

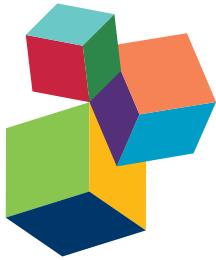
# MELATONIN IN PLANTS

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# MELATONIN IN PLANTS

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This topic focuses on distribution, synthesis, metabolism, and the *in vivo* roles of melatonin in plants, with 1 editorial, 3 reviews, 21 original research studies and 1 corrigendum.

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# Table of Contents

- 06 Editorial: Melatonin in Plants**  
Haitao Shi, John Love and Wei Hu
- 08 Melatonin in Plants – Diversity of Levels and Multiplicity of Functions**  
Rüdiger Hardeland
- 22 Melatonin: Current Status and Future Perspectives in Plant Science**  
Muhammad A. Nawaz, Yuan Huang, Zhilong Bie, Waqar Ahmed, Russel J. Reiter, Mengliang Niu and Saba Hameed
- 35 Fundamental Issues of Melatonin-Mediated Stress Signaling in Plants**  
Haitao Shi, Keli Chen, Yunxie Wei and Chaozu He
- 41 Corrigendum: Melatonin: Current Status and Future Perspectives in Plant Science**  
Muhammad A. Nawaz, Yuan Huang, Zhilong Bie, Waqar Ahmed, Russel J. Reiter, Mengliang Niu and Saba Hameed
- 43 Exogenous Melatonin Mitigates Photoinhibition by Accelerating Non-photochemical Quenching in Tomato Seedlings Exposed to Moderate Light during Chilling**  
Fei Ding, Meiling Wang, Bin Liu and Shuoxin Zhang
- 54 Melatonin in Plants and Plant Culture Systems: Variability, Stability and Efficient Quantification**  
Lauren A. E. Erland, Abhishek Chattopadhyay, Andrew Maxwell P. Jones and Praveen K. Saxena
- 67 Alleviation of cold damage to photosystem II and metabolisms by melatonin in Bermudagrass**  
Jibiao Fan, Zhengrong Hu, Yan Xie, Zhulong Chan, Ke Chen, Erick Amombo, Liang Chen and Jinmin Fu
- 81 Effects of Melatonin on Anti-oxidative Systems and Photosystem II in Cold-Stressed Rice Seedlings**  
Qiao-Hong Han, Bo Huang, Chun-Bang Ding, Zhong-Wei Zhang, Yang-Er Chen, Chao Hu, Li-Jun Zhou, Yan Huang, Jin-Qiu Liao, Shu Yuan and Ming Yuan
- 95 Comparative Physiological and Transcriptomic Analyses Reveal the Actions of Melatonin in the Delay of Postharvest Physiological Deterioration of Cassava**  
Wei Hu, Hua Kong, Yunling Guo, Yuliang Zhang, Zehong Ding, Weiwei Tie, Yan Yan, Qixing Huang, Ming Peng, Haitao Shi and Anping Guo
- 107 Melatonin-Producing Endophytic Bacteria from Grapevine Roots Promote the Abiotic Stress-Induced Production of Endogenous Melatonin in Their Hosts**  
Jian Jiao, Yaner Ma, Sha Chen, Chonghuai Liu, Yuyang Song, Yi Qin, Chunlong Yuan and Yanlin Liu

- 120 Melatonin Protects Cultured Tobacco Cells against Lead-Induced Cell Death via Inhibition of Cytochrome c Translocation**  
Agnieszka Kobylińska, Russel J. Reiter and Małgorzata M. Posmyk
- 131 Exogenous Melatonin Confers Salt Stress Tolerance to Watermelon by Improving Photosynthesis and Redox Homeostasis**  
Hao Li, Jingjing Chang, Hejie Chen, Zhongyuan Wang, Xiurong Gu, Chunhua Wei, Yong Zhang, Jianxiang Ma, Jianqiang Yang and Xian Zhang
- 140 Melatonin Regulates Root Architecture by Modulating Auxin Response in Rice**  
Chengzhen Liang, Aifu Li, Hua Yu, Wenzhen Li, Chengzhi Liang, Sandui Guo, Rui Zhang and Chengcai Chu
- 152 Endophytic Bacterium *Pseudomonas fluorescens* RG11 May Transform Tryptophan to Melatonin and Promote Endogenous Melatonin Levels in the Roots of Four Grape Cultivars**  
Yaner Ma, Jian Jiao, Xiucai Fan, Haisheng Sun, Ying Zhang, Jianfu Jiang and Chonghuai Liu
- 167 Melatonin Application to *Pisum sativum* L. Seeds Positively Influences the Function of the Photosynthetic Apparatus in Growing Seedlings during Paraquat-Induced Oxidative Stress**  
Katarzyna Szafrarska, Russel J. Reiter and Małgorzata M. Posmyk
- 179 Melatonin Improves the Photosynthetic Apparatus in Pea Leaves Stressed by Paraquat via Chlorophyll Breakdown Regulation and Its Accelerated de novo Synthesis**  
Katarzyna Szafrarska, Russel J. Reiter and Małgorzata M. Posmyk
- 189 Melatonin Regulates Root Meristem by Repressing Auxin Synthesis and Polar Auxin Transport in Arabidopsis**  
Qiannan Wang, Bang An, Yunxie Wei, Russel J. Reiter, Haitao Shi, Hongli Luo and Chaozu He
- 200 Isolation and Functional Characterization of Bidirectional Promoters in Rice**  
Rui Wang, Yan Yan, Menglin Zhu, Mei Yang, Fei Zhou, Hao Chen and Yongjun Lin
- 211 Comparative Transcriptional Profiling of Melatonin Synthesis and Catabolic Genes Indicates the Possible Role of Melatonin in Developmental and Stress Responses in Rice**  
Yunxie Wei, Hongqiu Zeng, Wei Hu, Lanzhen Chen, Chaozu He and Haitao Shi
- 226 A Simple, Rapid Method for Determination of Melatonin in Plant Tissues by UPLC Coupled with High Resolution Orbitrap Mass Spectrometry**  
Tiantian Ye, Yan-Hong Hao, Lei Yu, Haitao Shi, Russel J. Reiter and Yu-Qi Feng
- 236 Comparative Transcriptomic Analyses of Differentially Expressed Genes in Transgenic Melatonin Biosynthesis Ovine HIOMT Gene in Switchgrass**  
Shan Yuan, Cong Guan, Sijia Liu, Yanhua Huang, Danyang Tian, Xin Cui, Yunwei Zhang and Fuyu Yang
- 247 RNA-seq Analysis of Overexpressing Ovine AANAT Gene of Melatonin Biosynthesis in Switchgrass**  
Shan Yuan, Yanhua Huang, Sijia Liu, Cong Guan, Xin Cui, Danyang Tian, Yunwei Zhang and Fuyu Yang
- 260 Genistein: A Novel Anthocyanin Synthesis Promoter that Directly Regulates Biosynthetic Genes in Red Cabbage in a Light-Dependent Way**  
Na Zhang, Yan Qi, Hai-Jun Zhang, Xiaoyun Wang, Hongfei Li, Yantong Shi and Yang-Dong Guo

