technology (Invitrogen, Waltham, MA, United States) into the pEarleyGate202 vector to generate final overexpression (OE) vectors pEG202-*HpSNAT1* and pEG202-*HpSNAT2*.

Genetic Transformation and Growth Conditions

For overexpressing *HpSNAT* in *Arabidopsis*, the pEG202-*HpSNAT* constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 (self-made) and transformed into *Arabidopsis snat* plants by the floral dip method (Zhang et al., 2006). According to the resistance of the expression vector to BASTA, the positive transgenic plants were screened out until T3 generation (**Supplementary Figure 2**).

[5]http://smart.embl.de/

For overexpressing *HpSNAT* in *H. perforatum*, the recombinant constructs were transferred into *Agrobacterium rhizogenes* strain K599 (Weidi, Shanghai, China). The specific steps follow the product instructions. A culture of *A. rhizogenes* with pEG202- *HpSNAT1* and pEG202-*HpSNAT2* was grown for 1 day at 28°C and 120 rpm in TY liquid media (0.5% tryptone, 0.3% yeast extract, 0.5% meat extract, 10 mM $CaCl_2$, 1.5% agar with 50 μg/ml kanamycin, and 50 μg/ml streptomycin; pH 7.0). The bacterial suspension was centrifuged at 5,000 rpm for 10 min and resuspended in sterile water, then diluted to an OD_{600} of 0.1.

Hairy Root Induction

The roots of 3-month-old aseptic seedlings used as explants were cut into 1.0–1.5 cm segments and placed on solid B5 medium (Solarbio; supplemented with 3% sucrose, 0.7% agar, and pH 5.8) without antibiotics in an incubator for 2 days (25°C, dark). Then the pre-cultured segments were put into the diluted bacterial solution for transfection at 25°C. During this period, the tube was slightly reversed several times to make the roots fully immersed. After 15 min, the root segments were taken out to absorb the remaining surface solution and placed on solid B5 medium for 2 days (25°C, dark). Afterward, the transfected explants were transferred to solid B5 medium containing 200 μg/ml cefotaxime and 2 μg/ml BASTA (25°C, dark) for transgenic explants selection.

Hairy Root Propagation

When growing to 6 weeks, samples of the transgenic roots weighing 100 mg were inoculated into 250 ml beaker flasks containing 100 ml liquid MS medium (supplemented with 0.2 μg/ml naphthyl acetic acid and 50 μg/ml cefotaxime) and then placed on an orbital shaker at 110 rpm/min, 25°C in the dark. After 1 week, the medium was changed to liquid MS medium only supplemented with 50 μg/ml cefotaxime, which were regularly sub-cultured (every 1 week). These lines cultured for 2 months were used for DNA and RNA analysis. The positive transgenic lines were detected by amplifying the CaMV35S promoter sequence.

Calli Induction and Plant Regeneration

The positive and high expression of HR were cut into 1.0–1.5 cm root segments and put on solid MS medium (supplemented with 0.1 μg/ml thidiazuron and 50 μg/ml cefotaxime) for callus induction (25°C, dark). Two months later, the green calli were cut off and placed on solid MS medium (supplemented with 1 μg/ml 6-Benzylaminopurine and 0.1 μg/ml naphthylacetic acid) maintained at 25°C with a 16 h photoperiod at 108 μmol/m^2/s for the induction of adventitious buds.

Root Induction

When the adventitious buds grew to about 2 cm, they were cut off and placed on one-half strength solid MS medium without phytohormones for roots induction at the same growth conditions. The above-mentioned medium was changed every 2 weeks. After 4–5 months, the aboveground part of the seedlings

was used to detect the expression patterns of *HpSNAT* and the content of the metabolites.

PCR Analysis and Subcellular Localization Analysis

Genomic DNA and RNA were extracted using the FastPure Plant Total RNA/DNA Isolation Kit (Vazyme, Jiangsu, China). The specific extraction steps are by the product instructions. The CaMV35S promoter was amplified from the genomic DNA to detect whether the gene was integrated into the genome of the transgenic plants. PCR amplification was performed as previously described (Wang et al., 2018). Reverse transcription PCR (RT-PCR) was performed to explore the expression patterns of *HpSNAT* genes in *Arabidopsis snat*, Col-0, and T3 transgenic plants. An *Arabidopsis Actin* gene fragment is used as an internal control. Quantitative real-time PCR (qPCR) was used to investigate the expression patterns of *HpSNAT* genes in *H. perforatum* plant, and the *HpACT2* gene was used as an internal reference. The specific operation steps can be referred to in the previous detailed description (Zhou et al., 2021) and primer sequences used are shown in **Supplementary Table 1**. The transient transformation of *Arabidopsis* protoplasts is operated according to Dr. Sheen's operation manual[6] (Sheen, 2001). Briefly, the leaves of *Arabidopsis* plants aged 3–4 weeks were cut into 1 mm wide bands, completely soaked in 10 ml enzymatic hydrolysate and digested in the dark at 55°C water bath for 10 min. Then, digestion was continued at 25°C for about 5 h and gently shaken in the dark. The samples were washed with solution W5 and filtered through a 300-mesh sieve. The obtained protoplasts were resuspended in MMG solution for microscopic examination. After PEG-mediated transfection, the fluorescence was observed by Nikon C2-ER laser confocal microscope.

Measurement of Melatonin and Its Precursors

Two-month-old *Arabidopsis* OE, Col-0, and *snat* plants were collected. According to the extraction and detection methods established in our laboratory, melatonin in *Arabidopsis* leaves was determined by LC/MS (Zhou et al., 2021). The leaves of OE and WT (the line infected by *A. rhizogenes*, K599 only) lines of *H. perforatum* were freeze-dried, and a 200 mg dry sample was extracted by ultrasonic treatment in 500 μl 80% methanol (Sigma, St. Louis, MO, United States) (Zuo et al., 2015). The sample preparation and HPLC detection of melatonin and its precursors were performed as previous described (Zhao et al., 2013).

Physiological Assays

One-week-old OE, Col-0, and *snat* aseptic seedlings of *Arabidopsis* under drought and high salt stress were to observe their phenotypes. One-month-old plants without watering to create drought stress for physiological analysis. The concentration of H_2O_2 and Malondialdehyde (MDA) were measured using the Hydroperoxide/Malondialdehyde Fluorometric Assay Kit (Sigma–Aldrich). Reactive oxygen species (ROS) concentration was examined by histochemical staining with CM-H_2DCFDA fluorescent dye (Solarbio, Tongzhou District, Beijing, China) (Oparka et al., 2016). Fluorescence images were taken with a Leica stereomicroscope (Leica, Germany).

Statistics

All experiments were performed with three biological replicates unless otherwise specified. The data were the average of three technical repetitions expressed as mean ± standard error. ANOVA analysis was used for statistical analysis; the probability value $P < 0.05$ was considered statistically significant.

RESULTS

Isolation and Bioinformatics Analysis of *Hypericum perforatum* Serotonin N-Acetyltransferase

A total of 48 HpSNAT members were screened, all of which contained GNAT conserved domain. The MW of the predicted HpSNAT proteins ranged from 14.92 kDa (HpSNAT26) to 132.67 kDa (HpSNAT21), the PIs ranged from 4.98 (HpSNAT23) to 9.97 (HpSNAT1), and protein lengths ranged from 129 (HpSNAT26) to 1,178 (HpSNAT21) amino acids (**Supplementary Table 2**). Hierarchical clustering of expression profiles of HpSNAT genes in different tissues (root, stem, leaf, and flower) were shown in **Supplementary Figure 3**. Among them, two full-length HpSNAT showed homology to the four bait proteins, and they were named *HpSNAT1* (Scaf 151.204) and *HpSNAT2* (Scaf 64.495) according to the phylogenetic analysis (**Figure 1A**). As shown in **Figure 1B**, two HpSNATs and four bait proteins have the same motif, showing that the functional proteins have similarities. The qPCR results were consistent with the expression pattern of *HpSNAT1* and *HpSNAT2* in different tissue transcriptome sequencing results, indicating that the two genes were highly expressed in leaves (**Figure 1C**). Through the analysis of *cis*-acting elements in the promoter region of the two genes, it was found that both two genes contain gibberellic acid (GA), methyl jasmonate (MeJA), salicylic acid (SA), auxin (IAA), abscisic acid (ABA), and stress response elements of low temperature, drought, and injury. In addition, *HpSNAT1* also contains flavonoids biosynthesis and elements related to plant circadian rhythm regulation, while *HpSNAT2* also contains ethylene response and elements related to gene expression regulation in plant meristem.

Subcellular Localization of HpSNAT

To determine the subcellular location of HpSNAT1 and HpSNAT2 *in vivo*, HpSNAT-GFP fusion proteins expression vector driven by the CaMV 35S promoter was constructed, and the empty vector PBI221-GFP was used as a positive control. The fused expression vector was used to perform a transient expression assay in *Arabidopsis* protoplasts. From **Figure 2A**, the GFP fluorescence of positive control was almost distributed in the whole cell. In contrast, the GFP fluorescence of HpSNAT1/2 was observed only in the chloroplasts in cytoplasm, which was

[6]https://molbio.mgh.harvard.edu/sheenweb/main_page.html

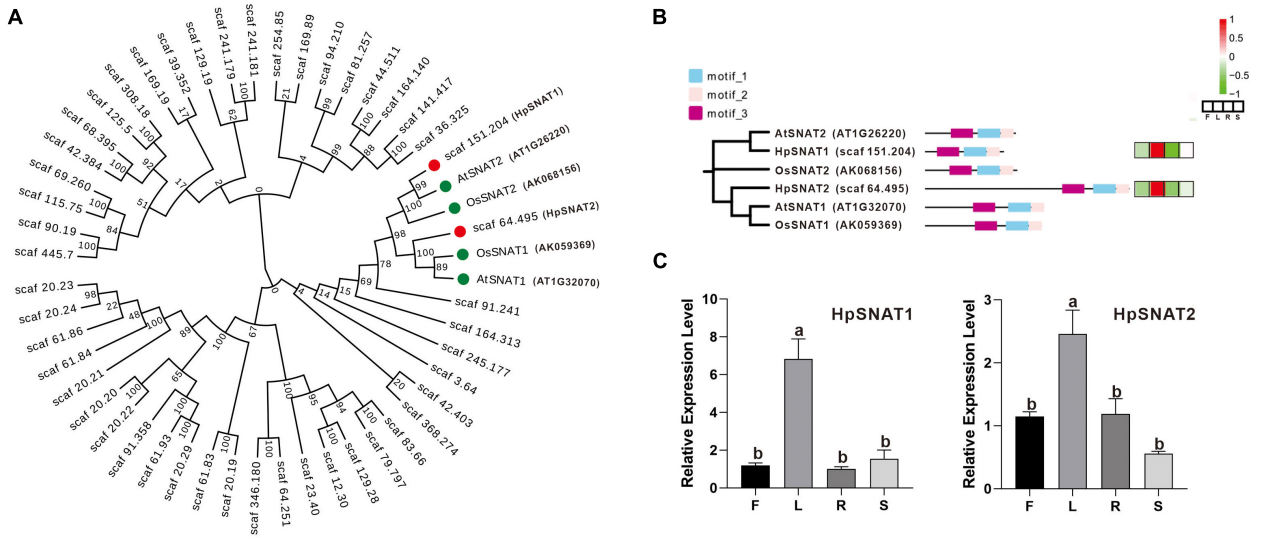

FIGURE 1 | Bioinformatics analysis of HpSNAT. **(A)** The phylogenetic tree of HpSNAT and the reference proteins AtSNAT1, AtSNAT2, OsSNAT1, and OsSNAT2 from *Arabidopsis thaliana* and *Oryza sativa*. The sequences were analyzed by MEGA 10.0 using bootstrap analysis with 1000 replicates. **(B)** The distribution of three conserved motifs and heat maps of six SNAT proteins. The heatmaps were analyzed by the FPKM values (F: flowers, L: leaves, R: roots, and S: stems) for each protein (*Z*-score). **(C)** Relative quantitative analysis of *HpSNAT1* and *HpSNAT2* in different tissues of *Hypericum perforatum*. Data were normalized by *HpACT2* (MK054303), calculated with the equation $2^{-\Delta\Delta Ct}$. All data represent averages of three biological replicates, error bars indicate ± SD. Different letters indicate significant differences from the control ($P < 0.05$) tested by one-way ANOVA.

consistent with the previous subcellular localization of SNAT proteins in other plants (Byeon et al., 2013a; Lee et al., 2015a; Yeong et al., 2016).

Expression Patterns of HpSNAT

Melatonin has strong free radical scavenging and antioxidant ability to prevent cell oxidative damage. To determine the effects of drought and high salt stress on the expression of *HpSNAT1* and *HpSNAT2*, 3-month-old aseptic seedlings of *H. perforatum* were treated with 250 mM D-Mannitol and 200 mM NaCl for up to 32 h. The expression trend of the *HpSNAT1* and *HpSNAT2* in *H. perforatum* at five-time intervals (0, 4, 8, 16, and 32 h) was quantified by qPCR (**Figure 2B**). Under drought stress, the expression level of *HpSNAT1* reached the highest at 8 h, increased by about 2.5 times, while *HpSNAT2* had no obvious response to D-mannitol treatment. Similarly, the expression level of *HpSNAT1* increased nearly three-fold at 8 h under high salt stress, while *HpSNAT2* showed a slight response at 32 h.

Overexpression of HpSNAT Increased Melatonin Accumulation in *Arabidopsis*

By overexpression of *HpSNAT1* and *HpSNAT2* in the *Arabidopsis* mutant *snat*, the OE lines contained the expected 926 bp fragments of the CaMV35S promoter were confirmed by PCR. Five T3 homozygous transgenic lines of OE-*HpSNAT1* and OE-*HpSNAT2* were obtained for gene expression (**Figure 3A**) and melatonin detection (**Figure 3B**). Finally, three OE lines with high *HpSNAT1* (OE1, OE3, and OE5) and *HpSNAT2* (OE2, OE4, and OE5) gene expression and melatonin content were used for further analysis. The content of melatonin in OE1- *HpSNAT1* was 1.86 times higher than that in Col-0 plants, and that in OE5-*HpSNAT2* was 1.67 times higher. By analyzing the rosette leaves of different lines (*snat*, Col-0, OE1- *HpSNAT1*, and OE5-*HpSNAT2*) growing to 25 days, we found that the OE lines had more leaves than *snat* and Col-0. The dry and fresh weight of OE leaves was almost three times that of the *snat* and twice that of Col-0 (**Figure 3C**).

Overexpression of HpSNAT Enhanced Drought and Salt Tolerance in *Arabidopsis*

In order to explore whether *HpSNAT* genes can enhance the drought and salt resistance of plants, *Arabidopsis* root morphology of different lines was observed in one-half-strength MS medium with NaCl and D-mannitol, respectively. Results as shown in **Figure 4A**, after 10 days of treatment, there was no significant change in the length of the primary root of *snat*, Col-0, and OE, but the number of lateral roots of OE lines was significantly more than that of Col-0 and *snat* plants under drought and salt stress. Similarly, the growth of 4-week-old seedlings in soil was better than that of Col-0 and *snat* after without watering for 12 days (**Figure 4B**), and the MDA and H_2O_2 contents in OE lines were significantly lower than in Col-0 and *snat* plants (**Figure 4C**). From the CM-H_2DCFDA staining, it can be seen that the fluorescence intensity in OE leaves (**Figure 4D**) is lower than that in *snat* under drought treatment. The relative staining fluorescence intensities in *snat*, Col-0, OE-*HpSNAT1*, and OE-*HpSNAT2* leaves were 91.455, 71.502, 63.009, and 60.531, respectively. These results showed that the content of ROS in transgenic leaves was lower than that in Col-0 and *snat*. In

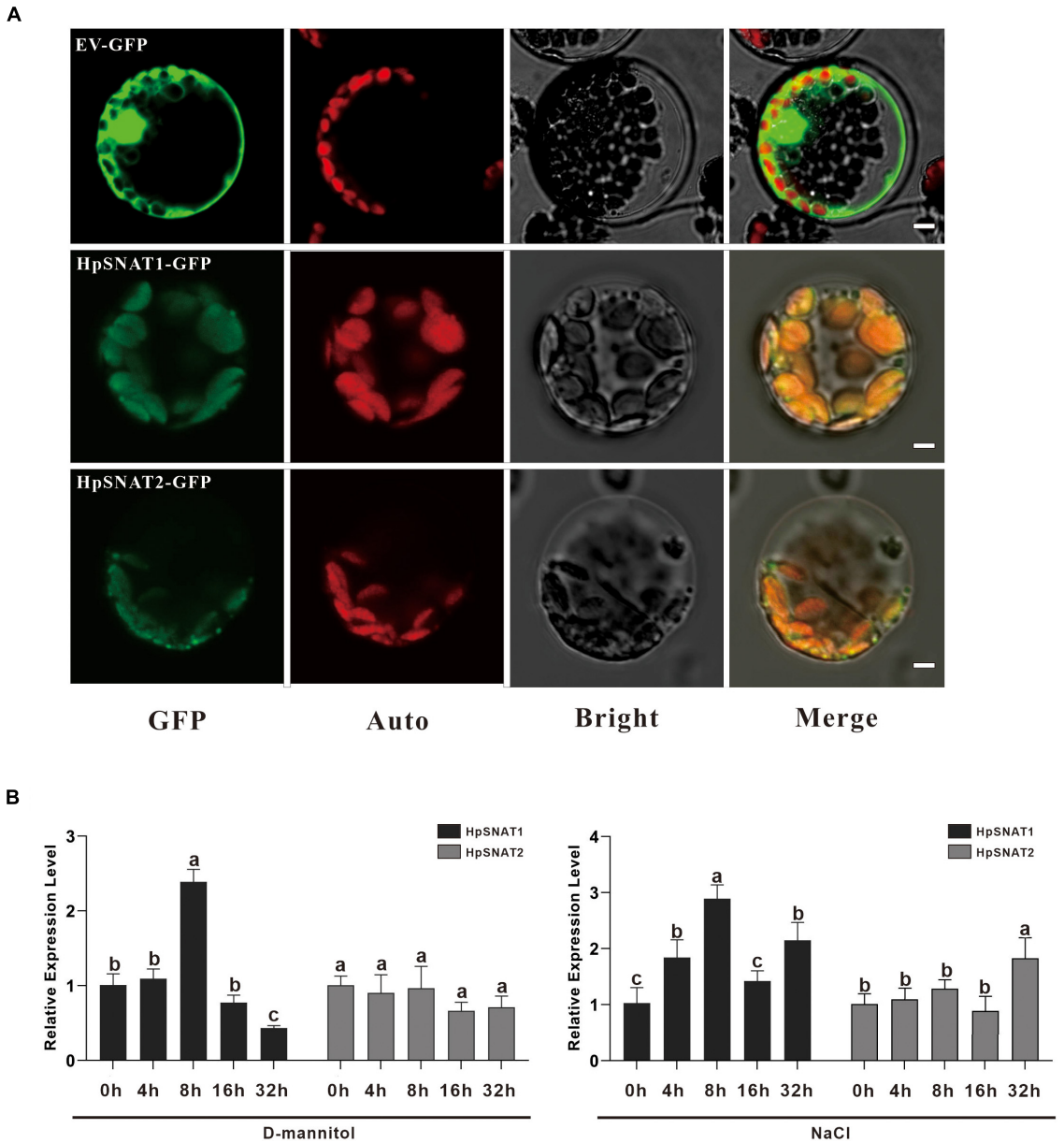

FIGURE 2 | Subcellular localization and expression patterns of HpSNAT. **(A)** From left to right are the subcellular localization of the target genes (GFP), chloroplast autofluorescence (Auto), bright-field (Bright), and Merged (Merge) images. Scale bars = 5 μm. **(B)** Relative quantitative analysis of *HpSNAT1* and *HpSNAT2* under drought (D-mannitol) and high-salt (NaCl) stress conditions. Data were normalized by *HpACT2* (MK054303), calculated with the equation $2^{-\Delta\Delta Ct}$. All data represent averages of three biological replicates, error bars indicate ± SD. Different letters indicate significant differences from the control ($P < 0.05$) tested by one-way ANOVA.

other words, the content of ROS in plants can be greatly reduced by overexpression of *HpSNAT* genes, so as to improve the drought tolerance of plants.

Hairy Root Induction in *Hypericum perforatum*

As shown in **Figure 5A**, the first candidate transgenic root tips emerged from the wounded areas of the explants about the 2nd week on the selective medium with antibiotics. When growing to the 6th week, 100 mg roots were put into a liquid MS medium containing phytohormones for expanded culture to obtain enough samples for molecular detection. After 10 weeks of growth, the positive lines carrying the *HpSNAT* gene were verified by PCR. In total, we obtained five positive transgenic HR lines for both constructs that were confirmed in *HpSNAT* gene expression. The expression levels of the two genes in the OE lines were significantly higher than those in the WT group, especially the OE2, OE4, and OE5 of *HpSNAT1* and OE1, OE2, and OE4 of *HpSNAT2* detected by qPCR (**Figure 5B**). Among them, the

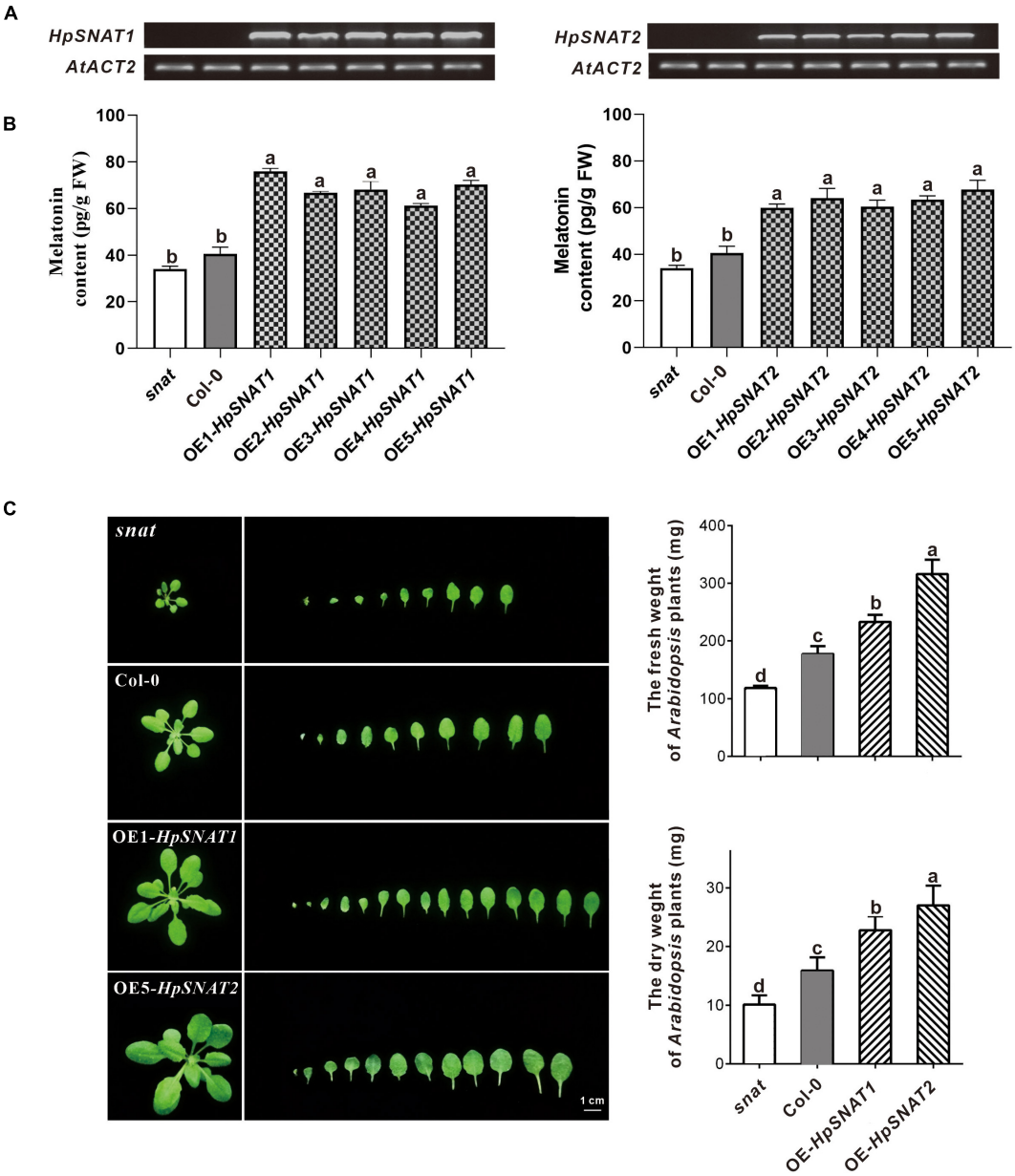

FIGURE 3 | The melatonin content and phenotype of *snat*, Col-0, and overexpressed *Arabidopsis* (OE). **(A)** and **(B)** Expression levels of HpSNAT and melatonin content in *snat*, Col-0, and five OE lines. HpSNAT expression was analyzed by RT-PCR. **(C)** Comparison of the rosette leaves in *snat*, Col-0, and OE lines. Each value represents the average of three replicates, and the error bars represent ± SD. Different letters indicate significant differences from the control ($P < 0.05$) tested by one-way ANOVA.

expression of *HpSNAT2* in the OE lines increased by about 1500 times. The above highly expressed HR lines were selected for callus, adventitious bud, and root induction to obtain a complete transgenic plant of *H. perforatum*.

Transgenic Lines Selection in *Hypericum perforatum*

As shown in **Figure 5C**, when explants were grown on MS medium containing thidiazuron for 2 months, a large amount of brown callus will be formed with a little green part in it. Then the green callus was separated and transferred to MS medium containing 6-Benzylaminopurine and naphthylacetic acid to induce adventitious buds. About 1 month later, the shoots were cut off and inserted into one-half strength MS medium for rooting induction to obtain complete plants. It began to take root slowly in about 2 weeks, and lots of HRs and branches could be found outside the medium after 1 month. qPCR analysis of HpSNAT expression showed that *HpSNAT1* expressed over 20-fold higher than the WT transformed lines,

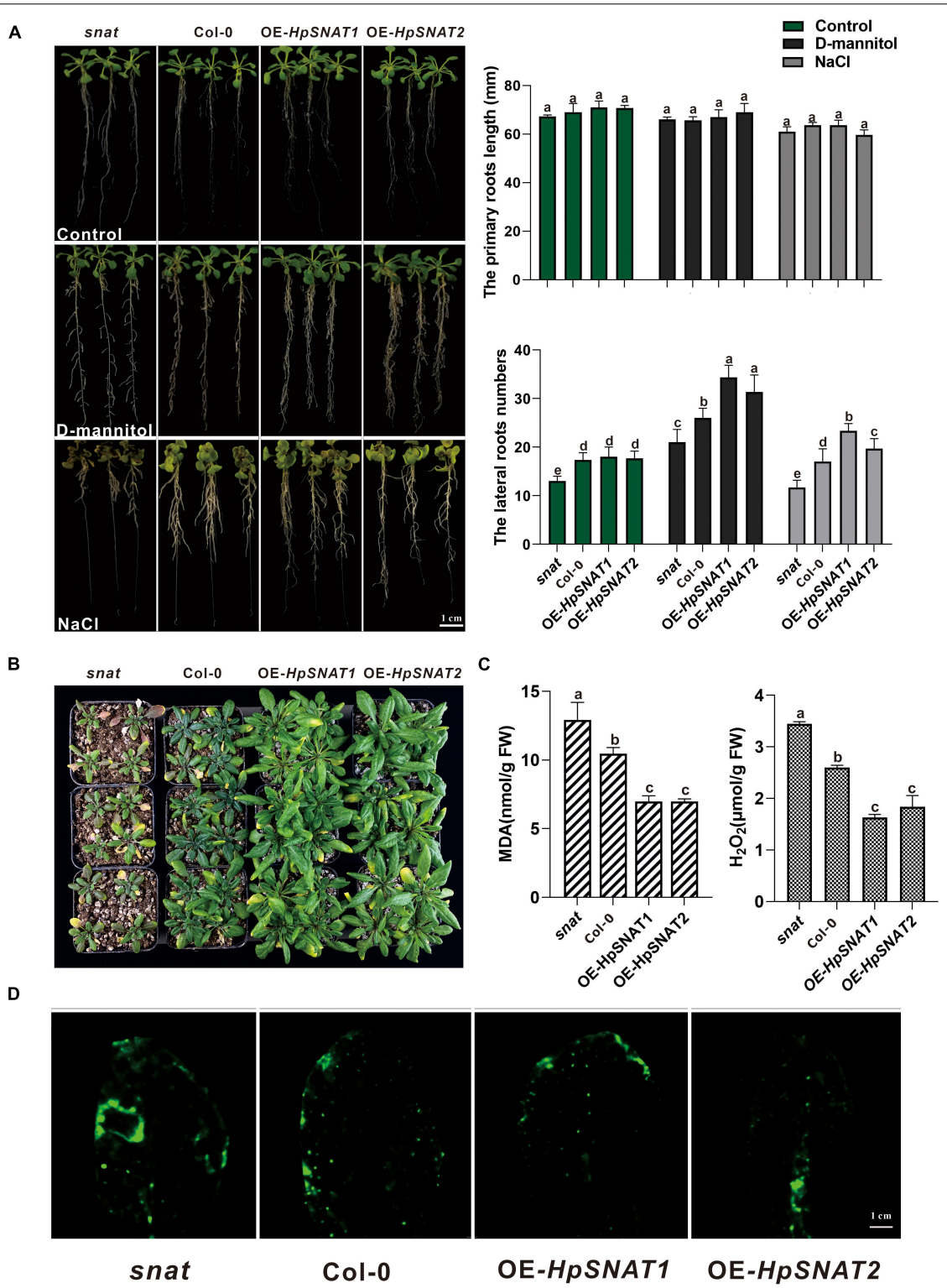

FIGURE 4 | Function of *HpSNAT1* and *HpSNAT2* when expressed in *Arabidopsis* plants. **(A)** The phenotype, primary root length, and lateral root number of *snat*, Col-0, and OE seedlings under normal condition (control), D-mannitol, and NaCl treatments. Each value represents the average of three replicates, and the error bars represent ± SD. Different letters indicate significant differences from the control ($P < 0.05$) tested by one-way ANOVA. **(B)** Drought tolerance test. Four-week-old plants of *snat*, Col-0, and OE in soil were dehydrated for 12 days. **(C)** MDA and H_2O_2 accumulation in *snat*, Col-0, and OE lines under drought stress. Each value represents the average of three replicates, and the error bars represent ± SD. Different letters indicate significant differences from the control ($P < 0.05$) tested by one-way ANOVA. **(D)** Histochemical staining with fluorescent CM-H2DCFDA to detect reactive oxygen species in *snat*, Col-0, and OE lines under drought stress.

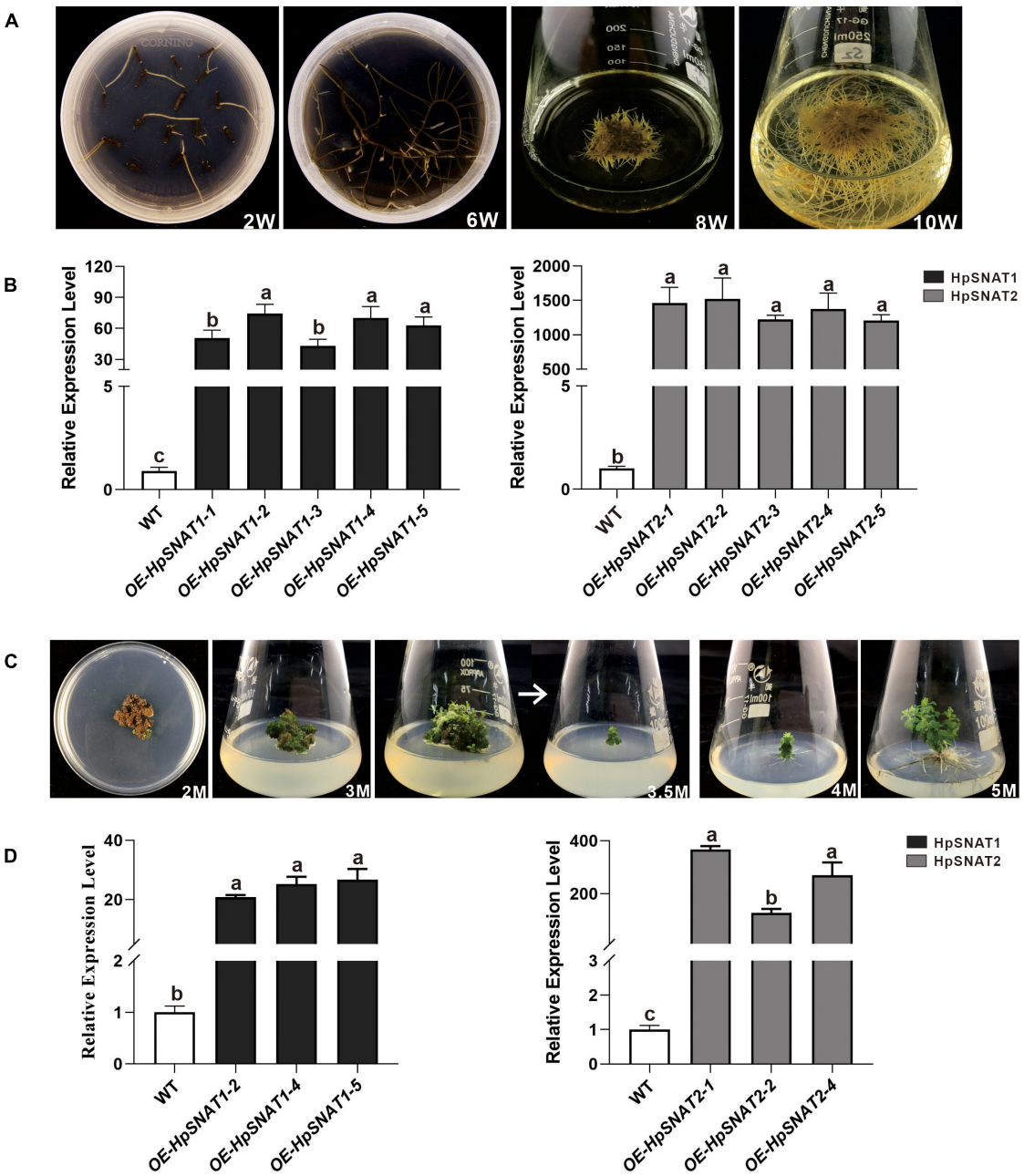

FIGURE 5 | Selection of positive hairy roots (HRs) lines and generation of adult transgenic *H. perforatum*. **(A)** The phenotypes of HRs induction at different time (W: week). **(B)** Relative quantitative analysis of *HpSNAT1* and *HpSNAT2* expression in overexpression (OE) and control of *H. perforatum* HRs (WT: the line infected by *A. rhizogenes*, K599 only). Data were normalized by *HpACT2* (MK054303), calculated with the equation $2^{-\Delta\Delta Ct}$. All data represent averages of three biological replicates, error bars indicate ± SD. **(C)** Plant regeneration from *H. perforatum* HR cultures (M: month). **(D)** Relative quantitative analysis of *HpSNAT1* and *HpSNAT2* expression in OE and control of *H. perforatum* plants. Data were normalized by *HpACT2* (MK054303), calculated with the equation $2^{-\Delta\Delta Ct}$. All data represent averages of three biological replicates, error bars indicate ± SD.

and the expression of *HpSNAT2* was 100- to 400-fold higher in adult transgenic *H. perforatum* lines (**Figure 5D**). We further evaluate the content of melatonin and its precursors. Three OE and WT lines were used to examine four compounds, such as 5-HT, NAS, 5-MT, and melatonin in adult transgenic *H. perforatum*. The results showed that the contents of four compounds were significantly up-regulated, and the melatonin increased to 12.28–21.15 μg/g DW in OE lines (**Figure 6A**). Among them, OE-*HpSNAT2*-1 accumulated the highest content of melatonin, which was 4.21-folds higher than WT. These results confirmed the positive role of *HpSNAT1* and *HpSNAT2* in melatonin biosynthesis.

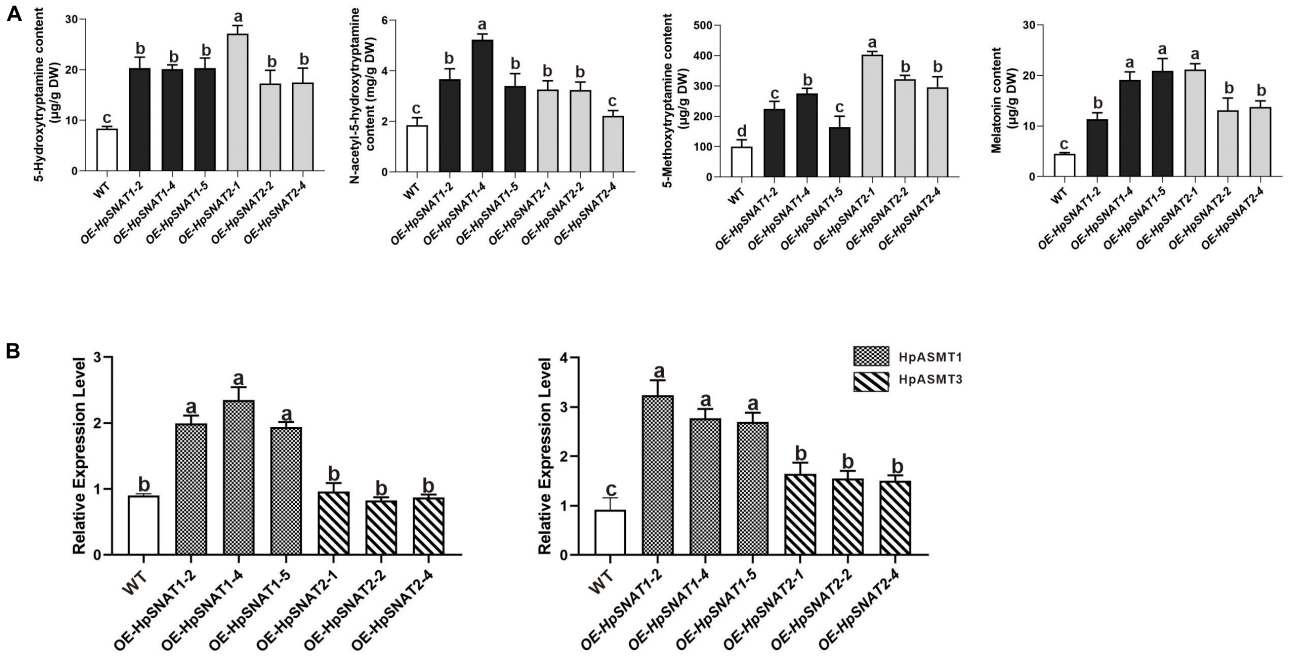

FIGURE 6 | Secondary metabolites contents and expression patterns of *HpASMT* in *H. perforatum*. **(A)** Comparisons of 5-HT, 5-MT, NAS, and melatonin in OE and control of *H. perforatum* plants. **(B)** Relative quantitative analysis of *HpASMT1* and *HpASMT3* in transgenic lines and control of *H. perforatum* plants. Data were normalized by *HpACT2* (MK054303), calculated with the equation $2^{-\Delta\Delta Ct}$. All data represent averages of three biological replicates, error bars indicate ± SD.

DISCUSSION

In the past 20 years, the research of phytomelatonin mostly focused on the content detection in the early stage. However, due to its diverse and important physiological functions, melatonin soon attracted extensive attention in the research of herbal medicine, horticultural plants, and crops. Although great progress has been made in the research of phytomelatonin, there are still many problems to be clarified compared with plant hormones such as auxin, abscisic acid, ethylene, polyamine, and brassinolide. Many studies have proved that applying of a certain concentration of exogenous melatonin could enhance the resistance of plants to the extreme environments and improve the disease resistance of plants (Li et al., 2012; Yin et al., 2013; Xian et al., 2018; Lin et al., 2019). Therefore, how to improve the content of endogenous phytomelatonin through modern breeding methods to improve stress resistance will be the key research field of using phytomelatonin to improve yield and product quality in the future. SNAT is a key rate-limiting enzyme in melatonin synthesis and plays an important role in regulating the balance of melatonin accumulation (Lee et al., 2015b; Yeong et al., 2015). The related research of the SNAT gene family mainly focuses on the model plants *Arabidopsis* and rice, while there is a scarce study on the medicinal plants such as *H. perforatum*. Therefore, it is necessary to expand the research object for the in-depth exploration and interpretation of many physiological processes.

In this study, 48 SNAT genes were screened based on the *H. perforatum* whole-genome database. There was high homology of amino acid sequence between the HpSNAT and AtSNAT/OsSNAT (homology from 42.34 to 70.59%). Phylogenetic analysis was also consistent with amino acid homology analysis, indicating that *HpSNAT* may have similar gene functions with *AtSNAT* and *OsSNAT*. In order to preliminarily explore the possible functions of *HpSNAT1* and *HpSNAT2*, we measured their subcellular localization and expression patterns in different tissues and under different stress. The two proteins were both located on the chloroplast, and the high expression of *HpSNAT1* and *HpSNAT2* in leaves also complements the results of subcellular localization. Chloroplast is the place of photosynthesis and also produces many free radicals at the same time. The production of melatonin in chloroplasts can be explained from an evolutionary point of view (Tan et al., 2013).

Increasing the content of bioactive compounds is the main aim of *H. perforatum* genetic engineering. Although the overexpression of genes involved in biosynthesis will enable us to achieve the above objectives, the pathway engineering of this species is still in the primary stage, mainly due to the lack of genetic information about these biosynthetic pathways and effective transformation methods. By overexpressing *HpSNAT1* and *HpSNAT2* in *Arabidopsis* mutant *snat*, we observed that the melatonin level in OE lines was nearly two times higher than that of Col-0 and mutant plants. When plants are under stress, the balance of the active oxygen metabolism system is broken, and the production of ROS increases. Accordingly, transgenic plants also showed stronger drought and salt tolerance compared with Col-0 and *snat*. These results indicate that

overexpression of *HpSNAT* genes in *Arabidopsis* improves the ability of plants to resist adverse environments, which is directly related to the strong antioxidant function of melatonin. Although *A. tumefaciens*-mediated transformation of *H. perforatum* has not been reported, the study of inducing HRs after co-culture with *A. rhizogenes* has been comparatively mature. In this study, when the HRs of positive transgenic *H. perforatum* were successfully obtained, complete transgenic plants were formed through dedifferentiation and redifferentiation induced by plant hormones. Finally, a stable and efficient genetic transformation system of *H. perforatum* was successfully established, which solved the problem of restricting the genetic transformation of *H. perforatum*. The contents of melatonin and its precursors (5-HT, 5-MT, and NAS) in different lines were detected. The results showed that the contents of these substances in OE lines were significantly higher than those in WT plants. It is well known that SNAT protein converted 5-HT and 5-MT into NAS and melatonin, while ASMT protein converted 5-HT and NAS into 5-MT and melatonin. Therefore, we further detected *HpASMT1* and *HpASMT3* in the OE lines and control group, and the results showed that the expression of *HpASMT* genes was also obviously up-regulated (**Figure 6B**). It indicated that the overexpression of *HpSNAT1* and *HpSNAT2* would not only increase the content of NAS and melatonin in *H. perforatum*, but also up-regulate the expression of *HpASMT1* and *HpASMT3* in the melatonin metabolic pathway to increase the content of melatonin and other precursors. It is also confirmed that these enzyme genes in the melatonin biosynthesis pathway have synergistic regulation (Byeon et al., 2015c).

CONCLUSION

This study defines a foundation for identifying and functional characterization of the role of SNAT genes in the species *H. perforatum*. It not only clarified the role of *HpSNAT* genes in phytomelatonin biosynthesis, but also provided an important basis for the molecular mechanism of melatonin regulating plant growth and stress response. In addition, the successful establishment of Agrobacterium-mediated transformation system of *H. perforatum* lays a foundation for the follow-up study of key genes functions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA588586.

AUTHOR CONTRIBUTIONS

WZ and ZW designed the study. SY, WZ, and QZ performed the experiments and analyzed the data. RX, DW, SW, and JN contributed analytical tools and provided technical support. WZ wrote the first draft. ZW approved the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.781717/full#supplementary-material

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Melatonin Participates in Selenium-Enhanced Cold Tolerance of Cucumber Seedlings

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Melatonin is an important and widespread plant hormone. However, the underlying physiological and molecular mechanisms of melatonin as a secondary messenger in improving cold tolerance by selenium are limited. This study investigated the effects of selenite on the cold stress of cucumber seedlings. The results showed that exogenous application of selenite improved the cold tolerance of cucumber seedlings, which was dependent on the concentration effect. In the present experiment, 1 μM of selenite showed the best effect on alleviating cold stress. Interestingly, we found that in the process of alleviating cold stress, selenite increased the content of endogenous melatonin by regulating the expression of melatonin biosynthesis genes (*TDC, T5H, SNAT,* and *COMT*). To determine the interrelation between selenite and melatonin in alleviating cold stress, melatonin synthesis inhibitor *p*-chlorophenylalanine and melatonin were used for in-depth study. This study provides a theoretical basis for cucumber cultivation and breeding.

Keywords: selenium, melatonin, cucumber, cold stress, *COMT*

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INTRODUCTION

Selenium was discovered by Berzelius in 1817, which plays vital roles in humans with functions in preventing cancer, boosting immunity, detoxifying cells, and even in combatting COVID-19 infections (Santi and Bagnoli, 2017; Moghaddam et al., 2020; Zhang et al., 2020). Selenium is generally beneficial for plants in appropriate concentrations and has been found to influence photosynthesis (Jiang et al., 2017), root architecture (Zhao et al., 2019), senescence (Hajiboland et al., 2019), vegetable quality (Mckenzie et al., 2019), defense, and stress response (Alves et al., 2020; Wen, 2021). With the interest in the roles of selenium in plants, reports of selenium have dramatically increased in recent years and it is anticipated that mechanism studies related to plant selenium will flourish in the near future (Wrobel et al., 2020; Tran et al., 2021; Wen, 2021).

Melatonin is an important and widespread plant hormone. In 1995, reports have demonstrated the presence of natural melatonin in the plant kingdom (Dubbels et al., 1995; Hattori et al., 1995). Scientists have conducted more comprehensive and in-depth studies on the synthesis, content, distribution, and function of melatonin in plants. Melatonin as plant master regulator plays vital

roles in plant growth (Erdal, 2019), crop yield (Zahedi et al., 2020), senescence (Tan et al., 2020), storage and fresh-keeping (Onik et al., 2021), root development (Wen et al., 2016), stress response, and so on (Arnao and Hernández-Ruiz, 2019; Khan et al., 2020). Although several researchers have studied the effects of exogenous melatonin on enhancing plant cold tolerance (Li et al., 2018; Liu et al., 2020), not much is known about the mechanism of melatonin associated with selenium as well as the related signal transduction events under the condition of cold stress.