**271 Melatonin Improved Anthocyanin Accumulation by Regulating Gene Expressions and Resulted in High Reactive Oxygen Species Scavenging Capacity in Cabbage**

Na Zhang, Qianqian Sun, Hongfei Li, Xingsheng Li, Yunyun Cao, Haijun Zhang, Shuangtao Li, Lei Zhang, Yan Qi, Shuxin Ren, Bing Zhao and Yang-Dong Guo

**288 Melatonin Improves Waterlogging Tolerance of *Malus baccata* (Linn.) Borkh. Seedlings by Maintaining Aerobic Respiration, Photosynthesis and ROS Migration**

Xiaodong Zheng, Jingzhe Zhou, Dun-Xian Tan, Na Wang, Lin Wang, Dongqian Shan and Jin Kong



# Editorial: Melatonin in Plants

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**Keywords:** melatonin, plant, reactive oxygen species (ROS), development, stress responses

## Editorial on the Research Topic

### Melatonin in Plants

Melatonin (*N*-acetyl-5-methoxytryptamine) is an important pleiotropic molecule with multiple physiological and cellular actions in animals and plants. In 1958, melatonin was identified in the pineal gland of bovine. In 1995, melatonin was discovered in higher plants. Thereafter, the numerous functions of melatonin in animals have shown its great potential in plant physiology.

The plant melatonin field is dynamic as evidenced in the increasing number of publications in all disciplines, including its involvement in seed germination, primary root and lateral root architecture, photoprotection, circadian rhythm, flowering time, biomass production, leaf senescence and fruit ripening. Compelling evidence suggests that melatonin is also involved in various stress responses. Most of these studies indicate that melatonin may act as the first barrier in response to reactive oxygen species (ROS) burst by scavenging free radical, and as the second step for defense by regulating the expression of several stress-responsive genes.

This topic focuses on distribution, synthesis, metabolism, and the *in vivo* roles of melatonin in plants. We aim to ask whether and how melatonin functions as an important regulator during plant development and plant stress responses, and how melatonin network connects with different signaling pathways. This topic contains 3 reviews, 21 original research studies and 1 corrigendum.

The first section is the review and quantification of melatonin. Nawaz et al. and Nawaz et al. provided a review update the available information about the presence and actions of melatonin in different plant species including important crops, and highlighted the untraceable value of melatonin-rich food crops (cereal, fruit and vegetables). Hardeland summarized the diversity of levels and multiplicity of functions of melatonin in plants, including the precursor, catabolism, isoenzymes, rate limitation and remarkable pleiotropy of melatonin biosynthetic pathway under various functional aspects, as well as the effects of melatonin on plant growth and stress response. Shi et al. highlighted the changes of endogenous melatonin levels under various stress conditions, melatonin-mediated stress responses through modulating several transcription factors, physiological mechanism, and the extensive reprogramming of transcriptome, proteome and metabolome. Erland et al. described a validated method for the quantification of melatonin, serotonin and the underlying biosynthetic precursors (tryptophan, tryptamine and *N*-acetylserotonin) in diverse plant culture systems. Ye et al. reported a simple and rapid quantification of plant endogenous melatonin by UPLC coupled with high resolution Orbitrap mass spectrometry.

The *in vivo* roles of melatonin were also revealed in different plant species in this topic. In bermudagrass, Fan et al. found that exogenous melatonin treatment alleviated cold damage by maintaining cell membrane stability, improving the process of photosystem II and increasing antioxidant enzyme activities. In cabbage and *Arabidopsis*, Zhang et al. found that melatonin improved anthocyanin accumulation and benefited cabbage growth, by increasing the expression

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levels of anthocyanin biosynthetic genes and ROS scavenging capacity. Similarly, genistein promotes anthocyanin synthesis in red cabbage in a light-dependent way, through directly regulating anthocyanin biosynthetic genes (Zhang et al.). Wang et al. found that high concentration of melatonin represses root meristem through modulation of both auxin synthesis and polar auxin transport in *Arabidopsis*. Consistently, Liang et al. found that melatonin regulates root architecture including both embryonic root and lateral root through modulation of auxin response in rice. In rice, Han et al. found that melatonin alleviated the inhibition of cold-mediated seedling growth by regulating anti-oxidative systems and photosystem II; and they also highlighted the dose dependent response of melatonin on plant physiological, biochemical and photosynthetic parameters. In cassava, Hu et al. found that melatonin delayed postharvest physiological deterioration (PPD) of cassava tuberous roots, through modulating ROS metabolism and transcriptomic reprogramming including metabolic-, ion homeostasis-, and enzyme activity-related genes as well as calcium signaling-, mitogen-activated protein kinase (MAPK) cascades-, and starch degradation-related pathways, etc. In rice, based on comprehensive transcriptional profiling of 11 melatonin related genes in different periods, tissues, in response to different treatments using published microarray data, Wei et al. provided new insight into the direct relation among melatonin biosynthesis and catabolic pathway, plant development, circadian rhythm, stress and defense responses in rice. Wang et al. provided a new method for selecting and identifying bidirectional promoters and underlying regulatory regions in rice; and they also found that almost all these promoters and novel *cis*-sequences are melatonin independent. In switchgrass, Yuan et al. and Yuan et al. identified a large number of differentially expressed genes (DEGs) in the melatonin-rich switchgrass through RNA-seq, providing some clues of melatonin metabolism on transcriptome reprogramming in switchgrass. Szafranska et al. and Szafranska et al. found that melatonin can enhance oxidative stress tolerance in growing seedlings of *Pisum sativum* L., through regulating photosynthetic apparatus, water content, ROS accumulation and chlorophyll degradation. Jiao et al. identified the endophytic bacterium *Bacillus amyloliquefaciens* SB-9, which displayed high level of *in vitro* melatonin secretion as well as melatonin biosynthesis pathways. This study showed the occurrence of melatonin biosynthesis pathway in endophytic bacterial and the novel role of the endophytic bacterial in counteract the adverse effects of