In the present study, melatonin-participated selenium enhanced plant cold tolerance of cucumber seedlings. Pharmacologic method was used in which *p*-chlorophenyl alanine (CPA) reduced melatonin biosynthesis (Murch et al., 2001; Ramakrishna et al., 2009; Park, 2011; Feng et al., 2021). Fluorescence quantitative technique was used to identify the expression of key genes in this process. Levels of melatonin biosynthesis genes *TDC* (tryptophan decarboxylase), *T5H* (tryptamine 5-hydroxylase), *SNAT* (serotonin *N*-acetyltransferase), and *COMT* (caffeic acid *O*-methyltransferase) were detected (Tan et al., 2015). *T5H*, which is the key gene that regulates the pathway from tryptamine to biosynthesis of melatonin rather than auxin (Back, 2021), significantly changed in exogenous application of selenium with cold stress of cucumber seedlings. *COMT,* as the key gene, which regulates the last step of melatonin synthesis (Byeon et al., 2015; Sun et al., 2020), also changed significantly. The present study deepens the understanding of selenium and melatonin in plant cold response as well as signaling transduction in plants.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

Cucumber (Jinyan No. 4) seeds were sterilized in 2.5% NaClO and washed three times for 5 min in sterile distilled water, then soaked in distilled water for 6 h at 28°C. After the seeds were germinated on filter paper in Petri dishes, they were transferred to the growth chamber filled with vermiculite maintained at 28°C/18°C (day/night) with a 12 h photoperiod (photosynthetically active radiation = 400 μmol/m^2/s). Cold stress condition maintained at 10°C/ 8°C (day/night), other conditions remain unchanged. Control, T1, T2, T3, T4, and T5 are the treatment of 0, 1, 10, 50, 100, and 1,000 μM sodium selenite (Sinopharm Chemical Reagent Co., Ltd), respectively. The concentration of exogenous application of sodium selenite was 1 μM under cold stress and normal conditions in the follow-up trial. The concentrations of melatonin (Tokyo Chemical Industry) and CPA (Tokyo Chemical Industry) were 1 μM each.

Determination of Plant Growth Index

After 10 days of treatment, the fresh shoot weights of cucumber seedlings were measured. Plant height was the distance from the bottom of the stem to the apical meristem. They were measured using a ruler. SPAD value was measured at the third leaf from the apical meristem after 5 and 10 days treatments using SPAD-502 Plus meter, with 5 replicates for each treatment.

Element Content Measurement

For analysis of element contents of N, P, and K, the roots of each seedling in the same treatment of five plants were taken, and then were rinsed with deionized water and dried at 70°C to a constant weight, leaves of 5 plants in one pot were mixed together, which was considered as one replicate. There were three replicates for each treatment. After pulverizing, a mixture of 0.2 g of powdered dry cucumber leaf was digested in a solution of H_2SO_4-H_2O_2, and the extract was used to determine N, P, and K content. N was determined by the Kjeldahl method and P was determined by vanadomolybdate colorimetric procedure (Wang et al., 2010; Gong et al., 2013). K was determined by a flame photometer (Wang and Zhao, 1995).

The Net Photosynthetic Rate Analysis

The net photosynthetic rate (Pn) was determined on the third fully expanded leaves by a photosynthesis system (LI-6400, Lincoln, United States). The measurement was performed after the treatments, at 5 d and 10 d between 9:00 and 11:00 A.M. while maintaining the air temperature CO_2 concentration, and PPFD at 25°C, 400 μmol/m^2/s, and 1,000 μmol/m^2/s, respectively.

Transmission Electron Microscopy Analysis

The transmission electron microscopy analysis of chloroplast ultrastructure was carried out as described by Fukuda et al. (2013). Samples were taken from the third leaves, counting from the tip stem, of control, selenium application, cold stress, and selenium application under cold stress conditions after treatment for 10 days. Samples were harvested at 9:00 to 11:00 A.M. Samples were rapidly cut into 1-mm × 1-mm squares and inserted into 0–4°C pre-cooled fixative within 1 min. The ratio of the sample and the liquid was 1:20–40. Every 100 mL fixative contained 10 mL 25% glutaraldehyde, 50 mL 0.2 M phosphate buffer (pH 7.4), and 40 mL double-distilled water. The samples were air exhausted in a vacuum after being immersed in the fixative liquid. The tissues were washed using 0.1 M PB (pH 7.4) for three times, 15 min each. Post-fix: Tissues were post-fixed with 1% OsO_4 in 0.1 M PB (pH 7.4) for 7 h at room temperature under the condition of light avoided. After removing from OsO_4, the tissues were rinsed in 0.1 M PB (pH 7.4) three times, 15 min each. Dehydrated at room temperature as follows: 30% ethanol for 1 h; 50% ethanol for 1 h; 70% ethanol for 1 h; 80% ethanol for 1 h; 95% ethanol for 1 h; 100% ethanol for 1 h; 100% ethanol for 1 h; ethanol:acetone=3:1 for 0.5 h; ethanol:acetone = 1:1 for 0.5 h; ethanol:acetone = 1:3 for 0.5 h; and pure acetone for 1 h. Resin penetration and embedding as follows: acetone:EMBed 812 = 3:1 for 2–4 h at 37°C; acetone:EMBed 8121 = :1 overnight at 37°C; acetone:EMBed 812 = 1:3 for 2–4 h at 37°C; pure EMBed 812 for 5–8 h at 37°C; poured the pure EMBed 812 into the embedding models and inserted the tissues into the pure EMBed 812, and then kept in 37°C oven overnight. Polymerization: The embedding models with resin and samples were moved into 65°C oven to polymerize for more than 48 h. And then the resin blocks were taken out from the embedding models for standby application at room temperature. Ultrathin section: The resin blocks were cut to 60–80 nm thin on the ultramicrotome

(Leica UC7, Leica), and the tissues were fished out onto the 150-mesh cuprum grids with formvar film. Stained with 2% uranium acetate saturated alcohol solution for 8 min under the condition of avoid light, rinsed with 70% ethanol for 3 times and then with ultra-pure water for 3 times. Lead citrate (2.6%) stained for 8 min by avoiding CO_2, and then rinsed with ultra–pure water three times. After drying by the filer paper, the cuprum grids were put into the grids board and dried overnight at room temperature. Observation and image capture: The cuprum grids were observed under transmission electron microscope (HT7800/HT7700, HITACHI) and images were taken.

Analysis of Reactive Oxygen Species Accumulation

The histochemical staining of O_2^- was performed using nitroblue tetrazolium (NBT) according to Xia et al. (2009). For the histochemical staining of O_2^-, leaves were vacuum-infiltrated with 0.1 mg/mL NBT in 25 mM K-Hepes buffer (pH 7.8) and cultivated at 25°C in the dark for 2 h. Leaves were rinsed in 80% (v/v) ethanol for 20 min at 70°C, mounted in lactic acid/phenol/water (1:1:1; v/v/v), and photographed.

Determination of Enzymatic Activity

Three hundred milligrams of cucumber leaves were ground with 3 mL of cold 50 mM phosphate buffer solution buffer (pH 7.8), which included 0.2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ascorbate, and 2% polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 4°C for 20 min at 12,000 g and the supernatants were used for the determination of antioxidant enzymatic activities. Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (Stewart and Bewley, 1980). Catalase (CAT) activity was measured as the decrease in absorbance at 240 nm because of the decrease in H_2O_2 extinction (Chamnongpol et al., 2010). Ascorbate peroxidase (APX) activity was measured by the decrease in absorbance at 290 nm as the ascorbic acid (ASA) was oxidized (Durner and Klessig, 1995). Peroxidase (POD) activity was measured as the increase in absorbance at 470 nm because of guaiacol oxidation (Nickel and Cunningham, 1969).

Quantification of Melatonin

According to Byeon and Back (2014) and (Yan et al., 2019b), 0.2 g samples were ground to a powder in liquid nitrogen and then extracted with 1.5 mL methanol at 4°C for 30 min. After centrifugation of the extraction mixture for 5 min at 8,000 g, the supernatant was taken. The precipitation was repeatedly extracted once. The two extracts were combined, then filtrated with 0.22 μm filter membrane, evaporated to dryness and dissolved in 0.2 mL of 40% methanol. Aliquots of 10 μL were subjected to HPLC using a fluorescence detector system (Waters). The samples were separated on a Sunfire C18 column (4.6 × 150 mm; Waters) and the mobile phase constitution was water:methanol = 6:4. The flow rate was 1 mL/min. Melatonin was detected at 286 nm excitation and 352 nm emission wavelengths. The melatonin was eluted at 8.9 min under these conditions. The standard curve was Y=963.3X+0.7044; R = 0.9999, with the melatonin concentrations of 0.03, 0.05, 0.1, 0.5, 1, 5 μg /mL were used.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from cucumber leaves using the TRIzol method according to the supplier's instructions (Invitrogen, Carlsbad, CA, United States). DNase was used during RNA extraction to reduce DNA contamination. cDNA synthesis was performed according to standard procedures of a Revert Aid First Strand cDNA synthesis kit (Fermentas, Ontario, Canada). The cucumber actin gene was used as the internal control for the quantification of transcripts. Real-time quantitative PCR using an aliquot of cDNA (1/500), Power SYBR Green PCR Master Mix (*ABI*), and 200 nM each primer on an ABI Prism 7900 HT machine. Data were analyzed using SDS 2.500 software (*ABI*), and relative expression was calculated using the comparative cycle threshold method with normalization of data to the geometric average of the internal control genes (Pfaffl, 2001).

The primers of *TDC* (*Csa3G611340*; F: 5′-ACCATCG TCGTCTTCGTTATC-3′ and R: 5′-CATTTCTCTGCTCGGAC TTCT-3′), *T5H* (*Csa6G501350*; F: 5′-GCCTGGTTCACACCAT CATA-3′ and R: 5′-ATGCTGGAAGTGTGGATTAGG-3′), *SNAT* (*Csa4G336250*; F: 5′-CGGGTAGCTGAAGAAGAAG AAG-3′ and R: 5′-AAATGGCCGGAGCAAAGA-3′), *COMT* (*Csa4G091880*; F: 5′-TCCGACCATTCCACCATTAC-3′ and R: 5′- CCGACATCCACCACTGAATTA-3′), and *ACTIN* (*Csa6G484600*; F:5′-CAGGAACTTGAGACTGCTAAGA−3′ and R: 5′-CGATGAGAGATGGCTGGAATAG-3′). Primer search method: the protein encoded by the gene in *Arabidopsis thaliana* was obtained by comparing tomato genes in the literature (Xu, 2016; Ahammed et al., 2018). Gcorn plant (http://www.plant.osakafu-u.ac.jp/~kagiana/gcorn/f/19/) was used to find the protein encoded by the direct homologous gene of this protein in cucumber, and then compared in cucumber genome to obtain the most similar gene. Tomato Genome website: https://solgenomics.net/tools/blast. Cucumber Genome website: http://cucurbitgenomics.org/. Arabidopsis Genome website: https://www.arabidopsis.org/.

Statistical Analysis

Data were plotted using Microsoft Excel 2010 software. Data were presented as the mean ± standard deviation of three replicates (five plants in each replicate). Statistical analyses were carried out by ANOVA using SAS software. Differences between treatments were determined by the least significant differences with $p < 0.05$.

RESULTS

The Dose-Dependent Curves for Plant Growth in Response to Exogenous Selenium

To determine the appropriate level of selenium for cucumber seedlings growth, plant height and element content of 30-day-old cucumber seedlings on 0, 1, 10, 50, 100, and 1,000 μM sodium selenite treatments for 10 days were compared as shown in **Figure 1**. Compared with no selenium treatment, treatment with exogenous selenium significantly enhanced plant height

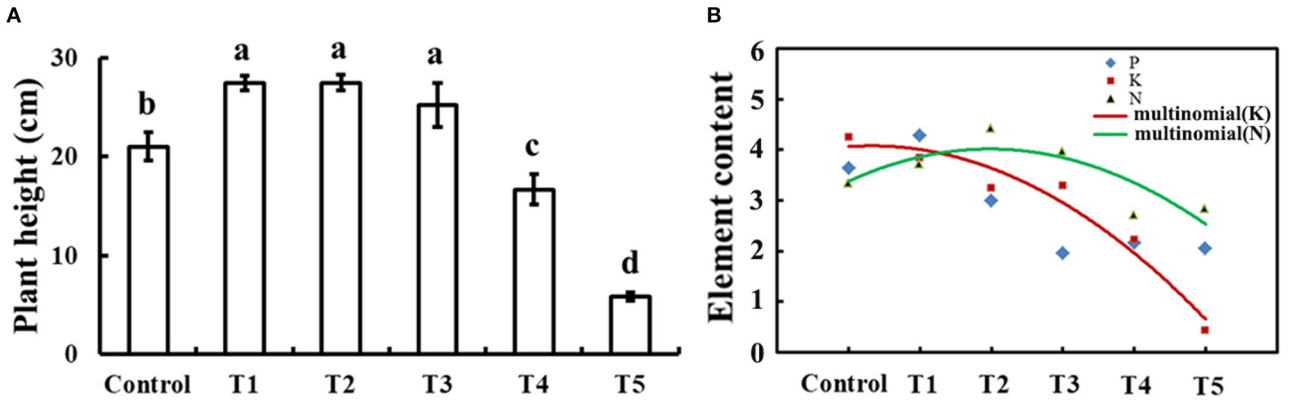

FIGURE 1 | Plant height and element contents in response to exogenous selenium with different concentrations. **(A)** Plant height of cucumber seedlings treated with 0, 1, 10, 50, 100, and 1,000 μM exogenous selenium for 10 days. These values are expressed as means of three replicates ± SD, and different letters are significantly different ($P < 0.05$). **(B)** Element (N, P, and K) content with 0, 1, 10, 50, 100, and 1,000 μM exogenous selenium for 10 days in cucumber seedlings root. These values are expressed as means of three replicates.

within the concentrations applied from 1 to 50 μM sodium selenite ($p < 0.05$). However, 100 and 1,000 μM sodium selenite treatments significantly inhibited plant growth (**Figure 1A**). A dose-dependent response was observed with low concentrations of selenium promoting plant growth and high concentrations inhibiting plant growth. Based on the different concentrations of selenium treatments in cucumber seedling root, results showed that the relative content of nitrogen presented a trend of rise and then decline, and in 10 μM sodium selenite of T2 treatment, the nitrogen content peaked. 1, 10, and 50 μM concentrations of sodium selenite promoted the cucumber seedling root nitrogen accumulation. The relative contents of potassium decreased with the content of sodium selenite increased. Phosphorus showed a trend of rise, then reduced, and the final leveling off, and 1 μM sodium selenite treatment showed phosphorus accumulated in cucumber seedling roots the most. Fitting the trend line of nitrogen and potassium, the trend lines on the intersection under 1–10 μM sodium selenite treatments, and meet 1 μM sodium selenite treatments closer. Under the condition of no significant difference in plant growth, the higher root nutrient element contents, the more plant growth potential (Kulcheski et al., 2015). According to a comprehensive estimation for these parameters, the most powerful concentration of selenium (1 μM) was used for further studies.

The Response of Exogenous Selenium to Cold Tolerance of Cucumber Seedlings

Figure 2A shows that 1 μM sodium selenite treatment significantly alleviated cold tolerance of cucumber seedlings, shoot weight, and leaf area had been detected (**Figures 2B,C**). Selenium application significantly increased leaf size and shoot fresh weight of cucumber seedlings. Not only that, but selenium application significantly alleviated the decrease of leaf size and shoot fresh weight caused by cold stress. Thus exogenous selenium application significantly alleviated the inhibition of cold stress on cucumber seedling growth. Under normal culture conditions, the SPAD value and the net photosynthetic rate of cucumber leaves were significantly increased by selenium application (**Figures 2D,E**). Exogenous selenium application significantly increased the SPAD value of cucumber leaves compared with no selenium application (**Figure 2D**). Compared with 5 days of cold stress, SPAD value of 10 days of cold stress decreased significantly. The net photosynthetic rate of cucumber leaves decreased significantly under cold stress, and the net photosynthetic rate of cucumber leaves under 10 days of cold stress was significantly lower than that of the leaves under 5 days of cold stress.

Exogenous Application of Selenium Protected the Photosynthetic Apparatus and Alleviated the Oxidative Stress Induced by Cold Stress

As a photosynthetic apparatus, chloroplast ultrastructures of cucumber leaves were significantly affected by exogenous application of selenium compared with control conditions. Exogenous selenium application increased the number and the size of starch grains (**Figure 3A**). However, cold stress decreased the number of starch grains and increased the number of osmiophilic granules. Under cold stress condition, exogenous application of selenium increased starch grains compared with no selenium application treatment under cold stress condition (**Figure 3A**). Histochemical observation of O_2^- in leaves using NBT staining corroborated the biochemical analysis, indicating that the accumulation of reactive oxygen species (ROS) was significantly increased, especially under cold stress condition after 10 days. However, the exogenous application of selenium increased the ROS-scavenging capability in cold stressed cucumber leaves (**Figure 3B**). The activities of the investigated ROS scavenging-related enzymes were greater in the treatment of selenium application than no application of selenium under cold stress (**Figure 3C**). Under control conditions, some enzymes, such as SOD, POD, CAT, and APX,

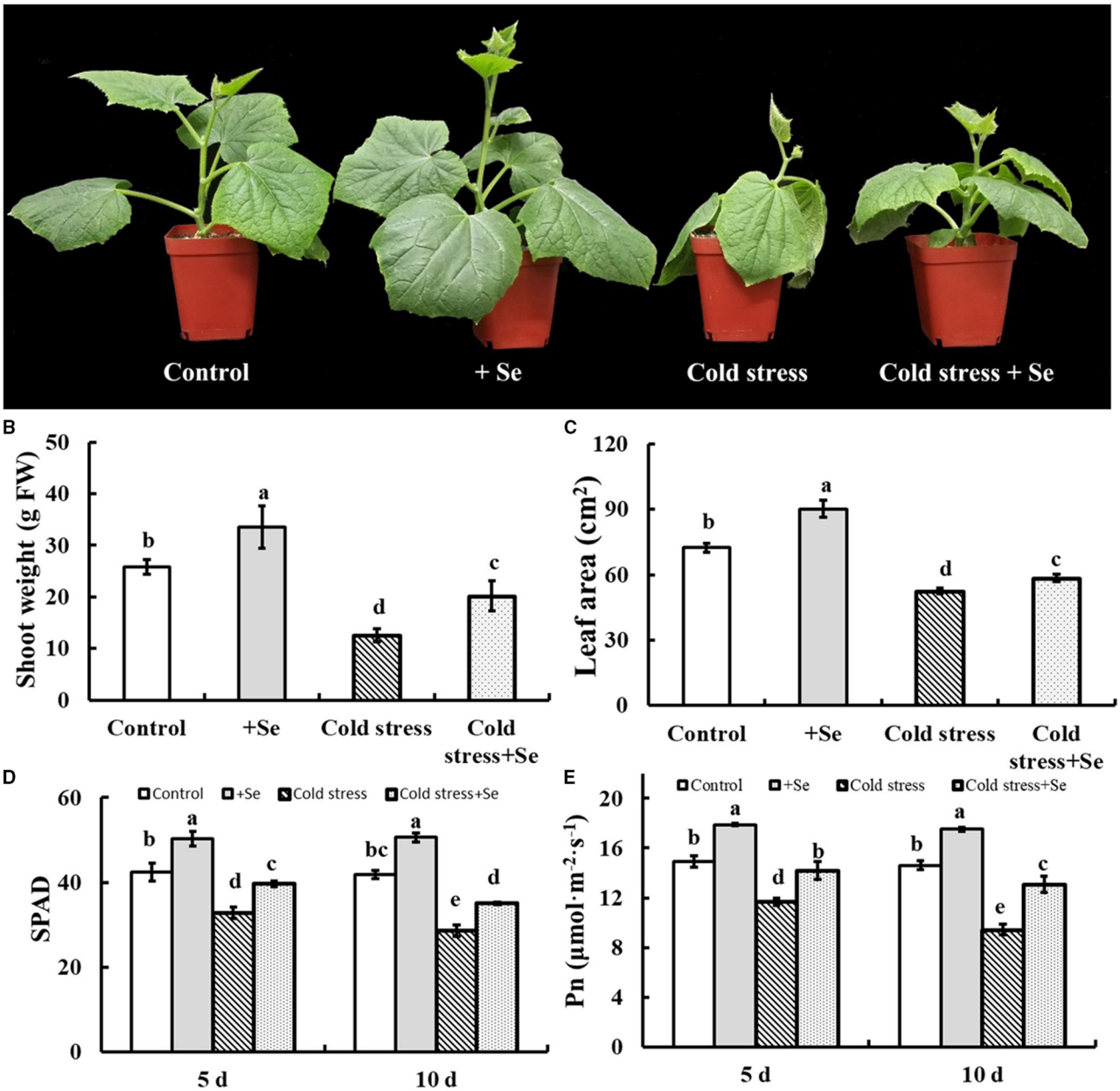

FIGURE 2 | Effects of exogenous selenium on cold stress of cucumber seedlings. **(A)** The phenotype of control, selenium application, cold stress, and selenium application under cold stress. **(B)** Shoot weight of control, selenium application, cold stress, and selenium application under cold stress. **(C)** Leaf area of control, selenium application, cold stress, and selenium application under cold stress. **(D)** SPAD value of control, selenium application, cold stress, and selenium application under cold stress with 5 and 10 days treatments, respectively. **(E)** Net photosynthetic rate of control, selenium application, cold stress, and selenium application under cold stress with 5 and 10 days treatments, respectively. Values are expressed as means of three replicates ± SD, and different letters are significantly different ($P < 0.05$).

also showed greater activity levels in the treatment of exogenous selenium application (**Figure 3C**).

Effects of Exogenous Selenium on the Endogenous Melatonin

To discover whether melatonin participates in selenium-enhanced cold tolerance of cucumber seedlings, the pharmacologic method was used. Application of selenium significantly increased the growth of cucumber seedling, which was shown as the shoot weight significantly increased with selenium application compared with normal condition (**Figures 4A,B,G**). Under cold stress condition, plant shoot weight decreased significantly, whereas plant shoot weight loss reduced when treated

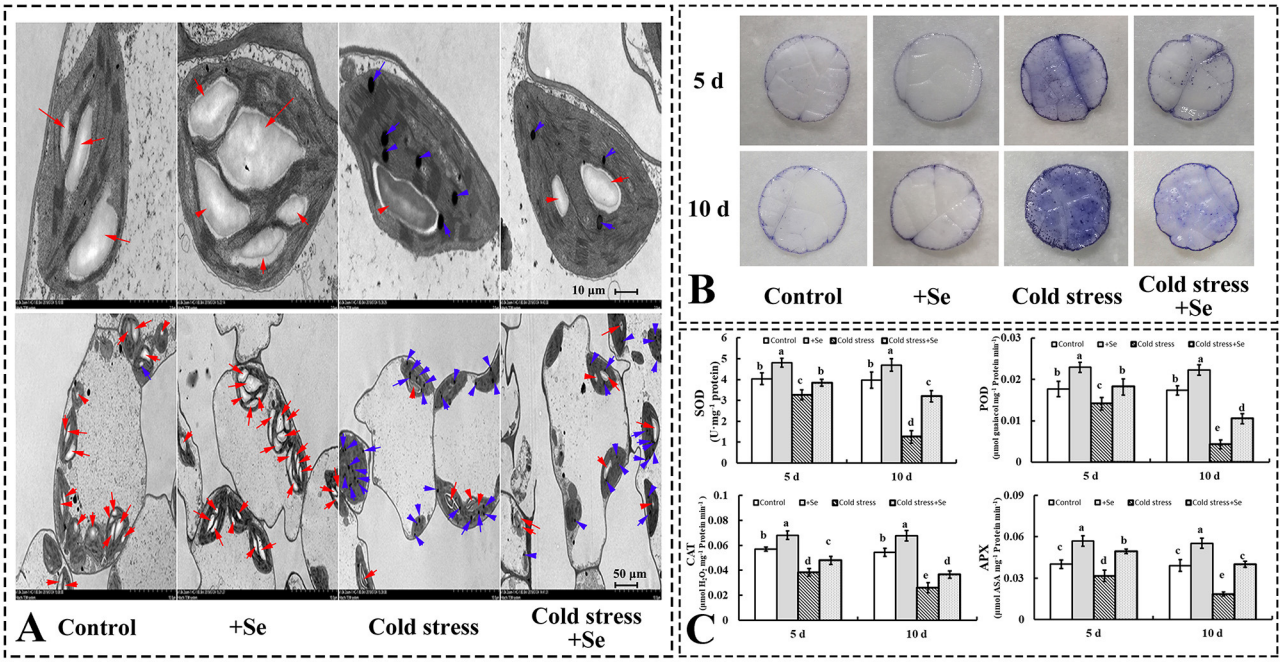

FIGURE 3 | Exogenous application of selenium protected the photosynthetic apparatus and alleviated the oxidative stress induced by cold stress. **(A)** Ultrastructures of cells of cucumber seedlings leaf of control, selenium application, cold stress, and selenium application under cold stress. Red arrow points to starch grain, blue arrow points to osmiophilic granule. **(B)** O_2^- in leaves was detected by NBT staining. **(C)** The activity levels of SOD, POD, CAT, and APX of control, selenium application, cold stress, and selenium application under cold stress with 5 and 10 days treatments, respectively. Values are expressed as means of three replicates ± SD, and different letters are significantly different ($P < 0.05$).

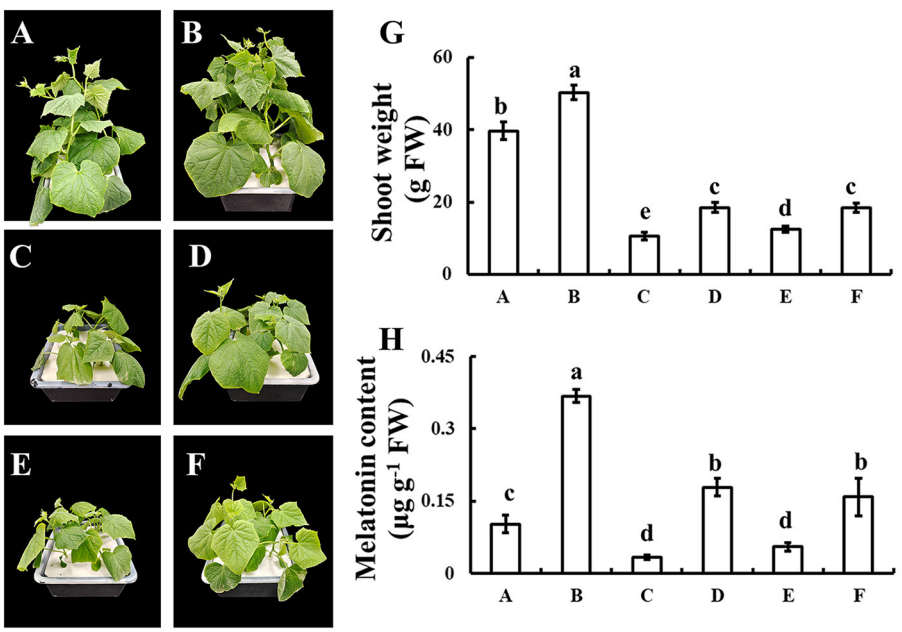

FIGURE 4 | Effects of exogenous selenium on endogenous melatonin. **(A–D)** The phenotype of control, selenium application, cold stress, and selenium application under cold stress condition. **(E)** The phenotype of selenium and melatonin biosynthesis inhibitor application under cold stress condition. **(F)** The phenotype of selenium, melatonin, and melatonin biosynthesis inhibitor application under cold stress condition. **(G)** Shoot weight of A-F. **(H)** Melatonin content of A-F. Values are expressed as means of three replicates ± SD, and different letters are significantly different ($P < 0.05$).

with selenium (**Figures 4C,D,G**). Treatment with both selenium and CPA showed only a partial alleviation of cold stress effects (**Figures 4D,E,G**). Adding selenium, melatonin, and melatonin biosynthesis inhibitor under cold stress condition restored phenotype to only treated with selenium under cold stress condition (**Figures 4D,F,G**).

Genes Expression Related With Melatonin in Cucumber Seedlings

It was demonstrated that melatonin plays a vital role in enhancing cold tolerance by the application of exogenous selenium. A series of genes related to melatonin biosynthesis was analyzed by RT-QPCR to detect the expression of those genes that showed more response to selenium under cold stress condition (**Figure 5**). The expression of *T5H* and *SNAT* increased after the application of selenium under normal conditions. However, under cold condition, application of selenium significantly increased the expression of *T5H* and *COMT*. These indicated that selenium induced higher expression of *T5H*, with the effect further enhanced by the cold stress condition. The expression of *COMT* increased even hundredfold with the application of selenium under cold stress condition compared with normal conditions. **Figure 5** also shows that the expression of *TDC* increased significantly under cold stress condition, while there seemed to be no significant changes with selenium application neither under cold stress nor under normal conditions. These suggested that *T5H* and *COMT* might be vital in enhancing melatonin content with selenium application under cold stress condition.

DISCUSSION

Selenium has been studied in plants undergoing adaptation to unfavorable stresses and fruit quality (Zhu et al., 2018; Wen, 2021). A previous study showed that the treatments with 0.5 and 1.0 mg/kg Se significantly increased biomass and chlorophyll content of wheat seedlings (Chu et al., 2010). Approximately 5 mg/L of sodium selenite solution had the greatest stress-alleviating effects that assist in protecting strawberry seedlings in a low-temperature environment (Huang et al., 2018). Cucumber, as a warm-loving vegetable, is vulnerable to cold stress in winter and spring. No studies have shown that the exogenous application of selenium enhances cold tolerance of cucumber, let alone explore its application concentration. Because of the difference in selenium concentration apply to different crops, the article reported that 1–50 μM selenium concentration significantly increases the plant height of cucumber seedlings under normal conditions. Meanwhile, through the determination of root element contents of cucumber seedlings, it was determined that the content of N, P, and K in the root system was optimal under the treatment of 1 μM selenium. The high content of N, P, and K in the root system not only indicated that the overall development of the root system was good, but also indicated that the root system had a good potential to transport nutrients to the shoot in the future. When there was no significant difference in the shoot height, the comparison of element content in the root system could effectively distinguish the subsequent development direction and difference in plants.

Hence, 1 μM selenium was used and our study also showed that 1 μM selenium effectively enhances cold tolerance of cucumber seedlings. This study showed that the exogenous selenium effectively reduced the inhibition on shoot weight of cucumber seedlings under cold stress condition (**Figure 2B**). The accumulation of fresh shoot weight depends on plant photosynthesis (Wen et al., 2021), and selenium effectively reduced the inhibition of the net photosynthetic rate of a plant under cold stress. On the one hand, the application of selenium effectively increased the leaf area of functional leaves of cucumber seedlings under cold stress condition, and then increased the photosynthetic area of the plant. On the other hand, selenium application significantly increased SPAD value, chlorophyll content, and net photosynthetic rate of leaves under the same leaf area under cold stress condition. Interestingly, there was no significant difference in the SPAD value of cucumber leaves under 5 days of cold stress and 10 days of cold stress when exogenous selenium was applied, and the net photosynthetic rate of cucumber leaves under 10 days of cold stress was significantly higher than that under 5 days of cold stress. These results indicated that selenium application significantly enhanced the increase of net photosynthetic rate under cold stress, and the increase of selenium not only increased the content of chlorophyll in leaves but also involved other regulatory pathways.

Because exogenous selenium application significantly affected the photosynthesis of plants under cold stress, transmission electron microscopy was used to observe the subcellular structure of cucumber seedling functional leaves (**Figure 3A**). Starch grains representing nutrient accumulation decreased significantly under cold stress, but the exogenous application of sodium selenite could reduce the decrease of starch grains. Stress easily leads to photoelectron transfer spilt and produces ROS (Wen et al., 2019). The detection of O_2^- showed that exogenous application of selenium reduced the content of superoxide anion. It was found that exogenous selenium could effectively improve the activity of antioxidant enzymes, and then reduce the production of ROS that damage the cells. Therefore, exogenous selenium application promoted the accumulation of organic matter produced by photosynthesis while also reducing the damage caused by the generation of ROS to cells under cold stress condition.

Melatonin plays an important role in plant resistance (Li et al., 2016; Wen et al., 2016; Gong et al., 2017; Yan et al., 2019a). Melatonin maintained cell membrane stability, increased antioxidant enzymes activities, improved the process of photosystem II, and induced alterations in Bermudagrass and rice metabolism under cold stress (Fan et al., 2015; Han et al., 2017). It has been reported that exogenous melatonin alleviates cold stress by upregulating the expression of C-repeat-binding factors, a cold-responsive gene, *COR15a*, et al., and stimulate the biosynthesis of cold-protecting compounds (Bajwa et al., 2014). There are also

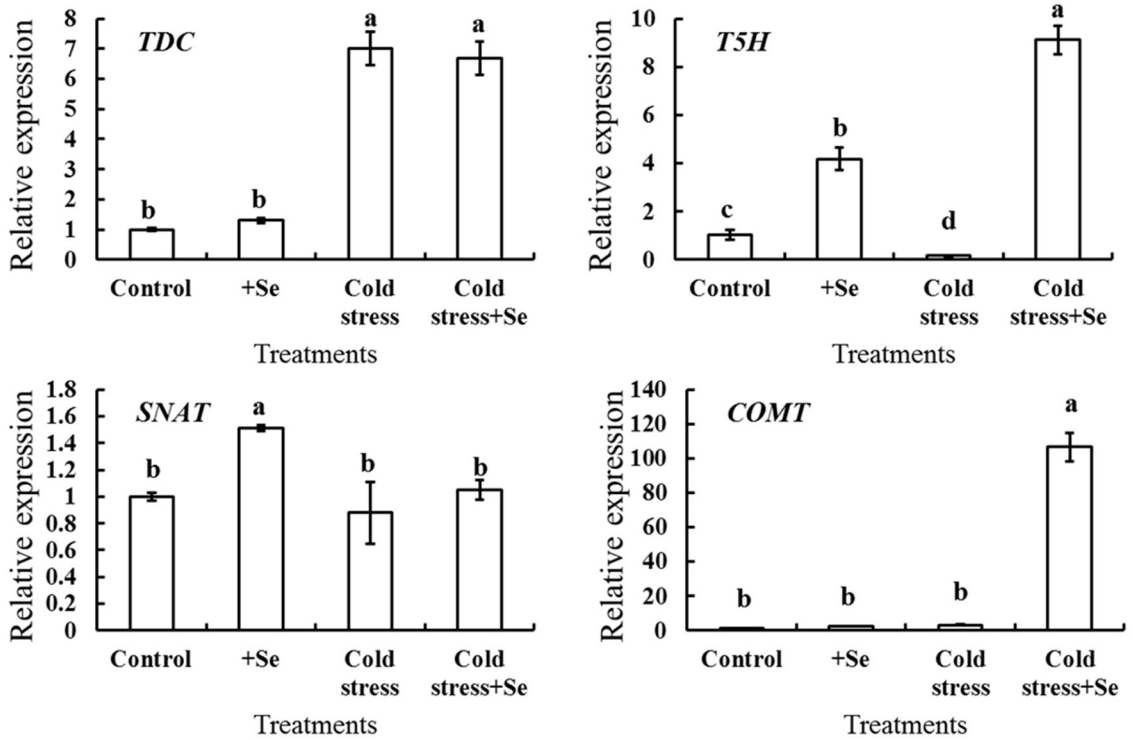

FIGURE 5 | Relative expression of melatonin biosynthesis genes in response to selenium treatment. RT-QPCR analyses was used to assess the relative expression of *TDC*, *T5H*, *SNAT,* and *COMT* in cucumber seedlings leaf of control, selenium application, cold stress, and selenium application under cold stress. Values are expressed as means of three replicates ± SD, and different letters are significantly different ($P < 0.05$).

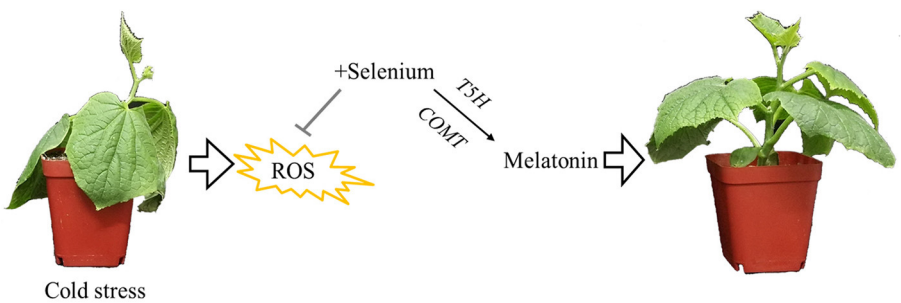

FIGURE 6 | Schematic illustration for melatonin participated in enhancing cold tolerance by exogenous application of selenium. Cold stress inhibited plant growth and increased the production of reactive oxygen species; however, selenium reduced cold stress damage to the plant. Meanwhile, melatonin as the downstream signaling participated in selenium-enhanced cold tolerance of cucumber seedlings. Melatonin biosynthesis genes *T5H* and *COMT* play vital roles in exogenous selenium application enhancing the melatonin content, promoting plant cold tolerance.

reports that melatonin enhances cold tolerance by regulating energy and proline metabolism (Liu et al., 2020). Most studies focused on melatonin alleviating plant cold stress, and our studies reported that melatonin as a downstream signal was involved in the improvement of exogenous selenium application by enhancing tolerance of cucumber seedlings under cold stress condition (**Figure 4**). The present study provided several lines of evidence that melatonin, as a downstream signal, was involved in selenium enhanced cold tolerance of cucumber seedlings. First, selenium increased the content of melatonin, both under normal and cold stress conditions. Second, the alleviating effect of exogenous selenium application on cold stress was significantly inhibited by melatonin synthesis inhibitors. Third, the expression of melatonin biosynthesis genes *T5H* and *COMT* increased significantly with selenium treatment under cold stress of

cucumber seedlings, which might lead to the increase of melatonin content. It has been reported in tomato plant that *COMT* silencing aggravates heat stress leading to the reduction in photosynthesis (Ahammed et al., 2018), which confirms our results in another way. At the same time, these give the hypotheses, whether the combination application, the precursor of melatonin tryptophan and selenium, effectively improves the cold tolerance of cucumber seedlings, which has important reference and theoretical support for the application of improving plants' cold tolerance in agriculture, needs to be verified by more experiments.

Based on our results and analyses, a schematic illustration of a possible mechanism for melatonin involvement in enhancing cold tolerance by exogenous selenium application in cucumber seedlings was prepared as seen in **Figure 6**. Cold stress inhibited plant growth and increased the production of ROS; however, selenium reduced cold stress damage to the plant. Meanwhile, melatonin as the downstream signaling participated in selenium-enhanced cold tolerance of cucumber seedlings. Melatonin biosynthesis genes *T5H* and *COMT* play vital roles in exogenous selenium application enhancing melatonin content promoting plant cold tolerance. However, research on selenium and melatonin signals in plants is just at the beginning, and more work is needed to gain a more accurate understanding of the signal pathway in cold tolerance regulation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

DW, NY, and KS: conceived and designed the research. NY and XK: performed the research. NY and DW: analyzed the data. KS, XW, KW, and JG: contributed materials/analysis tools. NY: wrote the first draft of the manuscript. DW: improved the first draft of the manuscript. All the authors have read and approved this manuscript.

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Jasmonate and Melatonin Act Synergistically to Potentiate Cold Tolerance in Tomato Plants

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Both jasmonic acid (JA) and melatonin (MT) have been demonstrated to play positive roles in cold tolerance, however, whether and how they crosstalk in the cold responses in plants remain elusive. Here, we report that JA and MT act synergistically in the cold tolerance in tomato plants (*Solanum lycopersicum*). It was found that JA and MT were both substantially accumulated in response to cold stress and foliar applications of methyl jasmonate (MeJA) and MT promoted cold tolerance as evidenced by increased Fv/Fm, decreased relative electrolyte leakage (EL) and declined H_2O_2 accumulation in tomato plants. Inhibition of MT biosynthesis attenuated MeJA-induced cold tolerance, while inhibition of JA biosynthesis reduced MT accumulation in tomato plants under cold conditions. Furthermore, qRT-PCR analysis showed that the expressions of two MT biosynthetic genes, *SlSNAT* and *SlAMST*, were strongly induced by MeJA, whereas suppression of *SlMYC2*, a master JA signaling regulator, abated the expressions of *SlSNAT* and *SlAMST* under cold stress. Additionally, suppression of *SlMYC2* reduced MT accumulation, decreased Fv/Fm and increased EL in cold-stressed tomato plants. Interestingly, exogenous MT promoted JA accumulation, while inhibition of MT biosynthesis significantly reduced JA accumulation in tomato plants under the cold condition. Taken together, these results suggest that JA and MT act cooperatively in cold tolerance and form a positive feedback loop, amplifying the cold responses of tomato plants. Our findings might be translated into the development of cold-resistant tomato cultivars by genetically manipulating JA and MT pathways.

Keywords: jasmonic acid, melatonin, crosstalk, cold tolerance, tomato

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INTRODUCTION

Unlike animals, plants are sessile and are unable to escape unfavorable growth conditions. Thus, they have to cope with diverse environmental challenges through their life cycles, such as pathogens, extreme temperatures, salinity and drought. Cold, consisting of chilling (0–15°C) and freezing (<0°C), is a recognized environmental stress factor that impairs plant growth and development,

restricts geographical distribution of plants in nature, and threatens agricultural productivity of many crop species, especially those of tropical or subtropical origin, including tomato (*Solanum lycopersicum*), maize (*Zea mays*), and soybean (*Glycine max*), among others (Lee et al., 1986; Chinnusamy et al., 2007; Ding et al., 2019). There are several adverse effects of cold stress on plant cells, including (1) cold stress leads to overproduction of reactive oxygen species (ROS), which subsequently causes oxidative damages to nucleic acids, proteins and membranes, and finally disrupts cell functions (Apel and Hirt, 2004; Ruelland et al., 2009); (2) cold stress changes membrane rigidification, which has been proved a key event that induces cold responses (Örvar et al., 2000); (3) cold stress disturbs stability of proteins and inactivates key enzymes involved in essential biological processes. For instance, cold stress impairs photosynthesis by reducing the activity of a Calvin-Benson cycle enzyme sedoheptulose-1,7-bisphosphatase (Ding et al., 2017c). To survive under the cold condition, plants have evolved elaborate mechanisms that improve cold tolerance. One notable example of these mechanisms is the enhancement of antioxidant capacity, which is crucial for ROS homeostasis and mitigation of cold-induced oxidative damages to plant cells (Wang M. et al., 2020). Plants also accumulate more low-molecular-mass solutes under cold stress, including soluble sugars, proline and polyamines, to protect themselves from cold damages (Ruelland et al., 2009; Ding et al., 2017a).

Jasmonates (JAs) are a class of lipid-derived phytohormones, including jasmonic acid and its derivatives, such as methyl jasmonate (MeJA), jasmonoyl-isoleucine (JA-Ile), and 12-OH-JA (Gidda et al., 2003; Świątek et al., 2004; Suza and Staswick, 2008). A growing number of studies show that JAs play crucial roles in plant responses to cold stress (Sharma and Laxmi, 2016; Ding et al., 2020). Upon cold treatment, JA accumulation is increased in *Arabidopsis*, leading to the degradation of JA signaling repressors, JASMONATE-ZIM-DOMAIN PROTEIN 1 (JAZ1) and JAZ4 proteins, which interact with and repress the ICE1-CBF module. Thus, JA improves cold tolerance by activating ICE1-CBF cascade in *Arabidopsis* (Hu et al., 2013). JA also positively regulates cold tolerance in rice, as *HAN1*, which encodes an oxidase catalyzing active JA-Ile to inactive 12-OH-JA-Ile, reduces chilling tolerance (Mao et al., 2019). Emerging evidence indicates that JA also confers cold tolerance in multiple horticultural plant species. In apple (*Malus hupehensis*), JA signaling promotes cold tolerance through the JAZ-BBX37-ICE1-CBF pathway, in which MdJAZ1 and MdJAZ2 interact with BBX37 to repress MdICE1 and two MdCBFs (An et al., 2020). MYC2, a positive regulator of JA signaling, confers cold tolerance by interacting with ICE1 in banana (*Musa acuminata*) (Zhao et al., 2013). JA has also been shown to increase cold tolerance by promoting biosynthesis of osmolytes. In trifoliate orange (*Poncirus trifoliata*), MYC2 activates the transcriptional expression of *PtrBADH-l* to promote glycine betaine biosynthesis, thus conferring increased cold tolerance (Ming et al., 2020). More recently, JA is found to increase chilling tolerance of tomato plants and fruits through MYC2-mediated polyamine biosynthesis (Ding et al., 2021; Min et al., 2021). Though great progress has been made in the understanding of JA-induced cold tolerance, yet the underlying mechanisms by which JA regulates cold tolerance are still not fully understood.

Melatonin (*N*-acetyl-5-methoxytrytamine, MT), structurally similar to indole-3-acetic acid (IAA), is a multifunctional molecule in animals, humans, plants, and algae (Vivien-Roels and Pévet, 1993; Fuhrberg et al., 1996; Ding et al., 2018a,b; Arnao and Hernández-Ruiz, 2019b). The presence of MT in plants was confirmed in 1995 (Dubbels et al., 1995; Hattori et al., 1995) and afterward, plenty of studies have revealed the versatile roles of MT in plant growth, development and stress responses. Due to its multifunctionality and recent identification of a phytomelatonin receptor PMTR1, MT has been proposed as a master regulator and a potential new hormone in plants (Wei et al., 2018; Arnao and Hernández-Ruiz, 2019a). MT has also been shown to crosstalk with different phytohormones to act in a variety of biological processes. For instance, MT interacts with auxin (Weeda et al., 2014; Wang Q. et al., 2016; Wen et al., 2016), ABA (Li et al., 2012; Zhang et al., 2014; Fu et al., 2017; Jahan et al., 2021), gibberellins (Zhang et al., 2014; Jahan et al., 2021; Lv et al., 2021), ethylene (Sun et al., 2015, 2016; Chen et al., 2021), SA (Chen et al., 2021), and brassinosteroids (Hwang and Back, 2018) either by regulating their biosynthesis or signaling pathways. Recently, several lines of evidence suggest that MT is also related to phytohormone jasmonic acid. In watermelon plants (*Citrullus lanatus*), melatonin action on cold tolerance is partly ascribed to MT-induced accumulation of MeJA (Li et al., 2021). In another study, MT is shown to mediate defense response against Huanglongbing, a devastating citrus (*Citrus sinensis*) disease, via crosstalk with JA signaling pathway (Nehela and Killiny, 2020). Moreover, MT promotes root development of copper-stressed melon plants by inhibiting JA biosynthesis (Hu et al., 2020). In addition, MT suppresses JA-induced tomato leaf senescence (Wang et al., 2019). Finally, MT is involved in the MeJA-mediated delay of cassava deterioration during postharvest storage (Liu et al., 2019).

Melatonin has been found to improve cold tolerance in a number of plant species, such as tomato (Ding et al., 2017a,b; Zhou et al., 2019; Wang M. et al., 2020), tea plants (*Camellia sinensis*) (Li et al., 2019), watermelon (Li et al., 2021), bermuda grass (*Cynodon dactylon*) (Shi et al., 2015) and Arabidopsis (Shi and Chan, 2014), among others. As both MT and JA contribute to cold tolerance in plants, we hypothesized that there may exist crosstalk between MT and JA in plant responses to cold stress. In this study, we provide evidence that JA and MT act synergistically to potentiate cold tolerance in tomato plants and we propose that JA and MT form a positive loop that amplifies tomato responses to cold stress.