salt and drought stress in host plant roots. Ma et al. identified another endophytic bacterium *Pseudomonas fluorescens* RG11, which can transform tryptophan to melatonin and promote endogenous melatonin levels in grape roots. Ding et al. found the effect of exogenous melatonin on alleviating photoinhibition in tomato response to moderate light during chilling through accelerating non-photochemical quenching. Li et al. revealed the improved salt stress tolerance as well as photosynthesis and redox homeostasis of watermelon by exogenous melatonin treatment. Zheng et al. identified melatonin as an effective molecule to protect apple against waterlogging stress, through maintaining aerobic respiration, preserving photosynthesis and reducing oxidative damage. In *Nicotiana tabacum* L. line Bright Yellow 2 (BY-2) cell, Kobylińska, et al. found that proper dosage of melatonin increases cell proliferation and protects lead-induced cell death through inhibition of cytochrome c translocation.

We have to notice that several fundamental issues need to be resolved in the future. Besides this topic of melatonin in plants, we are looking forward to seeing more new findings.

## AUTHOR CONTRIBUTIONS

HS wrote and revised the manuscript. JL and WH provided suggestions and revised the manuscript. All authors approved the manuscript and the version to be published.

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# Melatonin in Plants – Diversity of Levels and Multiplicity of Functions

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Melatonin has been detected in numerous plant species. A particularly surprising finding concerns the highly divergent levels of melatonin that vary between species, organs and environmental conditions, from a few pg/g to over 20 µg/g, reportedly up to 200 µg/g. Highest values have been determined in oily seeds and in plant organs exposed to high UV radiation. The divergency of melatonin concentrations is discussed under various functional aspects and focused on several open questions. This comprises differences in precursor availability, catabolism, the relative contribution of isoenzymes of the melatonin biosynthetic pathway, and differences in rate limitation by either serotonin *N*-acetyltransferase or *N*-acetylserotonin *O*-methyltransferase. Other differences are related to the remarkable pleiotropy of melatonin, which exhibits properties as a growth regulator and morphogenetic factor, actually debated in terms of auxin-like effects, and as a signaling molecule that modulates pathways of ethylene, abscisic, jasmonic and salicylic acids and is involved in stress tolerance, pathogen defense and delay of senescence. In the context of high light/UV intensities, elevated melatonin levels exceed those required for signaling via stress-related phytohormones and may comprise direct antioxidant and photoprotectant properties, perhaps with a contribution of its oxidatively formed metabolites, such as *N*<sup>1</sup>-acetyl-*N*<sup>2</sup>-formyl-5-methoxykynuramine and its secondary products. High melatonin levels in seeds may also serve antioxidative protection and have been shown to promote seed viability and germination capacity.

**Keywords:** antioxidant, auxin-like, circadian, photoprotection, seeds, senescence, stress

## INTRODUCTION

Melatonin, once discovered in vertebrates as a hormone of the pineal gland, is now known to be formed in bacteria and numerous taxa of eukaryotes including various algae of different phylogenetic position and plants (Hardeland and Fuhrberg, 1996; Hardeland et al., 2007b; Tan et al., 2014b; Hardeland, 2015), whereas the presence of melatonin in archaea is still uncertain (Tan et al., 2014b). In addition to melatonin, recently discovered isomers of this molecule have been shown to also exist in some plants (Tan et al., 2014b). However, their full spectrum of abundance and their functional roles are not yet sufficiently known.

The fact that melatonin is an ancient molecule present in numerous phylogenetically distant organisms raises questions concerning the identity or similarity of functions. Some of them might be suspected to reflect fundamental cell biological requirements already existent before or at the basis of eukaryotic evolution. On the other hand, the billions of years since then may have been sufficient to allow the acquisition of secondary, additional functions that may entirely differ

between taxa. This can be assumed to be also the case within the viridiplantae and even the embryophyta. In fact, strong evidence exists for such a functional diversity.

After the first discovery of melatonin in a phototrophic organism, the dinoflagellate *Lingulodinium polyedrum* (syn. *Gonyaulax polyedra*; Poeggeler et al., 1989, 1991; Balzer and Hardeland, 1991) and, thereafter, in macroalgae (Fuhrberg et al., 1996) and plants (Dubbels et al., 1995; Hattori et al., 1995), investigators tried to identify classic functions of this compound known from vertebrates, such as transmission of the signal 'darkness' and regulation of seasonality and circadian rhythmicity. The idea of possibly having found an agent mediating information on the length of scotophase, as in mammalian seasonal breeders, led to hopes concerning a role in plant photoperiodism. However, the first precondition, namely, existence of a high-amplitude circadian melatonin rhythm with a nocturnal maximum, was not generally fulfilled. Such a rhythm was demonstrated in *L. polyedrum* (Poeggeler et al., 1991; Balzer et al., 1993) and in a few plants, in particular, a short-day ecotype of *Chenopodium rubrum* (Kolář et al., 1997), but not generally in macroalgae and plants. In *C. rubrum*, no flower induction by melatonin was observed (Kolář and Macháčková, 2005). Instead, flowering was partially suppressed when given late at night. A similar effect was obtained with a putative pharmacological agonist, CGP 52608, considered as a ligand of an assumed mammalian nuclear melatonin receptor (Kolář et al., 1999). However, this transcription factor is actually no longer regarded as a melatonin-binding protein (Slominski et al., 2014). Moreover, neither melatonin nor its bioactive metabolite 5-methoxytryptamine induced flowering in several lemnaceans and in the crassulacean *Kalanchoë tubiflora*, another short-day plant (Hardeland and Poeggeler, 2003; Hardeland et al., 2007b). Therefore, a role in photoperiodism of plants seems unlikely, although this cannot be ruled out for other, phylogenetically distant phototrophic organisms, such as dinoflagellates (Balzer and Hardeland, 1991; Hardeland et al., 1995).

In recent years, transcriptomic, proteomic and metabolomic studies conducted in different plants such as *Arabidopsis thaliana* (Weeda et al., 2014; Qian et al., 2015), *Malus hupehensis* (Wang et al., 2014a) and *Cynodon dactylon* (Shi et al., 2015b,f) have revealed a plethora of melatonin-induced changes in the expression of genes at mRNA and protein levels as well as in metabolite concentrations. A remarkable diversity of actions was observed in different fields of function, which indicates a pleiotropic, orchestrating role of melatonin reminiscent of that known from animals (cf. Hardeland et al., 2011).

Within plants, another strong hint for a diversity of functions can be deduced from the extreme species- and organ-specific differences in melatonin concentration, which range from almost or totally undetectable to levels of above 20 or 30 µg/g, as summarized elsewhere (Hardeland et al., 2007b; Arnao, 2014). Recently, melatonin contents of up to 200 µg/g were reported to exist in kernels of several Iranian *Pistacia vera* cultivars (Oladi et al., 2014). It seems highly unlikely that such extremely high amounts serve the same function as low concentrations of a few pg/g, which would be in a range of vertebrate levels and may be compatible with the role of a signaling molecule. In dry

seeds, under conditions of strongly reduced metabolism and gene expression and in the absence of circadian rhythmicity, melatonin might have a specific function differing from those in tissues with active metabolism (Balzer and Hardeland, 1996).

Even if the particular situation of seeds is left apart, other considerable differences exist, partially between organs, sometimes in the course of developmental processes, but most importantly between species and local variants. Notably, substantial variations were observed between specimens of the same or related species from habitats that differ with regard to temperature and light/UV exposure (cf. discussion in Hardeland et al., 2007b). It will be an important objective in the research of plant melatonin to understand why the levels of this compound can vary so profoundly and what the functional consequences of these divergencies are.

## IDENTITY OR DIFFERENCES IN PLANT MELATONIN METABOLISM?

In plants, the classic pathway of melatonin biosynthesis from tryptophan comprises four steps, decarboxylation by tryptophan decarboxylase (TDC), hydroxylation of the amine by tryptamine 5-hydroxylase (T5H) to serotonin, its N-acetylation by a serotonin N-acetyltransferase (SNAT), which catalyzes the same reaction as the nonhomologous aralkylamine N-acetyltransferase (AANAT) of vertebrates, and the final O-methylation to melatonin by N-acetylserotonin O-methyltransferase (ASMT, formerly known as hydroxymethyl O-methyltransferase, HIOMT). For summaries using the actual terminology see Tan et al. (2014b) and Hardeland (2015). Whether or not these enzymes are exclusively responsible for melatonin formation in plants may be not as certain as it appears at first glance.

The highly divergent melatonin levels of different plant species raise the question of whether this may be only caused by strongly deviating expression levels of the same biosynthetic enzymes. Alternately, this divergency may be explained by either (i) splice variants, (ii) homologous gene variants encoding enzymes that substantially differ with regard to substrate affinity and  $V_{max}$ , (iii) deviating precursor availability, or (iv) the involvement of enzymes of only moderate homology. This latter possibility is not generally unlikely, since this has been also discussed for melatonin synthesis in extrapineal sites of vertebrates (Hardeland, 2008), despite the observation that AANAT and ASMT are responsible for melatonin formation in the pineal glands and various other organs in these animals. For instance, another N-acetyltransferase, NAT-1, was reported to contribute to melatonin synthesis in the mammalian skin (Slominski et al., 2005). In insects, several AANAT subforms exist, which strongly differ in their substrate specificity and are also involved in other functions such as exoskeleton sclerotization or neurotransmitter catabolism (Han et al., 2012; Barberà et al., 2013; Hiragaki et al., 2015). In the future, isoenzymes of melatonin biosynthesis should be more generally considered in the botanical area. Evidence for such isoenzymes has been recently summarized (Hardeland, 2015). In principle, these may be isoforms coded by the same or

duplicated genes, but could also represent functionally deviating members belonging to the same protein family. In particular, a lower substrate specificity can be associated with side activities in the indoleamine metabolism, as will be illustrated below by the example of O-methylation of *N*-acetylserotonin in plants. On the other hand, relatively small genetic deviations, even point mutations, can profoundly change the substrate specificity within an enzyme family. Therefore, homologies in nucleotide or amino acid sequences should not be overinterpreted with regard to a possible involvement in melatonin formation, as recently illustrated for some examples from plants (Hardeland, 2015).