MATERIALS AND METHODS

Plant Materials

Tomato plants (*Solanum lycopersicum* "MicroTom"), including wild-type tomato plants and *MYC2-RNAi* transgenic tomato plants (Ding et al., 2021) were used in this study. Tomato seeds

were germinated and grown in plastic pots filled with peat and vermiculite. Tomato plants were grown in a growth chamber with the following settings: day/night temperature 25°C/20°C, 14-h photoperiod (200 μmol m^{-2} s^{-1} photosynthetic photon flux density), relative humidity ∼60%.

Treatments

For determination of JA and MT accumulations under cold conditions, at 4-leaf stage, tomato plants were subjected to cold stress at 4°C for 24 h. For determination of cold tolerance affected by exogenous MeJA and MT, tomato plants were pretreated with mock (1/10,000 ethanol), 100 μM MeJA, or 100 μM MT 12 h prior to cold treatment. Tomato plants were treated for 24 h for physiological assessment and 60 h for phenotype analysis. To block the biosynthesis of JA, a JA biosynthesis inhibitor DIECA (diethyldithiocarbamic acid) was used. Tomato plants were treated with 2 mM DIECA 12 h before being subjected to cold stress. To block the biosynthesis of MT, 100 μM CPA (*p*-chlorophenylalanine, a MT biosynthesis inhibitor) was used (Li et al., 2021) to treat tomato plants prior to cold stress.

To assess the induction of MT biosynthetic genes by MeJA, fully expanded young leaves were detached from tomato plants at 4-leaf stage and were incubated in 50 μM MeJA. Leaves were harvested at 0, 2, 4, 8, and 12 h following MeJA treatment and were used for qRT-PCR analysis. To determine the regulation of MT biosynthesis by SlMYC2, detached leaves from two *SlMYC2-RNAi* transgenic lines were incubated in 50 μM MeJA for 12 h and were collected for qRT-PCR analysis. Similarly, to assess the induction of JA biosynthetic genes by MT, detached leaves were incubated in 50 μM MT and leaves were collected at 0, 2, 4, 8, 12, and 24 h following MeJA treatment.

Cold Tolerance Assays

Cold tolerance was evaluated by measuring Fv/Fm, EL, and H_2O_2 accumulation. Fv/Fm was obtained with a portable chlorophyll fluorometer (PAM-2000, Walz, Germany) as described in a previous study (Ding et al., 2016). Tomato plants were first dark adapted for 30 min and the minimal fluorescence (Fo) was obtained. Then a saturating pulse was used to yield the maximal fluorescence (Fm). Finally, the maximum quantum efficiency (Fv/Fm) was calculated.

Electrolyte leakage measurement was performed following a previous study (Ding et al., 2018c). Leaf samples were collected and incubated in deionized water and the conductivity of the incubated solution was measured as C1. Leaf samples were then boiled and the conductivity of the solution was measured as C2. The relative EL was calculated as the ratio of C1/C2.

Quantification of H_2O_2 was performed according to previous studies (Patterson et al., 1984; Wang M. et al., 2020). Leaf samples were ground with 5% (w/v) trichloroacetic acid. The resulting homogenate was centrifuged and the supernatant was mixed with $TiCl_2$ to form the Ti–H_2O_2 complex, which was further precipitated using ammonia solution. The resulting precipitate was resuspended in H_2SO_4 and the absorbance of the solution was measured at 410 nm and was used for the calculation of H_2O_2 content.

Quantification of Melatonin

Quantification of MT was performed by High-Performance Liquid Chromatography (HPLC) as previously described (Ding et al., 2017a). Briefly, frozen leaf samples were homogenized in chloroform and melatonin was extracted at 4°C in the dark. The crude extraction was then centrifuged at 4,000 × *g* for 5 min and the chloroform phase was purified using a C18 solid-phase extraction (SPE) cartridge. The extract was evaporated under N_2 gas and the pellet was resuspended in methanol for HPLC analysis. The HPLC system was equipped with a 5 μm Hypersil ODS column (C18) and a fluorescence detector. The mobile phase was methanol and was delivered at a flow rate of 1.0 mL min^{-1}. Twenty μL samples were injected into the system. For melatonin detection, the excitation wavelength was set at 280 nm and the emission wavelength was set as 348 nm.

Quantification of Jasmonic Acid

Quantification of JA was performed following published procedures with slight modifications (Wang F. et al., 2016). Briefly, leaf samples were ground into fine powder in liquid nitrogen and homogenized in ethyl acetate. The homogenate was shaken at 4°C overnight. Then, the homogenate was centrifuged at 18,000 × *g* for 10 min. The supernatant was collected and the pellet was resuspended with ethyl acetate, followed by centrifugation for 10 min at 18,000 × *g* and the supernatant was collected. The supernatants were mixed and evaporated to dryness using nitrogen gas. The residue was resuspended in methanol and centrifuged at 18,000 × *g* for 2 min, and the supernatants were subjected to analysis by HPLC. HPLC analysis was conducted with a 3.5 μm Agilent ZORBAX XDB column (C18). The mobile phase was a mixture of 0.1% formic acid and methanol at a flow rate of 0.3 mL min^{-1}. The column temperature was set at 40°C and 20 μL sample was injected into the system.

Quantification of Transcript Abundance by Quantitative Real-Time PCR

Quantification of transcript abundance was performed by quantitative real-time PCR (qRT-PCR). Briefly, total RNA was extracted from detached leaves treated with MeJA using RNAprep Pure Plant Kit (QIAGEN) according to manufacturer's instructions. The RNA was then used for cDNA synthesis. qRT-PCR was performed using a Premix Ex Taq kit (TaKaRa, Dalian, China). Tomato *ACTIN2* was used as an internal control. Specific primers used in this study were listed in **Supplementary Table 1**.

Statistical Analysis

The experiments were performed with three independent replicates. The values were presented as means ± standard deviations (SDs). The data were analyzed by means of ANOVA, and p-values < 0.05 were considered significantly different according to Tukey's test. Different letters represent significant difference at $p < 0.05$ in each figure.

RESULTS

Cold Stress Promotes the Accumulation of Jasmonic Acid and Melatonin in Tomato Plants

To investigate the actions of JA and MT in the cold tolerance of tomato plants, we first examined the endogenous accumulation of JA and MT in tomato plants following a 24 h cold treatment. It was shown that tomato plants under cold conditions accumulated remarkably more JA than those under control conditions (**Figure 1A**). Likewise, MT production was significantly enhanced by cold treatment, with MT content being increased by 88% (**Figure 1B**). These results indicate that both JA and MT are involved in the responses to cold stress in tomato plants.

Exogenous Jasmonic Acid and Melatonin Enhance Cold Tolerance in Tomato Plants

Having found that accumulations of JA and MT were increased in response to cold stress, we next assessed the roles of JA and MT in cold tolerance in tomato plants by applying exogenous MeJA and MT to tomato leaves and measuring maximum photochemical efficiency (Fv/Fm), relative electrolyte leakage (EL) and accumulation of hydrogen peroxide (H_2O_2). It was observed that cold stress markedly decreased Fv/Fm in tomato plants, while exogenous MeJA and MT alleviated cold-induced inhibition of Fv/Fm. Under cold stress, compared with foliar application of mock, application of MeJA and MT increased Fv/Fm by 41.7 and 33.3%, respectively (**Figure 2B**). Membrane integrity is closely associated with cold tolerance in plants, so we next examined the relative electrolyte leakage (EL) to evaluate membrane integrity of tomato plants subjected to different treatments. The results showed that cold stress increased EL by 63% in tomato leaves; However, application of MeJA decreased EL by 33% and application of MT decreased EL by 27% in comparison with application of mock under cold stress (**Figure 2C**), indicating that MeJA and MT exerted protective effective on membranes in tomato leaves under the cold condition. As cold stress generally results in hyperaccumulation of ROS, we also examined the level of H_2O_2 in cold-stressed tomato plants. It was found that the content of H_2O_2 was enhanced in tomato plants exposed to cold stress compared with that in tomato plants under control growth conditions, whereas application of MeJA and MT significantly reduced the level of H_2O_2 in tomato plants under cold stress (**Figure 2D**). Altogether, these results support the crucial roles of MeJA and MT in the cold tolerance of tomato plants.

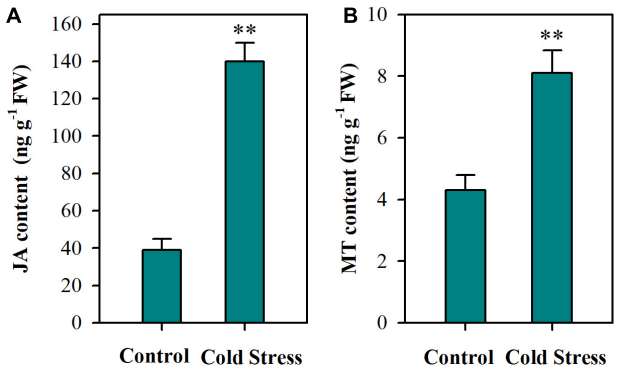

FIGURE 1 | Contents of JA and MT in tomato leaves under cold stress. **(A)** JA content; **(B)** MT content. At 4-leaf stage, tomato plants were subjected to cold stress at 4°C for 24 h. Following treatment, leaves were harvested for determination of JA and MT content. Data are mean values of three replicates ± SD. Asterisks represent significant difference at $P < 0.01$ according to Student's *t*-test.

Methyl Jasmonate Increases Cold Tolerance Partly Through Melatonin in Tomato Plants

To understand the role of MT in JA-mediated cold tolerance, we treated tomato plants with MeJA and a MT biosynthesis inhibitor (CPA), and assessed the changes in cold tolerance. Compared with application of mock under cold conditions, foliar application of MeJA significantly increased Fv/Fm by 30%, however, this MeJA-mediated increase in Fv/Fm was attenuated by application of CPA in tomato plants, with the increase being reduced by 14% (**Figure 3A**). We also performed EL analysis and the results showed that under cold stress, MeJA treatment led to decreased EL, while the combined treatment of MeJA and CPA abated the effect of MeJA on EL (**Figure 3B**). These results imply that MeJA-induced cold tolerance may be partly ascribed to MT.

As we have found that MeJA may act through MT in the cold tolerance of tomato plants, we next asked whether MeJA plays a role in the accumulation of MT in cold-stressed tomato plants. To answer this question, we treated tomato plants with MeJA and a JA biosynthesis inhibitor (diethyldithiocarbamic acid, DIECA) and determined the content of MT. It was observed that under normal growth conditions at 25°C, application of MeJA significantly increased MT content, while application of DIECA only marginally reduced MT content. Notably, under cold conditions at 4°C, MeJA largely boosted the accumulation of endogenous MT compared with mock, with the increase being 64%, whereas DIECA significantly inhibited MT accumulation of MT (**Figure 3C**). These results suggest that JA may have a crucial role in the biosynthesis of MT in tomato plants under cold stress.

Methyl Jasmonate Positively Regulates the Transcriptional Expression of Melatonin Biosynthetic Genes in Tomato Plants

To explore the potential mechanisms of JA-mediated MT accumulation, we incubated detached tomato leaves in 50 μM MeJA for 12 h and determined the transcript abundance of three tomato genes through qRT-PCR analysis, including *SlSNAT* (Solyc10g074910) (Wang X. et al., 2020), *SlTDC* (Solyc09g064430) (Li et al., 2016) and *SlAMST* (Solyc03g080180) (Xu et al., 2016), which have been demonstrated to act in MT biosynthesis in tomato plants. Following a 4 h MeJA treatment,

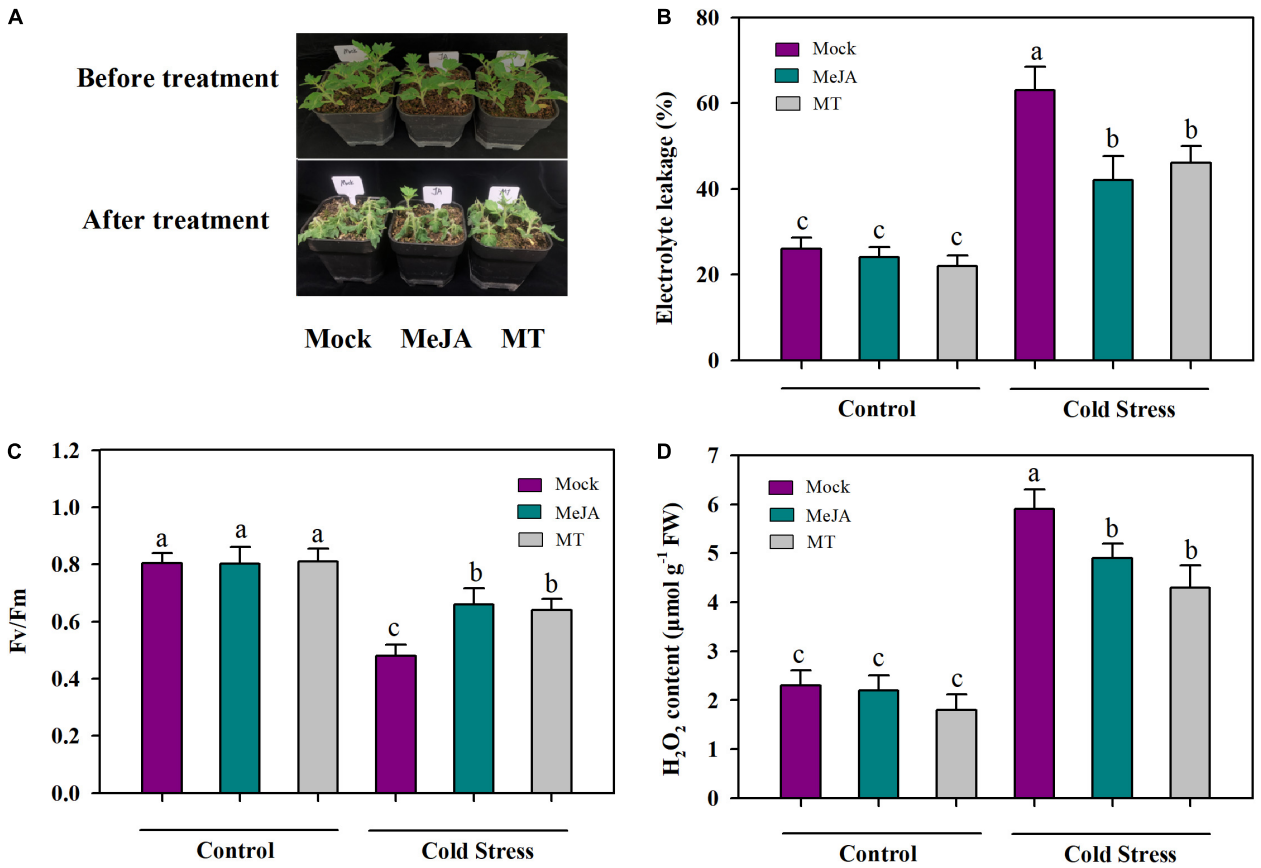

FIGURE 2 | MeJA and MT promote cold tolerance. **(A)** Representative MeJA- and MT-treated tomato plants after cold stress. **(B)** Fv/Fm; **(C)** EL; **(D)** H_2O_2 content. At 4-leaf stage, tomato plants were treated with mock, 100 μM MeJA or 100 μM MT 12 h before they were subjected to cold stress at 4°C. Tomato plants were treated for 24 h for physiological assessment and 60 h for phenotype analysis. Data are mean values of three replicates ± SD. Data for Fv/Fm are mean values from 12 leaves. Different letters represent significant difference at $P < 0.05$ according to Tukey's test.

the relative expression between *SlSNAT*, *SlTDC* and *SlAMST* did not exhibit significant difference. At 8 and 12 h, *SlSNAT* and *SlAMST* were intensively induced, while *SlTDC* remained at a low level of relative expression (**Figure 4**), suggesting that *SlSNAT* and *SlAMST* are two major MT biosynthetic genes responsive to MeJA. These results imply that the observed MT accumulation by MeJA may rely, in part, on JA-induced expression of *SlSNAT* and *SlAMST*.

Suppression of *SlMYC2* Represses Methyl Jasmonate-Induced Expression of Melatonin Biosynthetic Genes in Tomato Plants

To investigate whether MeJA-induced expression of *SlSNAT* and *SlAMST* depends on JA signaling, we utilized two *SlMYC2-RNAi* transgenic lines, in which MYC2, a master transcriptional regulator of JA signaling, was substantially suppressed (Ding et al., 2021). In response to MeJA, the relative expression of *SlSNAT* was dramatically increased in both wild-type plants and two *SlMYC2-RNAi* transgenic lines, however, the expression level was significantly reduced in *SlMYC2-RNAi* plants compared with that in wild-type plants (**Figure 5A**). Similar results were also observed for *SlAMST* (**Figure 5B**). These results suggest that *SlSNAT* and *SlAMST* may be subjected to regulation by MYC2-dependent JA signaling.

Suppression of *SlMYC2* Decreases Melatonin Accumulation and Cold Tolerance in Tomato Plants

To further verify that JA signaling is important for MT accumulation and the responses to cold stress in tomato plants, we analyzed the MT content, Fv/Fm and EL in wild-type and *SlMYC2-RNAi* transgenic plants under cold conditions. In accordance with decreased expression of *SlSNAT* and *SlAMST* that we observed in our last experiment, MT content was significantly reduced in *SlMYC2-RNAi* transgenic plants compared with that in wild-type plants under cold stress (**Figure 6A**). Consistently, *SlMYC2-RNAi* transgenic plants displayed lower Fv/Fm and higher EL than wild-type plants under cold conditions (**Figures 6B,C**). These results substantiate the idea that JA-mediated cold tolerance may depend on MYC2-regulated MT biosynthesis in tomato plants.

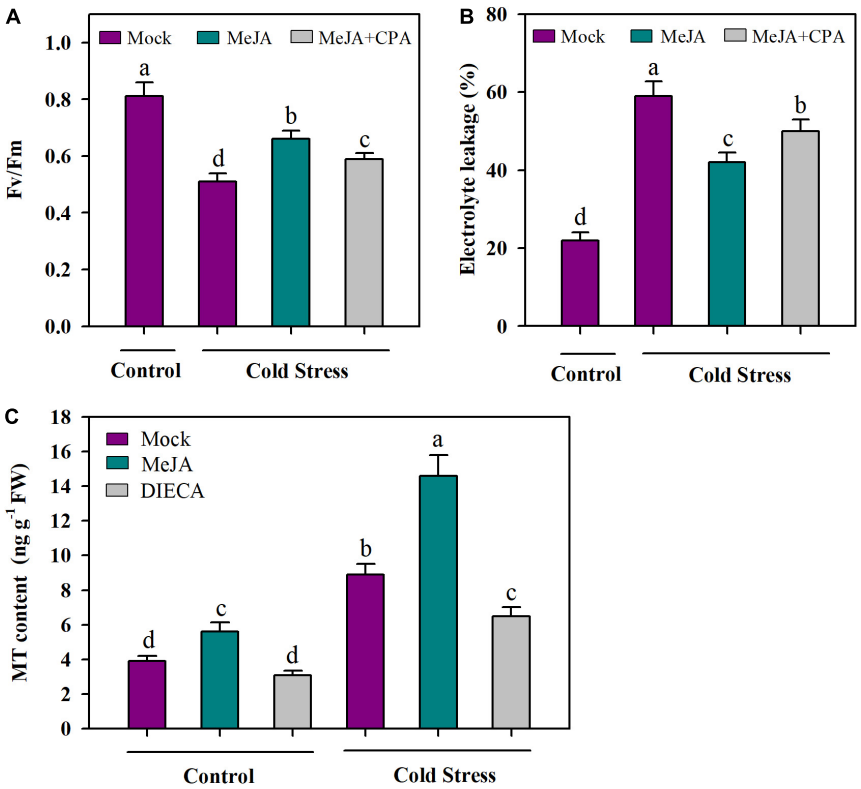

FIGURE 3 | MeJA-induced cold tolerance partly depends on MT. **(A)** Fv/Fm; **(B)** EL; **(C)** MT content. For determination of Fv/Fm and EL, tomato plants at 4-leaf stage were treated with mock, 100 μM MeJA or 100 μM MeJA plus 100 μM CPA (*p*-chlorophenylalanine, a MT biosynthesis inhibitor) 12 h before they were subjected to cold stress at 4°C for 24 h. For determination of MT content, at 4-leaf stage, tomato plants were treated with mock, 100 μM MeJA or 2 mM DIECA (diethyldithiocarbamic acid, a MT biosynthesis inhibitor) 12 h before they were subjected to cold stress at 4°C for 24 h. Data are mean values of three replicates ± SD. Data for Fv/Fm are mean values from 12 leaves. Different letters represent significant difference at $P < 0.05$ according to Tukey's test.

Melatonin Promotes the Accumulation of Jasmonic Acid in Tomato Plants Under Cold Conditions

The finding that JA enhanced MT biosynthesis in tomato plants under cold stress prompted us to ask whether MT promoted JA biosynthesis in tomato plants in a feedback manner. To resolve this question, we first incubated detached tomato leaves in 50 μM MT for 12 h and determined the transcript levels of three JA biosynthetic genes in tomato leaves, including *SlLOX* (Solyc03g122340), *SlAOC* (Solyc02g085730), and *SlOPR3* (Solyc07g007870). It was shown that following MT treatment, the relative transcript levels of three selected JA biosynthetic genes were increased (**Figure 7A**), suggesting the potential regulation of JA biosynthesis by MT.

To further ascertain that MT regulates the biosynthesis of JA, we treated tomato leaves with MT or its biosynthesis inhibitor CPA and determined the JA content. The results showed that foliar application of MT slightly increased JA accumulation under control growth conditions, while MT significantly enhanced JA accumulation under cold stress, with the increase being 24%. Of particular note is the inhibition of endogenous MT by its biosynthesis inhibitor CPA reduced JA accumulation in tomato plants, with the JA level being reduced by 17% under cold stress (**Figure 7B**). These results provide evidence that MT plays a positive role in the biosynthesis of JA in tomato leaves under cold conditions.

DISCUSSION

Cold is an adverse environmental factor that poses threats to plants of tropical or subtropical origin. Cold stress inhibits plant growth and development, and causes severe losses of crop yields. Understanding the responses to cold stress in plants is important for development of cold resistant crop cultivars. Breeding cold-hardy crops is the most effective strategy to mitigate cold stress in agricultural practices. JAs are a group of phytohormones and have been implicated in defense against pathogens and herbivores, as well as abiotic stresses, such as drought, salinity and cold (Kazan, 2015; Wang J. et al., 2020). A great many studies have demonstrated that JA plays a key role in cold tolerance in a diversity of plant species, including tomato (Ding et al., 2021), apple (An et al., 2020), trifoliate orange (Ming et al., 2020), rice (Lee et al., 1996) and Arabidopsis (Hu et al., 2013), among others. Melatonin (MT), a newly identified plant growth regulator, has been shown to play versatile roles in plant growth and development, and resistance to biotic and abiotic stresses.

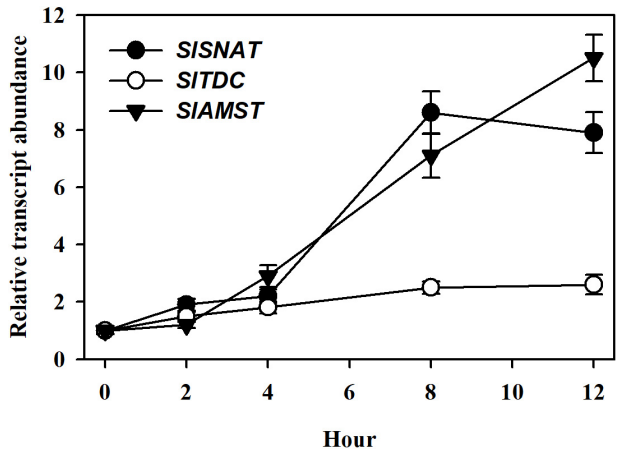

FIGURE 4 | Relative transcript abundance of MT biosynthetic genes, including *SlSNAT*, *SlTDC,* and *SlAMST* in response to MeJA. At 4-leaf stage, fully expanded young leaves were detached from tomato leaves and were incubated in 50 μM MeJA for 12 h. Following MeJA treatment, leaves were collected at 0, 2, 4, 8, and 12 h for relative expression analysis by qRT-PCR. The expression level of leaf samples collected at 0 h was set to 1, and the relative expression levels of the rest of samples were calculated accordingly. Data are mean values of three replicates ± SD.