Alternate pathways may already exist for the formation of serotonin from tryptophan. Serotonin biosynthesis in plants is usually believed to be carried out by the sequential actions of TDC and T5H and, therefore, to differ from the route in animals and dinoflagellates, which consists of tryptophan hydroxylation by a tryptophan 5-hydroxylase (TPH) followed by decarboxylation by an aromatic amino acid decarboxylase of usually broad substrate specificity (Hardeland, 2015). However, an animal-like pathway may be also present in some plant species. Although TDC and T5H have been shown to be decisive for serotonin synthesis in both monocots and dicots, such as rice (*Oryza sativa*) (Byeon et al., 2014c) and apple (*Malus × domestica*) (Lei et al., 2013), the rice TDC was also reported to decarboxylate 5-hydroxytryptophan (Park et al., 2008). The *T5H*-deficient Sekiguchi rice cultivar produced elevated levels of 5-hydroxytryptophan (Park et al., 2012), a finding that demonstrates the existence of an additional tryptophan hydroxylase activity independent of T5H. However, this rice variant did not exhibit an increase in melatonin because the more strongly accumulating tryptamine efficiently competed with serotonin at the SNAT enzyme. Surprisingly, transcriptional silencing of *T5H* in rice led to higher melatonin levels (Park et al., 2013a), a finding that, again, underlines the possibility of an alternate route of serotonin formation. Nevertheless, in rice, the classic plant pathway of TDC and T5H is obviously of higher relevance, since *TDC* overexpression causes an increase in melatonin (Byeon et al., 2014c). This may not be necessarily the case in all other plant species. In *Hypericum perforatum*, the expression of a tryptophan 5-hydroxylase was reported, in addition to TDC (He et al., 2012). Moreover, with reference to the statement made above that even point mutations can profoundly change the substrate specificity of some enzymes, it should be noted that the replacement of serine 372 by a glycine in the *Papaver somniferum* tyrosine decarboxylase generates a substantial affinity to 5-hydroxytryptophan (Torrens-Spence et al., 2014). In rice, three TDC isoforms were detected, TDC-1, TDC-2, and TDC-3, which seem to differ in their contribution to melatonin formation (Byeon et al., 2014c). The multiplicity of subforms and the general mutation-sensitive variability of substrate specificity observed in aromatic amino acid/amine decarboxylases (Torrens-Spence et al., 2013) may allow evolutionary changes in the serotonin pathway. Therefore, it may well be possible that, in some plants, the animal-type of serotonin formation has become prevalent. In the future, this

should be particularly tested in plants that produce extremely high levels of melatonin.

Overexpression of *TDC* in rice leads to increased melatonin levels (Byeon et al., 2014c), a finding that indicates a rate limitation by the availability of tryptamine in this species. However, a second bottleneck in melatonin formation seems to exist in a number of plants, as can be deduced from the observation that serotonin concentrations are often by one or more orders of magnitude higher than those of melatonin, not only in the transgenic (Byeon et al., 2014c) but also in wild-type rice (Park et al., 2013d), in *Datura metel* (Murch et al., 2009), *Punica granatum* and *Fragaria × ananassa* (Badria, 2002). In these cases, especially when levels of *N*-acetylserotonin do not substantially exceed melatonin, SNAT seems to catalyze a secondary rate-limiting step, a situation reminiscent of vertebrate melatonin synthesis. However, this is not generally the case. In *Echinacea purpurea*, concentrations of serotonin and melatonin were in the same range (Jones et al., 2007). Serotonin contents that only moderately exceed those of melatonin may not substantially change the metabolic throughput in this pathway and seem to be a matter of variability between sibling species, strains and, perhaps, environmental differences of biotopes. For instance, serotonin concentrations did not substantially differ from those of melatonin in *Vaccinium macrocarpon*, but were almost five times higher in *V. vitis-idaea* (Brown et al., 2012). Notably, in several cases with very high melatonin contents, serotonin was reported to only moderately exceed the methoxyindole, such as in beans of *Coffea canephora* and *C. arabica* (serotonin 10.5 and 12.5 µg/g; melatonin 5.8 and 6.8 µg/g, respectively; Ramakrishna et al., 2012), or even to remain strongly below, such as in ripening wine grapes (serotonin about or lower than 10 µg/g; melatonin between about 100 and 150 µg/g; Murch et al., 2010) and developing flowers of *H. perforatum* (serotonin maximally about 2 nmol/g; melatonin maximally 4000 nmol/g; Murch and Saxena, 2002). Interestingly, the serotonin peak preceded that of melatonin in the *Hypericum* flower buds, whereas serotonin was practically undetectable in an early stage of *Vitis* grapes, although melatonin had already reached the range indicated. These findings strongly suggest that, in these high-melatonin tissues, *N*-acetylation of serotonin is not rate limiting. It would be of interest to study this more systematically and to test the possibility that a major difference between low- and high-melatonin plants may consist in the limitation or non-limitation of melatonin formation by SNAT activity, which might contribute to understanding why melatonin contents of plants can be so exceptionally divergent over many orders of magnitude.

Whether or not the deduced divergencies of SNAT activity may be associated with differently regulated subforms or the involvement of nonhomologous serotonin-acetylating enzymes is unknown. Pertinent information is still restricted to a very few species and most molecular data are from rice (cf. Hardeland, 2015). Recently, a SNAT from *A. thaliana* had been cloned (Lee et al., 2014b) and another one from a gymnosperm, *Pinus taeda* (Park et al., 2014). SNAT from *Oryza sativa* displays homology to a cyanobacterial enzyme and is, in line with this information, plastidially located (Byeon et al., 2013, 2014b). However, its

vertebrate paralog, AANAT, is of alphaproteobacterial origin and thought to be inherited via mitochondria (Tan et al., 2013). To date, there is no existing evidence for mitochondrial serotonin acetylation in plants by an AANAT-like enzyme, but, on the small basis of respective knowledge, this should not yet be ruled out.