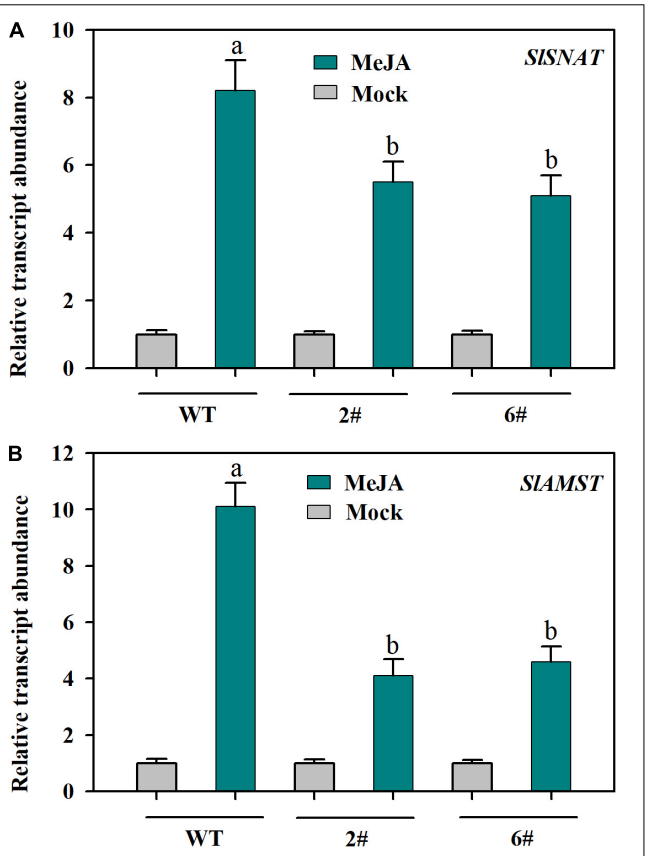

FIGURE 5 | Relative transcript abundance of *SlSNAT* and *SlAMST* as affected by knockdown of *SlMYC2*. **(A)** *SlSNAT*; **(B)** *SlAMST*. At 4-leaf stage, fully expanded young leaves were detached from wild-type and *SlMYC2-RNAi* transgenic tomato plants and were incubated in 50 μM MeJA for 12 h. Following MeJA treatment, leaves were collected for relative expression analysis by qRT-PCR. The expression level of mock-treated samples was set to 1, and the relative expression level of MeJA-treated samples was calculated accordingly. Data are mean values of three replicates ± SD. Different letters represent significant difference at $P < 0.05$ according to Tukey's test.

Accumulating evidence suggest that MT acts in the response to cold stress in plants (Wu et al., 2021). Both JA and MT regulate cold responses in plants, however, whether and how they crosstalk in the cold tolerance remains to be determined. In this study, we explored the potential crosstalk between JA and MT in the cold tolerance of tomato, which originated from tropical regions and is cold sensitive. We demonstrated that JA and MT form a positive feedback loop to promote respective biosynthesis and boost cold tolerance in tomato plants.

Previous studies have established that JA and MT are involved in the responses to cold tolerance in plants (Sharma and Laxmi, 2016; Tiwari et al., 2020). Our work provides several lines of evidence that both JA and MT are of importance in the cold tolerance in tomato plants. Firstly, levels of endogenous JA and MT were substantially increased by cold stress. Secondly, exogenous MeJA and MT significantly increased Fv/Fm and decreased EL. Finally, application of MeJA or MT led to reduced production of H_2O_2 under the cold condition. Though these results confirm the pivotal role of JA and MT in the cold tolerance, whether and how they crosstalk in tomato cold response is a yet-to-be answered question. In our attempt to address this question, we first found that MeJA enhanced cold tolerance, while CPA attenuated MeJA-induced cold tolerance (**Figures 3A,B**), suggesting that JA and MT are associated in the cold response, and that MT may act downstream of MeJA mediating MeJA-triggered cold tolerance in tomato plants. Additionally, MeJA increased MT accumulation, whereas a JA biosynthesis inhibitor DIECA decreased MT accumulation under the cold condition (**Figure 3C**). Together with previous reports that MeJA promotes MT accumulation to delay cassava deterioration and to improve watermelon cold tolerance (Liu et al., 2019; Li et al., 2021), these results support the interactions between JA and MT in tomato plants. These results also imply that JA-induced MT biosynthesis may be an important adaptive strategy in plants subjected to cold stress.

Though we observed that MeJA promoted MT biosynthesis, the relevant mechanisms or the major factors involved remain unclear. In plants, MT biosynthesis involves five major enzymes, catalyzing four main sequential steps. Briefly, tryptophan decarboxylase (TDC) first converts tryptophan to tryptamine, which is then catalyzed by tryptamine-5-hydroxylase (T5H) to produce serotonin. Conversion of serotonin to melatonin involves two independent pathways in plants. In one pathway, serotonin-*N*-acetyltransferase (SNAT) converts serotonin into *N*-acetylserotonin, which is then catalyzed to melatonin by *N*-acetylserotonin methyltransferase (ASMT) or caffeic acid O-methyltransferase (COMT). In the other pathway, ASMT/COMT first catalyzes serotonin into 5-methoxytryptamine, which is further converted to melatonin by SNAT (Wu et al., 2021). *SlSNAT*, *SlTDC* and *SlAMST* have

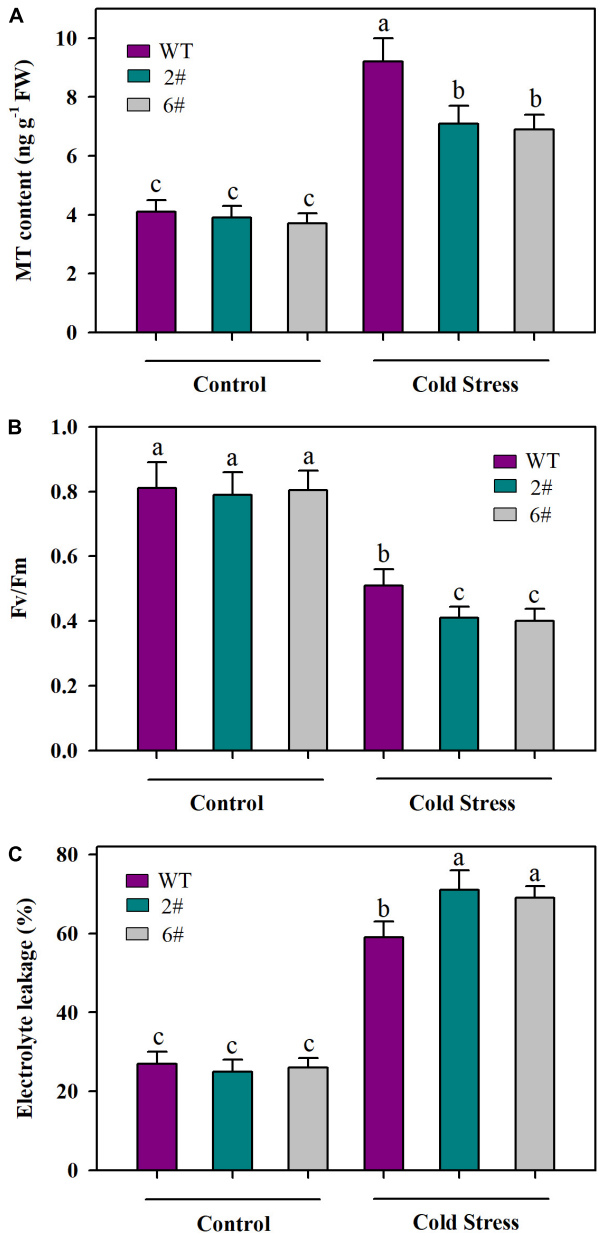

FIGURE 6 | MT content and cold tolerance as affected by knockdown of *SlMYC2*. **(A)** MT content; **(B)** Fv/Fm; **(C)** EL. At 4-leaf stage, wild-type and *SlMYC2-RNAi* transgenic tomato plants were subjected to cold stress at 4°C for 24 h. Following cold treatment, leaves were collected for determination of MT content, Fv/Fm and EL. Data are mean values of three replicates ± SD. Data for Fv/Fm are mean values from 12 leaves. Different letters represent significant difference at $P < 0.05$ according to Tukey's test.

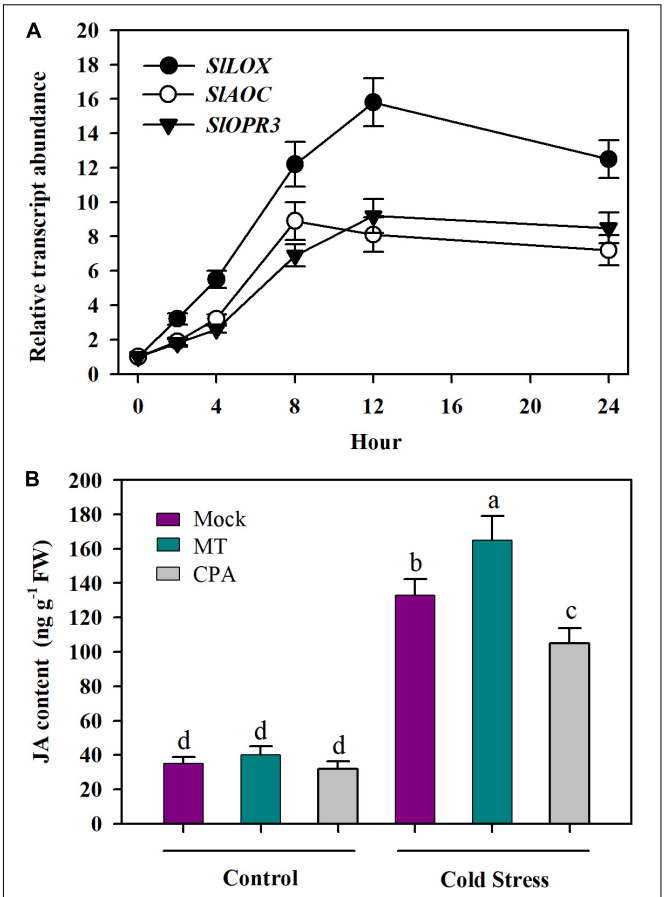

FIGURE 7 | MT promotes the accumulation of JA under cold stress. **(A)** Relative transcript levels of JA biosynthesis genes; **(B)** JA content. For determination of relative transcript levels of JA biosynthesis genes, fully expanded young leaves were detached from tomato leaves at 4-leaf stage and were incubated in 50 μM MT for 12 h. Following MT treatment, leaves were collected at 0, 2, 4, 8, 12, and 24 h for relative expression analysis by qRT-PCR. For determination of JA content, tomato plants at 4-leaf stage were treated with mock solution, 100 μM MT or 100 μM CPA 12 h before they were subjected to cold stress at 4°C for 24 h. Data are mean values of three replicates ± SD. Different letters represent significant difference at $P < 0.05$ according to Tukey's test.

been demonstrated to function in MT biosynthesis in tomato plants (Li et al., 2016; Xu et al., 2016; Wang X. et al., 2020). We found that *SlSNAT* and *SlAMST*, rather than *SlTDC*, were predominantly induced following MeJA treatment, implying that JA-induced expression of *SlSNAT* and *SlAMST* may contribute to cold-induced MT accumulation. It is thus reasonable to conclude that cold induces JA accumulation, as we observed in this study, which subsequently triggers the expression of *SlSNAT* and *SlAMST*, ultimately giving rise to increased melatonin accumulation. In an attempt to further understand how JA induces expression of *SlSNAT* and *SlAMST*, as well as MT biosynthesis, we took advantage of two *MYC2-RNAi* transgenic lines, in which a key JA signaling regulator, *SlMYC2*, was knocked down (Ding et al., 2021). Compared with their wild-type counterparts, *MYC2-RNAi* transgenic plants displayed lower transcript levels of *SlSNAT* and *SlAMST*, in response to MeJA treatment. Of particular note is that under cold conditions *MYC2-RNAi* tomato plants accumulated significantly less MT than wild-type plants. Furthermore, downregulation of *SlMYC2* attenuates JA-induced cold tolerance. Our results thus support that SlMYC2-dependent JA signaling is critical for MT biosynthesis in cold-stressed tomato plants, adding

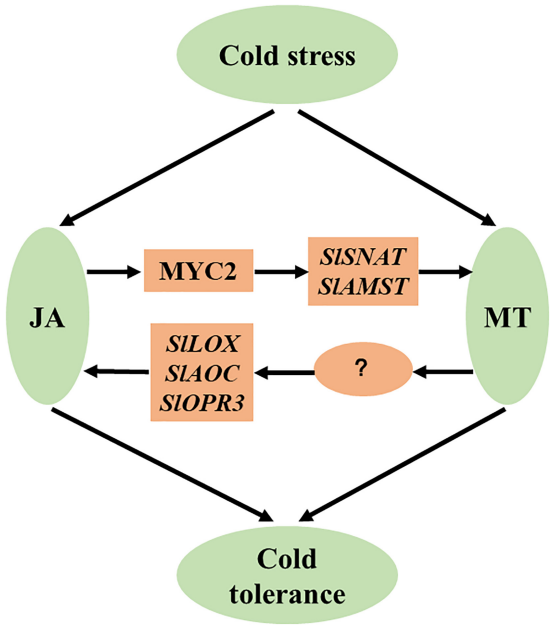

FIGURE 8 | A simplified model depicting the synergistic action of JA and MT in the cold responses of tomato plants. Cold stress increases JA accumulation, leading to elevated MT biosynthesis via MYC2-activated *SlSNAT* and *SlAMST*. Consequently, increased MT accumulation potentiates cold tolerance in tomato plants. JA-induced MT accumulation promotes JA biosynthesis, resulting in a positive feedback loop between JA and MT biosynthesis, which amplifies cold responses in tomato plants.

another line of evidence for JA-induced MT accumulation and cold tolerance in tomato plants. However, it is worth noting that further work on the mechanistic explanation of SlMYC2-regulated expression of *SlSNAT* and *SlAMST* is required to fully understand JA-induced accumulation of MT in tomato plants under cold conditions.

As discussed above, it is convincing that under the cold condition, JA is, at least partially, responsible for the observed increase in MT accumulation. JA and MT were both accumulated in response to cold stress. Having found that JA increases MT accumulation, we are not clear whether MT increases JA accumulation under cold conditions at this stage. It has been well studied that JAs are produced from α-linolenic acid through a series of steps catalyzed by LOXs, AOS, AOC, and OPR (Wasternack and Song, 2017). Our results revealed that expression of *SlLOX*, *SlAOC*, and *SlOPR3* was profoundly stimulated in response to MT, suggesting the involvement of MT in JA biosynthesis. Furthermore, MT increased JA accumulation, while CPA reduced its accumulation. Though it remains unclear how MT stimulated the expression of JA biosynthesis genes and subsequent increase in JA accumulation, these results verify that MT positively regulates the biosynthesis of JA in tomato plants under the cold condition. Regulation of JA biosynthesis occurs at multilayers, including at the transcriptional, post-transcriptional and post-translational levels. Previous studies have demonstrated that there exists a positive feedback loop in expression of JA biosynthesis genes by JA. In addition, MYC2, the master regulator of JA signaling, is able to directly bind to the promoter of JA biosynthetic genes (Wasternack and Song, 2017; Zhang et al., 2020), indicating the complexity of JA biosynthesis regulation. In our work, we did not examine the impact of MT on SlMYC2 expression, making it impossible to determine whether MT-induced JA accumulation depends on SlMYC2. Therefore, future studies should be directed to identify components that are regulated by MT and contribute to JA biosynthesis.

CONCLUSION

We demonstrate in the present study that JA and MT act synergistically in the cold tolerance of tomato plants. We propose a working model to describe the crosstalk between JA and MT in the enhancement of cold tolerance (**Figure 8**). Cold stress increases JA accumulation, which leads to elevated MT biosynthesis via MYC2-activated *SlSNAT* and *SlAMST*. Consequently, increased MT accumulation potentiates cold tolerance in tomato plants. Notably, JA-induced MT accumulation promotes JA biosynthesis, resulting in a positive feedback loop between JA and MT biosynthesis, which amplifies cold responses in tomato plants. Our work thus provides insight into the mechanisms of cold-induced accumulation of JA and MT and their crosstalk in the cold responses in tomato plants. Our findings could be translated into the development of cold-resistant tomato cultivars by genetically manipulating JA and MT pathways.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MW, FD, and SZ designed the study and performed the data analysis. FD, LR, FX, and MW conducted the experiments. MW and FD drafted the manuscript. MW and SZ edited the manuscript. All authors approved the publication of current version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.763284/full#supplementary-material

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Exogenous Melatonin Promotes the Salt Tolerance by Removing Active Oxygen and Maintaining Ion Balance in Wheat (*Triticum aestivum* L.)

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Melatonin (MT) is a small molecule indole hormone that plays an important role in the regulation of biological processes and abiotic stress resistance. Previous studies have confirmed that MT promotes the normal development of plants under stress by mediating physiological regulation mechanisms. However, the physiological mechanism of exogenous MT regulating seed germination and seedling growth of wheat under salt stress is still unclear. In this study, NaCl stress decreased germination rate and inhibited seedling growth of wheat, but shoot length, root length, and plant weight of SM15 did not change significantly. The addition of 300 μM MT in the cultivation solution directly promoted the germination rate of SM15 and ZM18, and lateral root production, but decreased the germination rate of JM22 and inhibited the length of germ and radicle of three varieties under salt stress. For wheat seedling, application of MT could increase proline content, soluble protein, soluble sugar, Ca^{2+} content, and vital amino acid content in leaves to keep high water content, low level of H_2O_2 content, and low $[K^+]/[Na^+]$ ratio. MT increased root vigor and $[K^+]/[Na^+]$ ratio and decreased H_2O_2 content in root induced by salt stress. In conclusion, MT enhanced salt tolerance in wheat seeds and seedlings by regulating the synthesis of soluble protein and sugar, ion compartmentation in roots and leaves, enhancement of enzymatic systems, and changes in amino acid levels. Salt resistance varied with different varieties under the same environmental condition. SM15 was a higher salt-resistant variety and JM22 was a salt-sensitive one. In wheat production, the application of exogenous MT should consider the differences among varieties of wheat during the sowing and seedling stages.

Keywords: salt stress, melatonin, wheat, germination, antioxidative activity

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INTRODUCTION

Soil salinization, a geologically environmental problem all over the world, is one of the main factors resulting in the decrease in grain production (Liang et al., 2018). According to incomplete statistics, the salinization of arable land globally is increasing at a rate of 0.3–1.5 million hectares year^{-1} (Harper et al., 2021). The total area of salinized soil in China is about 99.13 $\times$ 10^6 hm^2, which accounts for 1.03% of the total Chinese land area (Yang and Wang, 2015). It is expected that 50% of arable land will be under saline-alkali stress by 2050 due to environmental pollution, lack of freshwater, improper irrigation methods, and other factors (Dou et al., 2019; Yang et al., 2021). Wheat (*Triticum aestivum* L.), one of the medium salt-tolerant crops and the second-largest food crop in the world, also faces soil salinization in its growing area. Salt stress leads to the significant inhibition of seed germination and seedling growth (Shahi et al., 2015). Under salt stress, excessive reactive oxygen species (ROS) are accumulated which will cause cell damage, DNA damage, and oxidative stress to plants, consequently disrupting the physiological balance and leading to reduced photosynthesis and production yield (Zhang et al., 2016; Tripathi et al., 2020). High Na^+ accumulation in plant cells leads to osmotic stresses and limits uptake and utilization of other nutrition ions, which causes moisture loss and electrolytic leaching due to cell membrane damage (Abbas et al., 2015; Ahmed et al., 2015; Rebey et al., 2017).

Melatonin (MT), an amine hormone, has been confirmed extensively for enhancing plant tolerance to various abiotic stresses (Zhang et al., 2013; Turk et al., 2014; Ren et al., 2020). There are multiple mechanisms verified in MT helping plants to enhance abiotic stress tolerance (Huangfu et al., 2021; Sun et al., 2021). The application of MT has shown stimulating antioxidant enzymes and scavenging ROS in plants under stress conditions (Park et al., 2013; Li et al., 2018), preventing chlorophyll degradation and increasing photosynthetic efficiency, reducing ion toxicity (e.g., heavy metals, Na^+, and so on) and increasing osmotic substances to maintain water status (Gao et al., 2016; Cai et al., 2017; Siddiqui et al., 2019a, 2020; Al-Huqail et al., 2020), and enhancing secondary metabolite biosynthesis and upregulating defense genes to decrease cell injury (Li H. et al., 2017; Zhao et al., 2017; Elsayed et al., 2021). Under salt stress, MT alleviates directly ROS burst and cell damage for bermudagrass (Shi et al., 2015). Also, MT alleviates the inhibitory effects of NaCl stress on germination of cucumber seeds mainly by increasing antioxidant enzymes, affecting phytohormone biosynthesis and catabolism, and regulating storage protein degradation to vital amino acids during seed germination (Zhang H. J. et al., 2014; Zhang et al., 2017). MT has been reported to extend longevity by significantly reducing chlorophyll degradation, delaying the leaf senescence, and preventing cell death in rice plant and tomato seedlings under salt stress (Siddiqui et al., 2019b; Zhao et al., 2021). During wheat seedling growth, MT accelerates the transformation from arginine and methionine to polyamines in wheat seedlings (Ke et al., 2018). Salt tolerance enhanced in maize with MT is most likely due to the improvement in photosynthetic capacity, antioxidative capacity, and ion homeostasis (increased K^+ contents and K^+/Na^+ ratios) in leaves (Jiang C. et al., 2016). Also, Liu et al. (2020) demonstrate that MT improves salt tolerance in rice by reducing K^+ efflux induced by high NaCl concentrations in roots. Subsequent researches have partly revealed the mechanisms of MT, which affects directly or indirectly salt resistance in many plants. However, MT application had no effects on the growth of wheat and maize under normal conditions (Ke et al., 2018; Ren et al., 2020). It has not fully elucidated a universal pathway on whether or how MT launches crosstalk with vital regulators under stress.

Different concentrations of MT may have differential effects in mediating abiotic stress defense. MT at 200 μmol L^{-1} can effectively inhibit ROS production, improve the biomass and chlorophyll content, and regulate the photosynthetic characteristics of cotton seedlings under salt stress (Jiang et al., 2021). MT at 300 μmol L^{-1} alleviates the negative effect of water stress on wheat germination and promotes morphological development including radicle length, radicle number, and plumule length of germinated seeds (Li et al., 2020). About 800 μmol L^{-1} MT used for priming seed significantly improves germination energy, germination percentage, proline content, and total phenolic content of maize (Jiang X. et al., 2016). Application of exogenous MT (100 μmol L^{-1}) alleviates ROS burst and protects the photosynthetic activity in maize seedlings under salt stress (Chen et al., 2018). For some other reports, MT at a concentration of 100 μmol L^{-1} inhibits seed germination and seedling growth and enhances the toxic effect of copper on seedlings, but 1 or 10 μmol L^{-1} MT can eliminate the inhibitory effect of copper on the fresh weight of red cabbage seedlings (Posmyk et al., 2008). Pretreatment with 1 μmol L^{-1} MT was also found to partially mitigate the inhibition of shoot dry weight induced by salt stress (Ke et al., 2018). Li X. et al. (2017) showed that 10–500 μmol L^{-1} MT could help to recover seed germination potential, germination index, and vigor index on two varieties of rice treated with 120 mM NaCl to control levels. It seems that MT can regulate and enhance stress resistance by multiple mechanisms in a concentration-dependent manner.

In wheat production, different varieties show different resistances, adaptive areas, and production yields. So far, the roles of MT in mediating salt stresses on physiological regulation still need to be explored in different wheat varieties. In this study, seed germination and seedling growth of winter wheat experiments were conducted in a hydroponic solution with NaCl (100 mM) or without NaCl (100 mM), and the addition of MT. The purposes of this study were to (1) verify that MT enhances seed germination rate and antioxidant activities of seedling, (2) elucidate the regulatory effect of MT on ion exchange in the organelles, and (3) compare the regulatory effects of MT on the different varieties of wheat under salt stress. The results of this experiment provide theoretical support to promote the application of exogenous MT on saline-alkali wheat to increase arable land reserve and improve food security.

MATERIALS AND METHODS

Plant Material

The experiment was conducted in 2021 at the Hebei Agricultural University (38° 85′ N, 115° 30′ E), Hebei Province, China. Three commercial wheat (*T. aestivum* L.) cultivars, 'Jimai 22' (JM22), 'Shimai 15' (SM15), and 'Zhoumai 18' (ZM18), were used in this study. All varieties are high-yielding and extensively cultivated in Huang-Huai-Hai region. ZM18 is suitable for planting in south section of the Huanghuai winter wheat area, and JM22 is suitable for planting in north section of the Huanghuai winter wheat area. SM15 has comprehensive resistance and good adaptability.

Experimental Design

This experiment was performed in a growth chamber.

Seed Germination

In total, 450 seeds of each wheat variety were surface-sterilized with 75% ethanol for five minutes. After that, all surface-sterilized seeds were washed thoroughly with distilled water and were transferred into a glass plate for germination. Totally, two layers of filter paper saturated with three different treatment solutions: CK (distilled water), NaCl (100 mM), and NaCl-MT (100 mM NaCl + 300 μM MT) were placed in each plate. The concentrations of NaCl and MT solutions were selected referring to Li X. et al. (2017), Ke et al. (2018), and our former study (Li et al., 2020). The germination conditions were set at a light/dark shift (12/12h). The day/night temperature was 20/15°C. Light intensity was 600 $\mu mol/m^2/s^1$. Relative humility was 60%. The number of seed germinated was recorded daily till the 10th day. Water and other solutions were added into the germination plates to keep suitable humidity.

Hydroponic Experiments

At first, some other healthy seeds of three wheat varieties were germinated in a hole tray with moist vermiculite. The indoor environmental conditions were the same as those described above for the germination plate experiment. When the second leaf appeared, seedlings were removed out of hole tray gently and washed off vermiculite attached to the roots in tap water several times. Then, seedlings were transferred into Hoagland nutrient solution in plastic boxes covered with black film to avoid light. After about 1 week of recovery growth, seedlings were treated with fresh Hoagland nutrient solution as control, 100 mM NaCl solution as salt stress, and 100 mM NaCl + 300 μM MT. Each treatment was repeated three times. After 24 h, leaf and root samples were collected and quickly frozen using liquid nitrogen and then stored in a −80°C freezer until the measurements were taken.

Measurement of Morphological Parameters

Germinated seeds were recorded daily when radicle length exceeded 2 mm according to Liu et al. (2016). Germ and radicle length and radicle number of 10 randomly selected grains from each treatment were recorded with a ruler for 24 h after treatment.

Shoot and root length of five randomly selected seedlings from each replication were measured 24 h after treatment. Seedlings of each treatment with triplicates were dissected into roots, stems, and leaf and their fresh weights were recorded immediately. Then, the weighed fresh samples were kept in the paper bags to be dried in an oven at 105°C for 30 min and then at 75°C until the consistent weight was obtained.

Na^+, K^+, and Ca^{2+} Contents

Na^+, K^+, and Ca^{2+} contents of the whole-plant tissues were determined using a previous method with minor modifications (Chen et al., 2017). After the measurement of dry weight, samples were digested in a mixed solution of perchloric acid or concentrated nitric acid (volume ratio, $HClO_4$:HNO_3 = 1:5, v:v) in a glass test tube. More solution has replenished the solution to 12 mL. The ion content of a solution in a glass test tube was extracted in boiling water bath for 5 h and measured using an atomic absorption spectrophotometer (ZA-3000; Hitachi Instruments, Tokyo, Japan).

Hydrogen Peroxide Content and Detection of H_2O_2 and O_2^-

Hydrogen peroxide (H_2O_2) content in the leaf and root samples was determined using a H_2O_2 assay kit (A064, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's user manual. H_2O_2 accumulation in root samples was visualized according to what Liu (2020) described. About 1.5 cm root tip was soaked and stained in 1% (w/v) 3,3′-diaminobenzidine staining (DAB, dissolved with 10 mM MES buffer on pH value = 6.5) and incubated in dark at 25°C for 8 h. Then, the samples were washed several times and observed using an optical microscope (Leica, Wetzlar, Germany).

Superoxide anion (O_2^-) accumulation in root samples (about 1.5 cm) was also visualized according to Liu's (2020) method. Root samples were stained in 100 μM nitro blue tetrazolium chloride (NBT) dissolved with 50 mM phosphate buffer (pH value = 6.4) for 15 min and observed using an optical microscope (Leica, Wetzlar, Germany).

Antioxidant Enzymes, Soluble Sugar, Soluble Protein, and Malondialdehyde Content

Fresh leaf sample (0.5 g each) was triturated in 50 mM phosphate buffer (pH = 6.4). Homogenate solution was centrifuged at 20,000 *g* at 4°C for 15 min. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) activity, soluble sugar, soluble protein, and malondialdehyde (MDA) contents were determined using assay kits (A064, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Soluble sugar and protein contents were determined using a soluble sugar assay kit (A145-1-1, Nanjing Jiancheng Bioengineering Institute) and a BCA assay kit (BCAP-1-W, Suzhou Comin Biotechnology Co., Ltd.) according to the manufacturer's user manual.

Extraction and Measurement of Amino Acid Content

Sample Preparation

The leaf and root samples (0.20 g) were accurately weighed and placed in the sample bottle (12 mL), into which 10 mL 6 mol/L hydrochloric acid (containing 0.1% phenol) was added. The mixed solution was homogenized by ultrasound. The samples were hydrolyzed in the oven at 110°C for 24 h. After cooling, the filtrate was mixed and filtered by a 0.45 μm water membrane. One milliliter of the filtrate was put into a rotary evaporator and dried at 80°C. Finally, 2.00 mL 0.1 mol/L hydrochloric acid was added to evaporated samples in the rotary evaporator and then mixed evenly by the whirlpool and transferred to the sample bottle for further use.

Standard Curve Development

Seventeen amino acid standards including aspartic acid (Asp), glutamic (Glu), histidine (His), serine (Ser), arginine (Arg), glycine (Gly), threonine (Thr), proline (Pro), alanine (Ala), valine (Val), methionine (Met), cysteine (Cys), isoleucine (Ile), leucine (leu), phenylalanine (Phe), lysine (Lys), and tyrosine (Tyr) were calculated and accurately weighed in a volumetric flask. The mixed standard was diluted using 0.1 mol/L hydrochloric acid to obtain a final concentration of 500 mg/L of each amino acid. An appropriate amount of 500 mg/L mixed standard was diluted to make 10, 25, 50, 100, 150, 200, 300, and 400 mg/L mixed standard solution. The gradient elution procedures are shown in **Table 1**.

Aminoacyl Derivatization

Three solutions including 100 μL mixed standards, 200 μL buffer solution (0.5 mol/L sodium bicarbonate solution, 0.5 mol/L sodium carbonate solution, pH value = 9.0), and 100 μL 2,4-dinitrochlorobenzene (100 mg/mL) were swirly mixed and then reacted at 90°C for 90 min in dark. Thereafter, 50 μL of 10% acetic acid solution and 550 μL ultrapure water were added to 1.00 mL. The mixed solution was swirled and mixed and then filtered with 0.45-μm organic film and filter liquor prepared for measuring.

Chromatographic Analysis

An high-performance liquid chromatography (HPLC, Agilent 1200, United States) equipped with a C18 column (4.6 mm × 250 mm, 5 μm; Kromat Universil, catalog no. 35D5) and a diode array detector was used. The column temperature was 40°C. Mobile phase A was pure acetonitrile, and mobile phase B was acetic acid – sodium acetate buffer solution (2.50 g/L sodium acetate, 1.17 mL/L glacial acetic acid, 1.50 mL/L triethylamine, pH value = 5.25 ± 0.05). The flow rate was 1 mL/min. The sample injection amount was 10 μL with 10- to15-min intervals. The detection wavelength was 360 nm.

TABLE 1 | Gradient elution program.

Time (min)	Mobile phase (%)	
	A	B
0	18	82
10	18	82
15	29	71
25	34	66
30	55	45
37	60	40
39	18	82
45	18	82
60	18	82

Statistical Analyses

All data were processed using analysis of variance (ANOVA) in triplicate in Excel 2003 and IBM SPSS Statistics 17.0 (IBM Corp., Armonk, NY, United States). Duncan's new multiple range (DMR) test at a 5% probability level was used to test the differences among the means. Significant differences were labeled based on DMR.

RESULTS

Exogenous Melatonin Promotes Wheat Germination Trait

The effect of MT on wheat germination rate under salt stress is shown in **Figure 1**. The germination rate of CK reached the highest value on the third day and then showed a stable performance. The germination rate of wheat treated with NaCl increased rapidly in the first 3 days and then showed differences:

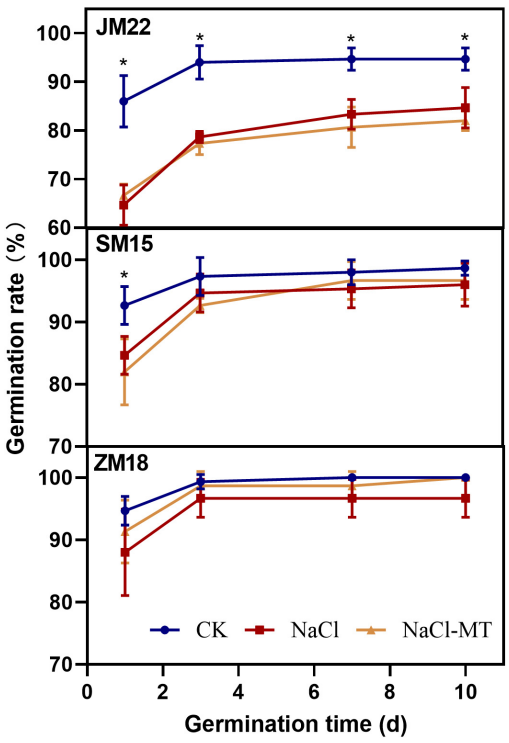

FIGURE 1 | Effects of MT on germination trait under salt stress. For each trait, asterisks represent significantly different according to Duncan's test at a $p < 0.05$ threshold.

TABLE 2 | Effects of MT on radicle number, root length, and germ length under salt stress.

Varieties	Treatments	Germ length (cm)	Root length (cm)	Radicle number
Jimai 22	CK	15.17a	22.52a	5.47c
	NaCl	11.53b	13.34c	6.00c
	NaCl-MT	10.47b	7.05d	7.93a
Shimai 15	CK	13.29a	21.77a	5.00b
	NaCl	9.5733b	12.02c	5.07b
	NaCl-MT	7.255c	5.38d	6.55a
Zhoumai 18	CK	13.94a	18.13a	5.00d
	NaCl	11.60c	12.07bc	5.27cd
	NaCl-MT	8.69d	5.39d	7.33a

For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

JM22 increased steadily, and SM15 and ZM 18 were basically stable. The germination rate of wheat decreased significantly under salt stress, especially for Jimai22, which was 11.09% lower than CK ($p < 0.05$), but there was no significant difference between Shimai 15 and Zhoumai18.

Exogenous Melatonin Inhibits Root Length and Germ Length and Promotes Radicle Number

Radicle number, root length, and germ length were also affected by salt stress significantly (**Table 2**). NaCl stress significantly decreased the length of germ and root of JM22, SM15, and ZM18 by 23.99 and 40.76, 27.97 and 44.79, and 16.79 and 33.43%, respectively. The radicle number did not change significantly under the NaCl treatment than that under CK. MT application further significantly decreased the length of germ and root of JM22, SM15, and ZM18 by 30.98 and 68.69, 45.41 and 75.29, and 37.66 and 70.27%, respectively. Meantime, the radicle number was significantly increased under NaCl-MT than the other two treatments.

Melatonin Application Relieve Inhibited Effect of NaCl on Shoot and Root Length

Figure 2 shows that shoot and root lengths of JM22 and ZM18 were both decreased significantly under NaCl stress compared with CK. MT application increased those indexes to some degrees, in which the shoot length of ZM18 and root length of JM22 were significantly increased under NaCl treatment than those under the NaCl-MT. Additionally, shoot and root lengths of ZM18 did not change significantly under NaCl and NaCl-MT treatments. These results indicated that SM15 showed higher salt resistance compared with the other two varieties.

Melatonin Application Increased Water Content in Plant Under NaCl Stress

Table 3 shows that fresh weight, dry weight, and water content in root, stem, and leaf of JM22 and ZM18 significantly decreased under NaCl stress. MT application had increased those indexes

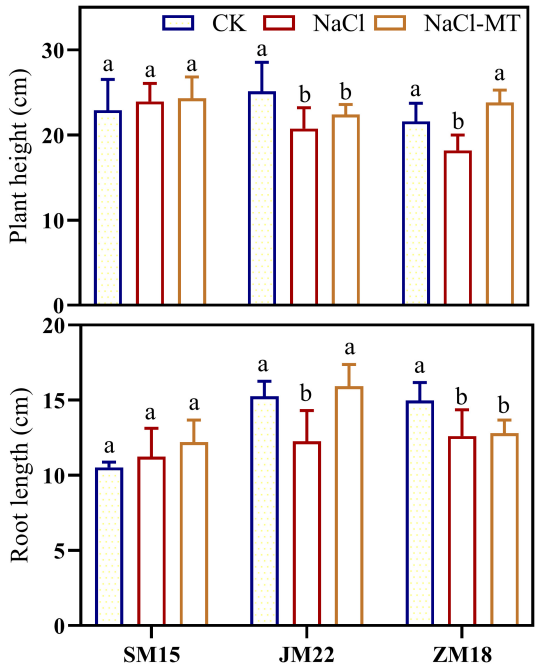

FIGURE 2 | Effects of MT on plant height and root length under salt stress. For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

to the level of CK. However, for SM15, the fresh weight, dry weight, and water content in plants did not change significantly under the NaCl and NaCl-MT treatments compared with CK. These results also indicated that SM15 contained a good ability on salt resistance.

Melatonin Application Regulates Na^+, K^+, Ca^{2+} Compartmentation, and $[K^+]/[Na^+]$ at Root and Leaf of Plant

Table 4 shows that K^+, Ca^{2+}, and Na^+ contents in different organelles and varieties followed different changing patterns. Under NaCl stress, compared with CK, Na^+ content significantly increased in root, stem, and leaf of three wheat varieties; K^+ and Ca^{2+} contents were both significantly decreased in root and stem (Ca^{2+} content in the stem of SM15 and ZM18 exception); when K^+ content increased, Ca^{2+} contents decreased significantly in the leaf of JM 22, but Ca^{2+} increased in the leaf of SM15; both K^+ and Ca^{2+} in leaf were not obviously changed in ZM18. NaCl-MT treatment significantly decreased Na^+ contents in root compared with that treated by NaCl alone, but increased in stem and leaf. This suggested that MT took effect first in the root, a higher Na^+ concentration formed in stem and leaf. Also, MT application further decreased K^+ content in stem and leaf and increased significantly Ca^{2+} content in the leaf of JM22; it decreased K content in the leaf of SM15 and decreased significantly K^+ content in stem and increased Ca^{2+} in root and leaf of ZM18. $[K^+]/[Na^+]$ ratio in root and stem + leaf was significantly decreased under NaCl stress compared with CK. MT application could

TABLE 3 | Effects of MT on fresh weight and dry weight of root, stem, and leaf of wheat under salt stress.

Variety	Treatments	Fresh weight (g/plant)			Dry weight (g/plant)			Water content (g/plant)		
		Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
JM22	CK	1.68c	0.89abcd	1.40ab	0.13bcd	0.15ab	0.26a	1.55c	0.74bc	1.14ab
	NaCl	1.33cde	0.63e	1.00c	0.08e	0.08d	0.15c	1.25cde	0.55d	0.84c
	NaCl + MT	2.07b	1.00abc	1.67a	0.15abc	0.15ab	0.28a	1.92b	0.85ab	1.39a
SM15	CK	1.26de	1.02abc	1.47ab	0.10de	0.14abc	0.24ab	1.16de	0.88ab	1.23ab
	NaCl	1.16e	0.87bcd	1.26bc	0.09de	0.11bcd	0.19bc	1.07e	0.76abc	1.07bc
	NaCl + MT	1.62cd	0.85cd	1.40ab	0.12cde	0.10cd	0.19bc	1.50cd	0.75bc	1.21ab
ZM18	CK	2.50a	1.08ab	1.57ab	0.17a	0.15ab	0.25a	2.33a	0.93a	1.32ab
	NaCl	1.38cde	0.78de	1.01c	0.11de	0.11bcd	0.16c	1.27cde	0.67cd	0.85c
	NaCl + MT	2.35ab	1.09a	1.51ab	0.16ab	0.16a	0.25a	2.19ab	0.93a	1.25ab

For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

TABLE 4 | Effects of MT on K^+, Na^+, Ca^{2+}, $[K^+]/[Na^+]$ in root, stem, and leaf of wheat.

Variety	Treatments	Root			Stem			Leaf			Root	Stem + leaf
		K^+	Na^+	Ca^{2+}	K^+	Na^+	Ca^{2+}	K^+	Na^+	Ca^{2+}	K^+/Na^+	
JM22	CK	47.77a	3.20c	12.57a	42.38a	3.00c	4.63a	41.95b	2.32c	7.11a	49.71a	16.05a
	NaCl	36.86b	16.23a	5.65b	39.10b	8.35b	3.87c	43.92a	10.32b	6.12b	7.55c	4.46b
	NaCl + MT	33.63b	13.40b	6.14b	35.13c	10.62a	4.43ab	40.85b	14.96a	6.20b	8.36b	2.97c
SM15	CK	46.06a	3.44c	7.62a	40.67a	1.75c	3.32b	41.89a	2.02c	5.77b	44.92a	21.96a
	NaCl	39.10b	16.51a	4.32 b	39.35b	10.62b	4.40 a	42.24a	12.83b	7.54a	7.89c	3.48b
	NaCl + MT	39.18b	10.98b	5.03b	39.21b	11.77a	4.50a	38.34b	16.94a	6.96a	11.90b	2.70c
ZM18	CK	43.04a	2.83	10.59a	42.23a	1.92c	4.40 a	38.78a	1.73c	7.01b	50.94a	22.21a
	NaCl	41.55b	17.09	6.20c	37.35c	6.38 b	4.17 a	38.58a	6.89b	6.81b	8.11c	5.73b
	NaCl + MT	38.10b	11.41	6.94b	39.74b	10.61a	4.51a	38.79a	13.36a	8.63a	11.14b	3.28c

For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

increase $[K^+]/[Na^+]$ ratio in the root, but decrease this value in stem + leaf.

Melatonin Application Enhance Activity of Antioxidant Enzymes

Figure 3 shows that NaCl stress-induced increase of activity for SOD, POD, and CAT in different degrees compared with CK. MT application could enhance further the activity of SOD and CAT in the leaves of three wheat varieties under NaCl treatments; POD activity was recovered to the level of CK. It is noted that SOD activity of JM22 was significantly decreased under NaCl stress, which was possibly explained by the fact that JM22 was salt-sensitive and biochemical characteristics experienced serious damage.

Melatonin Application Decreased Malondialdehyde Content and Increased Proline Content

As shown in **Figure 4**, MDA content and proline content in the leaves of three wheat varieties were both increased under NaCl stress. MT application decreased MDA content under NaCl condition, suggesting that the cell membrane structure had been improved significantly. Additionally, MT further increased the proline content in the leaf of SM15 significantly under NaCl + MT compared with that under the NaCl treatment alone.

Melatonin Application Increased the Contents of Soluble Protein and Soluble Sugar

As shown in **Figure 5**, the contents of soluble protein and soluble sugar were decreased by different degrees under NaCl stress compared with CK. MT application significantly increased the contents of soluble protein and soluble sugar, which helped plants keep good water status under salt stress. Soluble protein content in the leaf of ZM18 did not change significantly under different treatments.

Melatonin Application Increased the Root Vigor Under NaCl Stress

Figure 6 shows that the root vigor of JM22 and ZM18 decreased significantly under NaCl stress compared with CK. However, the root vigor of SM15 was increased significantly under NaCl conditions, suggesting higher salt resistance for this variety. MT application had increased the root vigor of JM22 and ZM18 significantly to alleviate the salt damage.

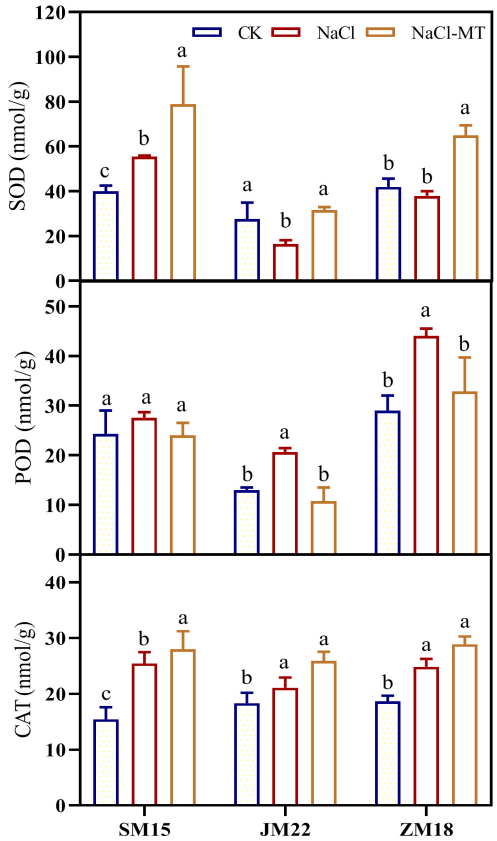

FIGURE 3 | Effects of MT on superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) under salt stress. For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

Melatonin Application Decreased H_2O_2 Content in Root and Displayed Less H_2O_2 and O_2^- Accumulation in Root

Figure 7A shows that H_2O_2 contents in root of JM22, SM15, and ZM18 were significantly higher under NaCl stress than that under CK by 23.74, 33.89, and 20.24%, respectively. MT application reduced H_2O_2 contents in the roots of three varieties under NaCl stress to the level of CK. H_2O_2 and O_2^- accumulation in roots of three varieties with three treatments were visualized by DAB and NBT staining. As expected, the detached roots of MT-treated wheat seedlings displayed less H_2O_2 and O_2^- accumulation than those of NaCl-treated seedlings (**Figure 7B**). However, H_2O_2 contents in the leaf of JM22, SM15, and ZM18 were decreased significantly compared with CK. MT application maintained a low level of H_2O_2 contents in the leaves of three varieties.