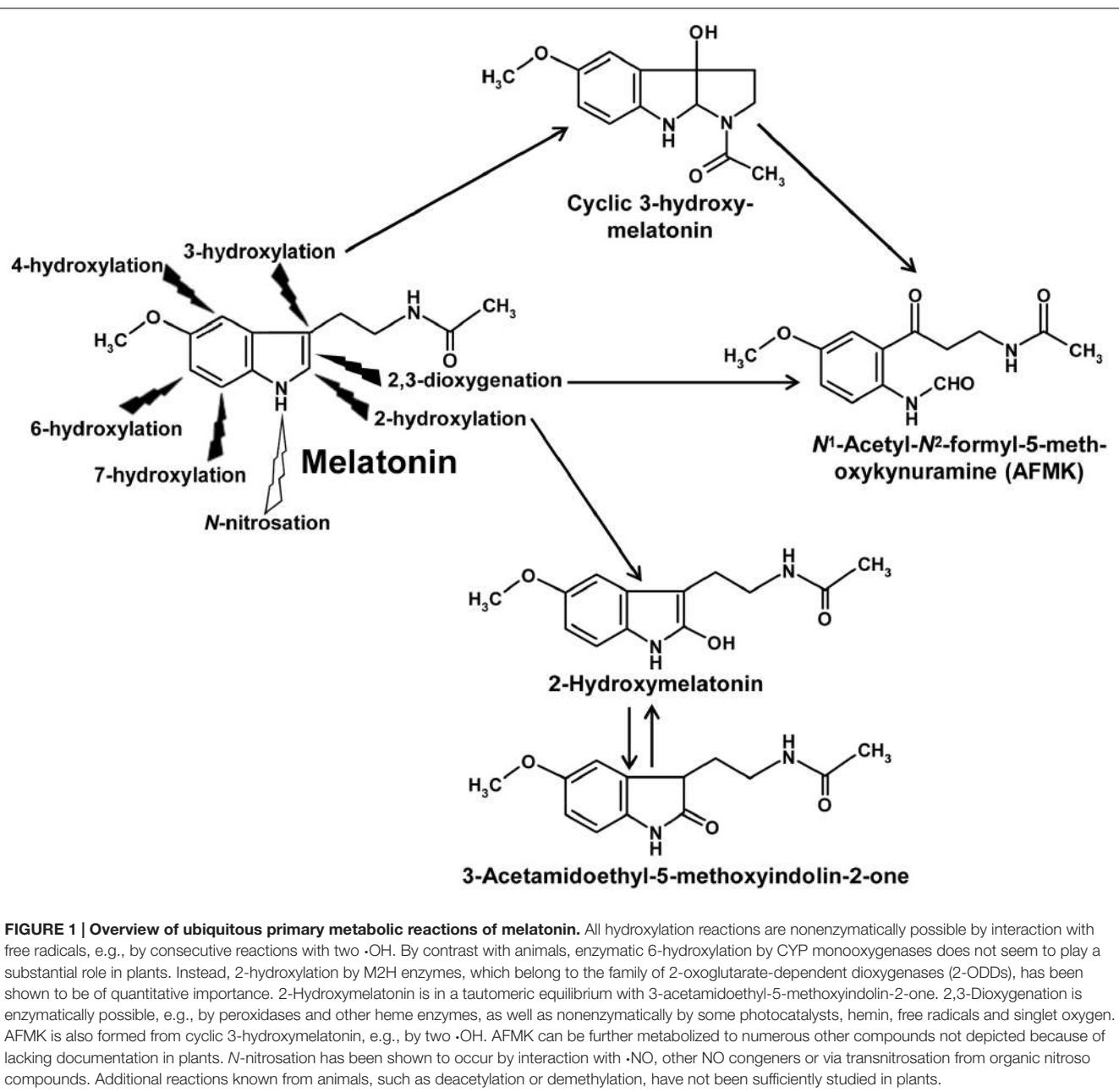
The final step of melatonin formation is catalyzed by cytosolic members of plant O-methyltransferases, which display differences to the mammalian ASMT (Park et al., 2013c) and do not seem to be closely related to the vertebrate enzymes. Obviously, several enzymes from plants are capable of catalyzing the O-methylation of *N*-acetylserotonin. In rice, three isoforms, ASMT1, ASMT2, and ASMT3 were reported to be encoded by different genes (Park et al., 2013b). In addition to these enzymes, ASMT activity was shown to be present in *Arabidopsis* caffeic acid O-methyltransferase (COMT). This activity was not negligible, although the nominal substrate, caffeic acid, displayed a higher affinity than *N*-acetylserotonin (Byeon et al., 2014a). Recently, *O. sativa* COMT was shown to methylate *N*-acetylserotonin at a 609-fold rate compared to ASMT1 from this species. Moreover, it was possible to increase or decrease melatonin synthesis in rice by overexpressing or suppressing OsCOMT (Byeon et al., 2015a). Nevertheless, it remains to be clarified to which degree other, competing substrates may reduce the ASMT-like activity of physiological OsCOMT levels. The situation seems to be entirely different in *Arabidopsis*, in which the specificity balance of COMT was anyway strongly on the side of caffeic acid. In fact, a recent study reported cloning and characterization of an AtASMT devoid of COMT activity (Byeon et al., 2016). However, this enzyme also methylated efficiently serotonin to 5-methoxytryptamine (5-MT), a finding that indicates the possible existence of an inverse route of melatonin formation, in which serotonin is first O-methylated and 5-MT, which is also accepted by SNAT, subsequently *N*-acetylated. Both routes, the traditional and the inverse one, may be used in parallel, as had been previously shown in *Saccharomyces*, in which melatonin can be formed from either *N*-acetylserotonin or 5-MT (Sprenger et al., 1999). According to the recent publication on AtASMT, ASMT-like sequences had been also detected in various other monocot and dicot species, however, with relatively low homology. An ASMT from *M. zumi* was cloned, characterized and also expressed in *A. thaliana* (Zuo et al., 2014). Overexpression of the transgene caused two to fourfold rises of melatonin in *A. thaliana*. With regard to the divergent findings on ASMT activities, three main conclusions should be drawn. (1) There may be considerable differences between species concerning the O-methyltransferases involved. These deviations may contribute to the remarkable differences in the melatonin levels detected in the various species. (2) The possibility of further enhancing melatonin formation by overexpressing ASMT transgenes indicates that ASMT activity can become rate limiting under certain conditions. This should not be seen as an unusual exception, since this is also known from mammals, in which AANAT is limiting at low or moderately elevated rates of melatonin synthesis, whereas ASMT can become limiting at highest rates of melatonin formation (Liu and Borjigin, 2005). (3) The variable substrate affinity and sometimes low specificity of O-methyltransferases should be taken as a caveat for not

precociously concluding from partial homology on functional identity.