Melatonin on Cluster Heatmap of Amino Acid Content of Wheat Seedlings Under Salt Stress

Figure 8 shows the variations in amino acid content in plants of three wheat varieties under different treatments. Principal component analysis extracted two major components that together accounted for 97.0% of the variance in the dataset. Principal component 1 (PC1, *x*-axis) explained 94.0% of the variation among the individual samples, and principal component 2 (PC2, *y*-axis) explained 3.0% of the variation. Under NaCl stress, the amino acid content in the roots and leaves of three cultivars shifted greatly along PC1 axis (**Figure 8A**). Glu content showed high content, but Met, Cys, His, and Tyr content were low in plants. Cys and Met content in the root of JM 22 and Cys content in the root of SM15 and ZM18 decreased under salt stress, but increased when MT application. Lys and Tyr content in leaf both increased under salt stress and further increased with MT application; His content in leaf increased significantly under both NaCl and NaCl-MT treatments. Other amino acids increased under NaCl and decreased under NaCl-MT without recovering to the level of control (**Figure 8B** and **Supplementary Table 1**).

DISCUSSION

Salinity, one of the major abiotic stresses, limits the growth and productivity of many field crops. For increasingly extreme climate events, it is becoming the most important scientific research and agricultural practices in improving the salt tolerance of crops and exploiting arable areas of saline soils (Zhang et al., 2011; Himabindu et al., 2016). MT plays significant roles in antisenescence and antistress (Arnao and Hernández-Ruiz, 2014). It is well documented that exogenous MT can also improve the salinity resistance of wheat seedlings (Ke et al., 2018; Liu et al., 2020). During seed germination, exogenous MT (20 and 1 μM) pretreatment enhanced the germination rate of cotton, wheat, cucumber, and so on (Zhang N. et al., 2014; Ke et al., 2018; Chen et al., 2021). In our result, however, the germination rate showed somewhat improvement on ZM18 and SM15, no improvement on JM22, and furtherly inhibited the length of radical and germ under salt stress (**Table 2** and **Figure 9**). On the one hand, different MT concentrations for different crops might be partly reasons. However, concentration was not the main and only reason because MT (0–500 μM) pretreatment recovered root vigor and growth of two maize varieties and decreased relative electrolytic leakage in roots and leaves during the period of seed germination and seedling cultivation under NaCl stress (Li X. et al., 2017). This is likely for different treatment modes, plant pretreated with MT often shows better stress resistance even under lower concentrations, but MT was added directly to the culture solution especially in high concentrations, the seed germination was inhibited, and the growth of radical and radicle appeared retardation because plants would trigger a defensive response as external stress elements. On the other hand, the radicle number increased significantly under salt and MT condition, which suggested that MT promoted lateral root produced and growth of plants under NaCl stress. This result was consistent with the newest report that MT promotes lateral root under salt stress (Javeed et al., 2021).

Exogenous MT partially mitigated the salt stress-induced inhibition of whole-plant growth by reducing the accumulation

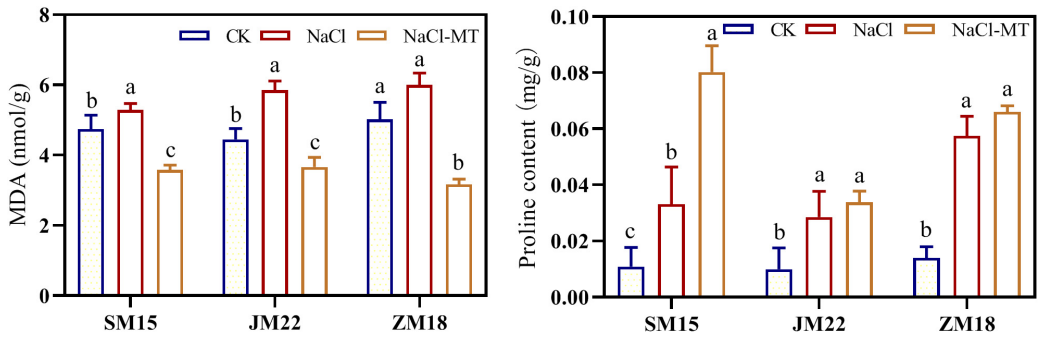

FIGURE 4 | Effects of MT on malondialdehyde (MDA) and proline content under salt stress. For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

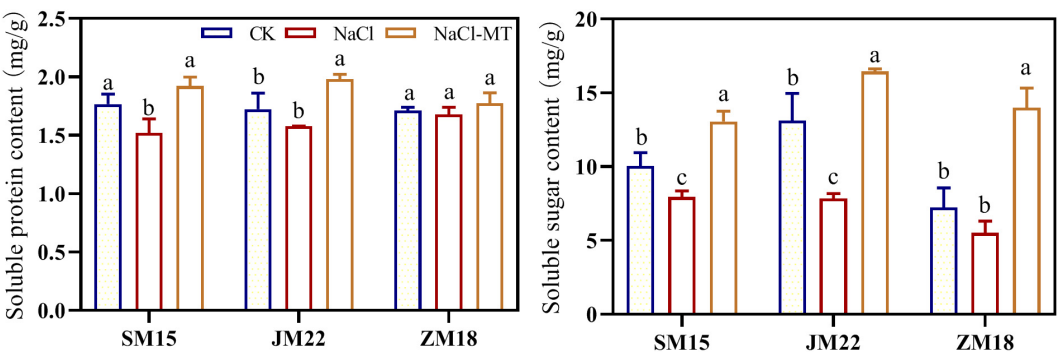

FIGURE 5 | Effects of MT on soluble protein and sugar content under salt stress. For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

of H_2O_2 in wheat leaf and increasing endogenous MT content and polyamine contents (Ke et al., 2018). Rice and cotton plants pretreated with MT also reduced H_2O_2 contents in leaf and roots, which accumulate high H_2O_2 concentration induced by salt stress (Li X. et al., 2017; Jiang et al., 2021). In our research, the changing rule of H_2O_2 content in root was also decreased with salt and MT treatments compared with NaCl alone treatment. However, H_2O_2 content in wheat leaf was significantly decreased under salt stress alone compare with CK, and MT application did not significantly recover H_2O_2 content to the level of control (**Figure 7**). The possible reason was that roots receive stress signals to turn on the defense system including recognizing and appointing exogenous MT as a stress regulator in a short time; for leaf, there was delayed effects for that MT has been reported as a potent long-distance signal to translocate stress message from roots to leaf (Li H. et al., 2017). Whether seeds of rice and wheat were pretreated with MT for 3–7 days, or the cotton seedling was treated with foliar spray MT every 24 h for 12 days, plants have been finished antistress training (Li X. et al., 2017; Ke et al., 2018; Jiang et al., 2021). Besides playing a vital signaling role during stress responses, H_2O_2 was induced by respiratory burst oxidase homologs (RBOH) to convert eventually into other ROS (OH) by the Fenton reaction and Haber–Weiss reaction (Liu et al., 2020; Michard and Simon, 2020). Additionally, MT treatment enhanced significantly the activities of antioxidant enzymes (especially SOD and CAT) and decreased MDA content in leaves resulted in a decrease in H_2O_2 level, which was conformed to this conclusion of Li X. et al. (2017). Not only does MT act as an endogenous antioxidant that enhanced the antioxidant capacity, but also MT was applied exogenously to modulate subcellular antioxidant systems in barley (Li et al., 2016). The possible response mechanism is still unknown comprehensively considering systematic reaction and crosstalk between organs.

Another effective strategy to resist salt stress employed by plants is to keep ion homeostasis and relieve ionic toxicity. One important approach that increases salt tolerance is to maintain a high level of $[K^+]/[Na^+]$ ratio in cells (Flowers and Läuchli, 1983). Many of glycophytes are subjected to NaCl stress which showed that Na^+ in roots and leaves significantly increased, and K^+ content clearly decreased compared with those of the control (Li X. et al., 2017; Ren et al., 2020). Na^+ is sensed possibly by Na^+ sensor glucuronosyltransferase, activates Ca^{2+} channels, and increases Ca^{2+} influx into the cytosol (Jiang et al., 2019). In our study, MT application significantly decreased Na^+ contents, increased $[K^+]/[Na^+]$ ratio in root induced by NaCl stress alone, but further increased Na^+ content, and decreased $[K^+]/[Na^+]$ in stem and leaf. This suggested that MT may take ameliorating effect first on the root, and meantime, a higher Na ion concentration formed along with

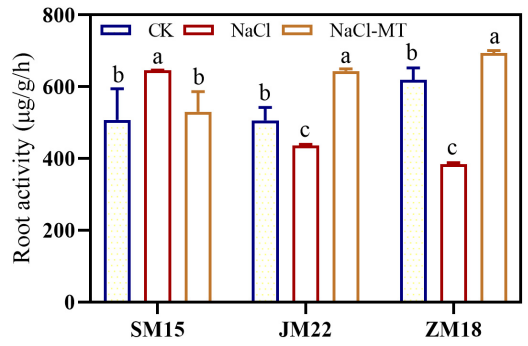

FIGURE 6 | Effects of MT on root activity under salt stress. For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

nutrient solution flows and circulates in stem and leaf. It is known that the ion transport system including ionic equilibrium of $[K^+]$ and $[Na^+]$ and $[K^+]/[Na^+]$ is often considered in a signaling network involving H_2O_2 and Ca^{2+}. Kaya et al. (2019) reported that MT treatments increase plant growth attributes to increased Ca^{2+} and K^+ in the leaves and reduced MDA, H_2O_2 in Cd-stressed wheat plants. MT also helps cold-stress plants that possessed higher Ca^{2+}-ATPase, which are important for the ATP formation (Sun et al., 2018). In our results, SM15 also showed a higher Ca^{2+} keeping ability in stem and leaf under salt stress alone; ZM 18 was more easily adjusted by MT to increase significantly Ca^{2+} content in root and leaf under salt stress. These results conform to that the increase in Ca^{2+} content in cytosol that triggers RBOH activity directly induces the formation of H_2O_2 that is eventually converted into other ROS (Michard and Simon, 2020), which is also interpreted as why H_2O_2 content decreased in leaves under salt treatment (**Figure 7**).

Salt stress promoted storage protein degradative, and thus, amino acid content changed accordingly (Zhang et al., 2017). Lysine (Lys) content is decreased with MT and transforms into other substances to raise the level of stress tolerance in wheat seeds during germination under drought stress (Li et al., 2020). MT also accelerates the metabolic flow from the precursor amino acids arginine and methionine to polyamines and decreases the degradation of salt-induced polyamines (Ke et al., 2018). From our study, the consistent results showed that salt stress increased Arg and Met contents, which decreased with MT application to some degrees. Additionally, Cys and Met contents in root of JM 22 and Cys content in the root of SM15 and ZM18 decreased under salt stress, but increased when MT application. This suggested that Cys is likely to participate in the H_2S-Cys cycle, which enhances its roles in the regulation of the antioxidant system (Huang et al., 2021). In our study, Lys and Tyr contents in leaf both increased under salt stress and further increased with MT application;

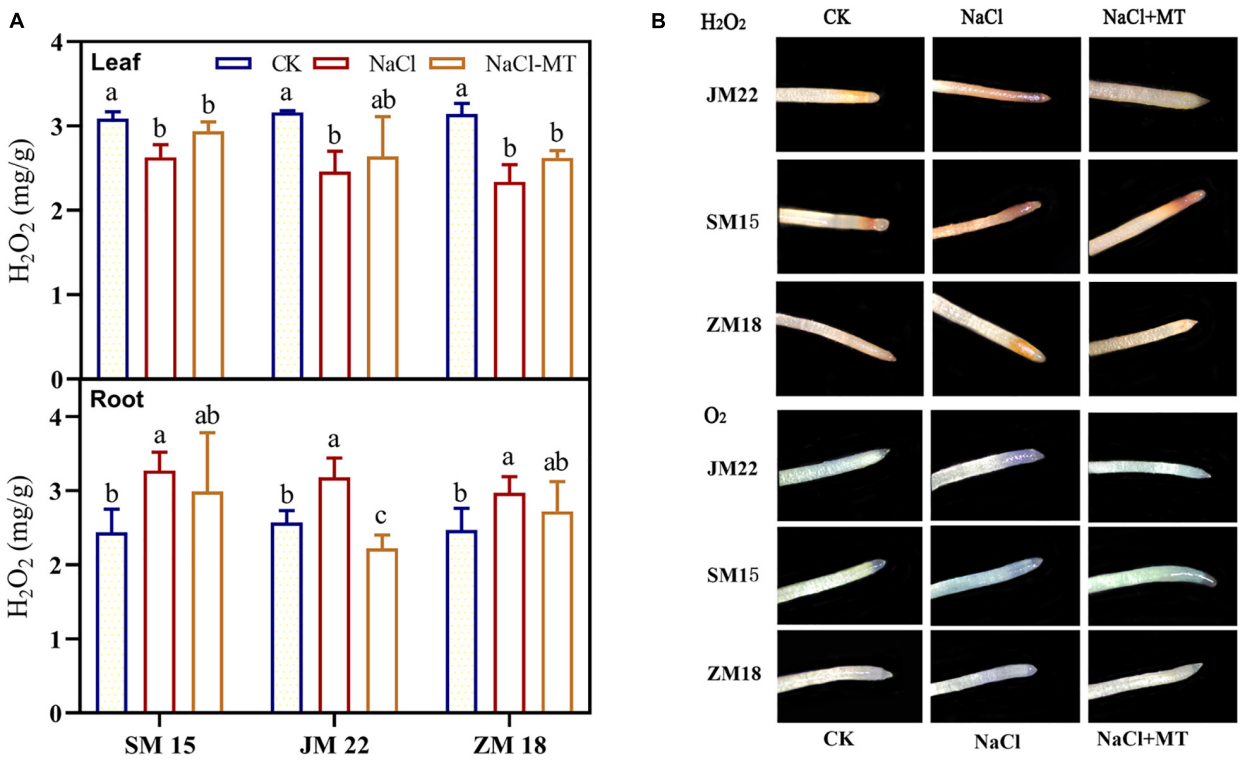

FIGURE 7 | Effects of MT on hydrogen peroxide (H_2O_2) content in plant **(A)** and H_2O_2 and upper-oxide anions (O_2^-) distribution in root **(B)** under salt stress. For each trait, bars with the same letter are not significantly different according to Duncan's test at a $p < 0.05$ threshold.

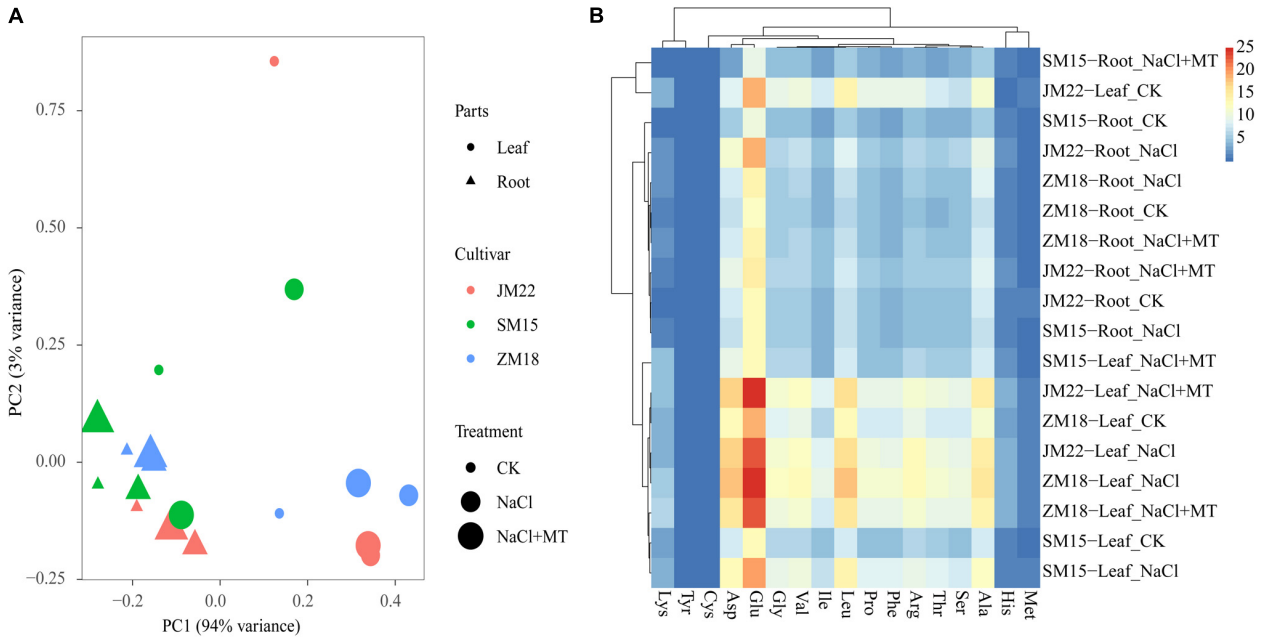

FIGURE 8 | The principal component analysis (PCA) **(A)** and cluster heatmap **(B)** of amino acid content of wheat seedlings under different treatments. Asp, aspartic acid; Glu, glutamic; His, histidine; Ser, serine; Arg, arginine; Gly, glycine; Thr, threonine; Pro, proline; Ala, alanine; Val, valine; Met, methionine; Cys, cysteine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Lys, lysine; Tyr, tyrosine.

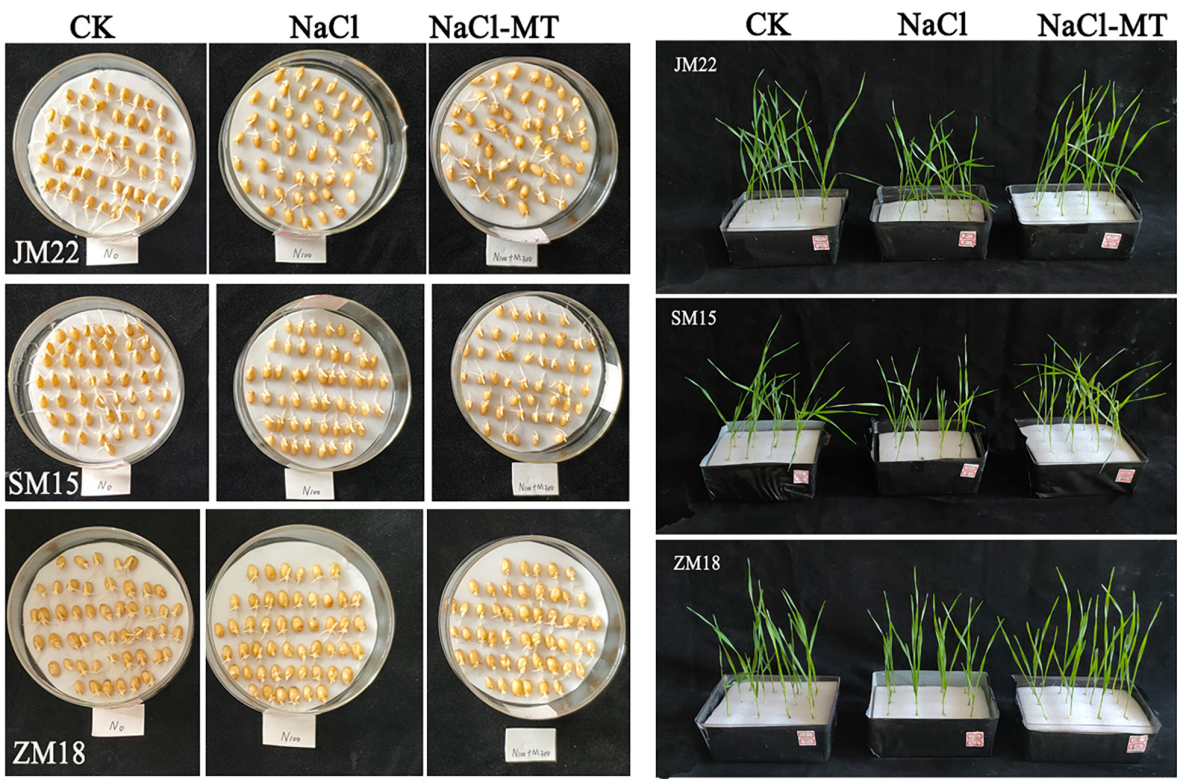

FIGURE 9 | Schematic diagram of seed germination and seedling growth with MT under salt stress.

His content in leaf increased significantly under both NaCl and NaCl-MT treatments. Other amino acids increased under NaCl and decreased under NaCl-MT without recovering to the level of control. It is known that MT often helps to induce amino acid accumulation in root and leaf to enhance the cellular osmotic potential (Cui et al., 2018). Lysine has been reported to be transformed into proline, aminobutyric acid, and polyamines during drought resisting processes (Hare and Cress, 1997; Klessig et al., 2000). So, our results also provided the evidence that MT increases the ability for the leaves of different wheat varieties to keep high water status and accumulate high soluble protein, soluble sugar, and proline content under NaCl stress (**Table 3** and **Figures 4**, **5**). On the other hand, lysine residues on histone terminus are deacetylated by histone deacetylase 14, which is involved in the biosynthesis of MT in *Arabidopsis thaliana* (Zhao et al., 2018). Both Lys and His contents increased in leaf under NaCl and NaCl + MT which indicated a complex biochemical process, during which the MT content changing the interaction between MT and amino acid, and involved mechanism would be the next important research point.

It is noted that MT application methods (pretreated coating or soaking, leaf pray, root or rhizospheric application, mixed application with other growth regulators) and varied experimental elements (MT concentration, plant species, varieties, and adversity types) appear to have different regulatory effects during stress-resistance process (Kostopoulou et al., 2015; Jiang C. et al., 2016; Li X. et al., 2017). In our research, different wheat varieties showed different salt-sensitivities. JM22, a widely adaptive super-high-yield variety, was not salt-resistant and shows an obvious decrease on germination rate and activity of SOD and CAT under NaCl stress. SM15 and ZM18 showed higher salt-resistant, considering that SOD activity of SM15 did not decrease but increased significantly and higher germination rate and proline accumulation under NaCl stress alone. MT at a concentration of 300 μmol L^{-1} played roles as regulated antioxygen to keep the physiological equilibrium of wheat seedling and to promote germination and lateral roots, but it took an inhibitory effect on the length of radicle and germ. The optimal concentration of MT on wheat seed germination and growth of wheat seedling and the dominant varieties of wheat for coping with salt stress should be further researched for meeting future production reality needs.

CONCLUSION

Exogenous MT promoted germination rate of SM15 and ZM 18 recovering control level under salt stress, but had no effect on that of JM22. SM15 and ZM 18 showed higher salt resistance than that of JM22, especially for SM15 that had higher proline content, root vigor, and higher antioxygen system under salt stress. JM22 was salt-sensitive. MT at 300 'μmol L^{-1} inhibited the length of germ and radicle, but promoted lateral root production of seed embryo, and increased shoot and root length of wheat seedling under NaCl stress. Salt stress-induced antioxygen system activity enhancing and H_2O_2 content increasing, decreased soluble protein, soluble sugar content, $[K^+]/[Na^+]$ in leaf and root vigor, and increased Na^+ content in root and leaf. MT application could increase proline content, soluble protein, soluble sugar, Ca^{2+} content, and vital amino acid content in leaf to keep high water status and maintain a low level of H_2O_2 content and $[K^+]/[Na^+]$ ratio in leaf. MT increased root vigor, $[K^+]/[Na^+]$ ratio, and decreased H_2O_2 content in root induced by salt stress. The findings of this study showed that the root as the first defense organization responded to salt stress was rapidly and earlier than the leaf in a short time (24 h), and the signal transmission and interaction between roots and leaf maintained to reduce stress injury as much as possible. So, in wheat production, the optimal concentration, varieties, and MT application method should be considered comprehensively at the sowing and seedling stages.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

DL and RL initiated and designed the research. ZZ, HL, XF, and SZ performed the experiments and collected the data. XZ, NY, and JS wrote the code and tested the methods. LL, ZZ, and DL analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.787062/full#supplementary-material

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