In plants, the understanding of catabolic melatonin metabolism is still in its infancy. From a general point of view, enzymatic and nonenzymatic routes of catabolism have to be distinguished. Nonenzymatic reactions with free radicals or singlet oxygen should be similar as in animals, but may be even more important, with regard to higher rates of oxidant generation in plants and more intense and poorly protected light exposure.

An overview of nonenzymatic hydroxylation (Tan et al., 2002) and dioxygenation reactions has been included in a recent review article (Hardeland, 2015). Nonenzymatic hydroxylations in different positions of the indole moiety are caused by interactions with free radicals of higher reactivity, usually hydroxyl radicals ( $\cdot\text{OH}$ ), e.g., by sequential actions of either two  $\cdot\text{OH}$  or another hydrogen-abstrating radical followed by combination with  $\cdot\text{OH}$ . Among the hydroxylated products, a metabolite carrying a third ring should be especially mentioned, cyclic 3-hydroxymelatonin (**Figure 1**), which is formed nonenzymatically in animal tissues under conditions of oxidative stress and displays, reminiscent of the parent compound, properties of an antioxidant (Tan et al., 1998, 2014a). Nonenzymatic dioxygenation of melatonin is mostly caused by sequential actions of an electron/hydrogen-abstrating free radical and a superoxide anion (Hardeland et al., 2003) or by interaction with a singlet oxygen (de Almeida et al., 2003). During dioxygenation, the pyrrole ring is cleaved to give a substituted kynuramine, *N*<sup>1</sup>-acetyl-*N*<sup>2</sup>-formyl-5-methoxykynuramine (AFMK; **Figure 1**). Further possibilities including pseudoenzymatic and photocatalytic reactions that lead to the same metabolite have been summarized elsewhere (Hardeland et al., 2009). AFMK can be also formed from cyclic 3-hydroxymelatonin by interaction with two  $\cdot\text{OH}$ . Reactions of AFMK with free radicals can lead to the formation of numerous other metabolites (Rosen et al., 2006; Hardeland et al., 2009), which have, however, not yet been studied in plants.

With regard to enzymatic melatonin catabolism, several possibilities known from animals and other eukaryotes may exist, such as hydroxylation, demethylation, deacetylation, and dioxygenation. Because of the high numbers of cytochrome P<sub>450</sub> (CYP) isoforms present in plants (Paquette et al., 2000; Zhong et al., 2002), hydroxylation and demethylation are highly likely. Among these possibilities, to date only one hydroxylation reaction has been detected, which was, however, not attributed to CYP enzyme, but rather to a melatonin 2-hydroxylase (M2H; Byeon et al., 2015b). M2H belongs to the 2-oxoglutarate-dependent dioxygenases (2-ODDs), which transfer the second O-atom to 2-oxoglutarate, which, thereafter, decomposes to succinate and CO<sub>2</sub>. Its gene from rice has been cloned and four different 2-ODD proteins were detected and shown to 2-hydroxylate melatonin (Byeon and Back, 2015). In plants, the M2H route seems to be of much higher importance than any other catabolic pathway of melatonin. In numerous plants such as rice, various dicots and also two gymnosperms, 2-hydroxymelatonin exceeded the melatonin levels, often by far (Byeon et al., 2015c). Notably, 2-hydroxymelatonin enters a tautomeric equilibrium



with the relatively stable 3-acetamidoethyl-5-methoxyindolin-2-one (**Figure 1**), which can be easily detected in chromatograms containing the 2-hydroxylated metabolite (Hardelein, 2010). This indolinone is much more lipophilic than its tautomer, 2-hydroxymelatonin. Therefore, it might be of interest to find out whether the very high amounts of the latter compound may be related to the entrance of the indolinone into lipophilic compartments or into vacuoles in which a further metabolization is less likely, but from which 2-hydroxymelatonin may be tautomERICALLY regenerated. The presence and functional capacity of M2H enzymes represents a major difference to melatonin catabolism in animals. With regard to the high levels of 2-hydroxymelatonin, it may be important to seek for eventual

physiological roles of this compound or, alternately, to identify it as waste molecule.

Apart from this metabolite, only one hydroxylated product of melatonin was to date discovered in plants, namely 4-hydroxymelatonin, which made up not more than 0.05% of these metabolites, whereas 6-hydroxymelatonin, the major metabolite in animals, remained below the detection threshold (Byeon et al., 2015c). Dealkylation by CYP isoforms, which is a rather common reaction type of these enzymes, has not yet been documented in plants, perhaps, because researchers did not study this directly, and since the resulting product *N*-acetylserotonin would not have been distinguished from the same molecule as a precursor. Deacetylation of melatonin to 5-MT, another bioactive metabolite

known from animals and also from phototrophic dinoflagellates (Hardeland et al., 1997, 2007b; Hardeland, 1999), has not yet been studied in plants. However, as mentioned above, 5-MT can be formed from serotonin by AtASMT (Byeon et al., 2016).

Enzymatic formation of the dioxygenated product, AFMK, has been shown to exist in rice, in which an indoleamine 2,3-dioxygenase (IDO) carrying out this reaction was identified and cloned. Upon overexpressing it as a transgene in tomatoes, melatonin levels were reported to decrease (Okazaki et al., 2010). This may indicate a certain physiological role of enzymatic AFMK formation, although the quantitative relevance cannot yet be easily judged. Quantification of AFMK in plant tissues was convincingly carried out in *Eichhornia crassipes* (Tan et al., 2007). Under natural light/dark conditions, the compound exhibited a 24-h rhythm with a maximum around the transition from photo-to scotophase, roughly in parallel with a rhythm of melatonin in this species. However, it is not yet clear to which extent this was due to an IDO and whether photochemical reactions, e.g., by singlet oxygen, contributed to the increase of AFMK over the photophase.

## AUXIN-LIKE EFFECTS

Morphogenetic and growth effects of melatonin, which comprise auxin-like actions, have been fully or partially reviewed a number of times in the last years (Arnao and Hernández-Ruiz, 2006, 2014, 2015; Paredes et al., 2009; Tan et al., 2012; Arnao, 2014; Hardeland, 2015). These observations have been made in various different species, both monocots and dicots, and different test systems, such as poacean coleoptiles (*Phalaris*, *Triticum*, *Avena*, *Hordeum*), *Lupinus* hypocotyls, root growth and formation or regeneration of adventitious and lateral roots (*Oryza*, *Lupinus*, *Prunus*, *Arabidopsis*, *Brassica*), and shoot multiplication by nodal segments (*Mimosa*), as recently summarized (Hardeland, 2015). Notably, concentration differences for stimulatory or inhibitory effects in roots, as known from auxins, were also observed with melatonin.

Although, in phenomenological terms, the auxin-like effects are well documented, the mechanistic understanding of melatonin's actions is still insufficient. The structural differences between the indolic compounds melatonin and indole 3-acetic acid (IAA) are too pronounced to assume affinity to the same binding sites. Indirect effects of melatonin on IAA levels have been reported, but did not reveal consistent changes. In *Brassica juncea*, 0.1  $\mu$ M melatonin elevated the IAA concentration in roots (Chen et al., 2009). However, overexpression of an ovine AANAT transgene in 'Microtom' tomato plants caused an increase in melatonin, but a reduction in IAA that was associated with a loss of apical dominance (Wang et al., 2014b). Therefore, melatonin is not just a stimulator of IAA formation, but the actions of the two signal molecules can be dissected. However, the inverse correlation between melatonin and IAA observed in tomato may not necessarily reflect a physiological relationship, if the melatonin and IAA biosynthetic pathways compete for precursors and AANAT overexpression favors the former. However, this assumption would require direct

experimental support. Another theoretically possible explanation might have been a catabolic route from melatonin via 5-MT and 5-methoxyindole 3-acetaldehyde to the auxin 5-methoxyindole 3-acetic acid, a pathway that exists in phototrophic dinoflagellates and animals (Hardeland et al., 2007b). However, this was ruled out in plants for reasons of product quantities (Arnao and Hernández-Ruiz, 2006). To date, it seems that the actions of melatonin and auxins are transmitted by different signal transduction pathways, which finally converge at some but not necessarily all regulatory checkpoints. The involvement of cytosolic calcium in both melatonin and IAA signaling, as recently discussed (Hardeland, 2015), would require further in-depth elaboration of mechanistic details. Differences in the actions of melatonin and auxins may be overlooked when studying a single or a very few growth-related or morphogenetic endpoints. In *A. thaliana*, melatonin as well as IAA and another auxin stimulated lateral root formation, but melatonin did not enhance the expression of an auxin-dependent GUS reporter (Koyama et al., 2013). An even more conflicting result was obtained in a transcriptome analysis of *A. thaliana*, according to which the genes of auxin signaling were preferentially downregulated by melatonin (Weeda et al., 2014). Under the impression of these findings, the auxin-like actions of melatonin appear more enigmatic than before. Another theoretical possibility is still devoid of direct experimental support and also seems to conflict with the transcriptome data. Melatonin was shown to downregulate IAA17 (indole-3-acetic acid inducible 17, alias auxin resistant 3, AXR3; Shi et al., 2015d), which acts as transcriptional repressor of various auxin-inducible genes. Therefore, melatonin might indirectly cause upregulations of auxin-dependent genes and, thus, cause effects known from IAA. However, these melatonin effects had been observed in the context of leaf senescence and may not be generally applicable.

Under the aspect of the highly divergent melatonin concentrations measured in plants, it should be underlined that relatively low concentrations of melatonin are required for auxin-like actions. This is precisely what one would expect from a signal molecule. The problem that arises is rather that of the role of melatonin in those species, their organs or seeds, which contain by orders of magnitude higher concentrations of melatonin. If, in these high-melatonin plants or parts thereof, melatonin were freely diffusible, regulation mechanisms based on high-affinity binding sites would no longer be functional, because of a persistent full saturation or even saturation-dependent desensitization. Therefore, the alternative seems to be that either melatonin is not freely diffusible, but sequestered by proteins, absorbed by oil droplets, perhaps otherwise hindered to leave certain compartments, or regulation mechanisms working at low concentrations are switched off. In the latter case, auxin-like effects of melatonin should not be expected.

The observation of growth inhibition at elevated levels of melatonin, especially in roots, has been forwarded as another argument for an auxin-like action. However, despite the similarity to the concentration dependence of auxin effects, this conclusion may not be entirely firm, because elevated levels of melatonin can interfere with the cytoskeleton including the mitotic spindle.

This is known from the earliest studies of melatonin in plants, in endosperm cells of *Scadoxus multiflorus* (syn. *Haemanthus katherinae*; Jackson, 1969) and onion roots of *Allium cepa* (Banerjee and Margulis, 1973).

## STRESS AND SENESCENCE

Melatonin effects in the complex of biotic and abiotic stress, defense, wound healing and senescence represent an emerging field, which seems to receive increasing attention. Notably, the transcriptome analysis by Weeda et al. (2014), which revealed down- rather than upregulations of auxin-related pathways in *A. thaliana*, demonstrated various stimulatory actions of melatonin on the pathways of ethylene, abscisic, jasmonic, and salicylic acids. However, in the context of stress, melatonin was also reported to conversely downregulate formation and upregulate catabolism of abscisic acid in *Malus* species (Li et al., 2015). In addition to the effects that seem to be mediated by these phytohormones, the role of melatonin as an antioxidant may play an additional role that could be in favor of stress resistance, healing and survival. In this regard, the situation is reminiscent of that in animals and comprises reduction of oxidant formation, direct scavenging of oxidants as well as induction of antioxidant enzymes and support of favorable redox balances of other antioxidants such as glutathione (Kostopoulou et al., 2015; Li et al., 2015; Shi et al., 2015f). This parallel to animals, which is not equally detectable in other functions of melatonin, might be seen as a consequence of a most ancient antioxidant role of melatonin (Hardeland et al., 1995), which is already demonstrable in unicellular organisms (Antolín et al., 1997) and includes mechanisms that reduce free-radical formation (Hardeland, 2005).

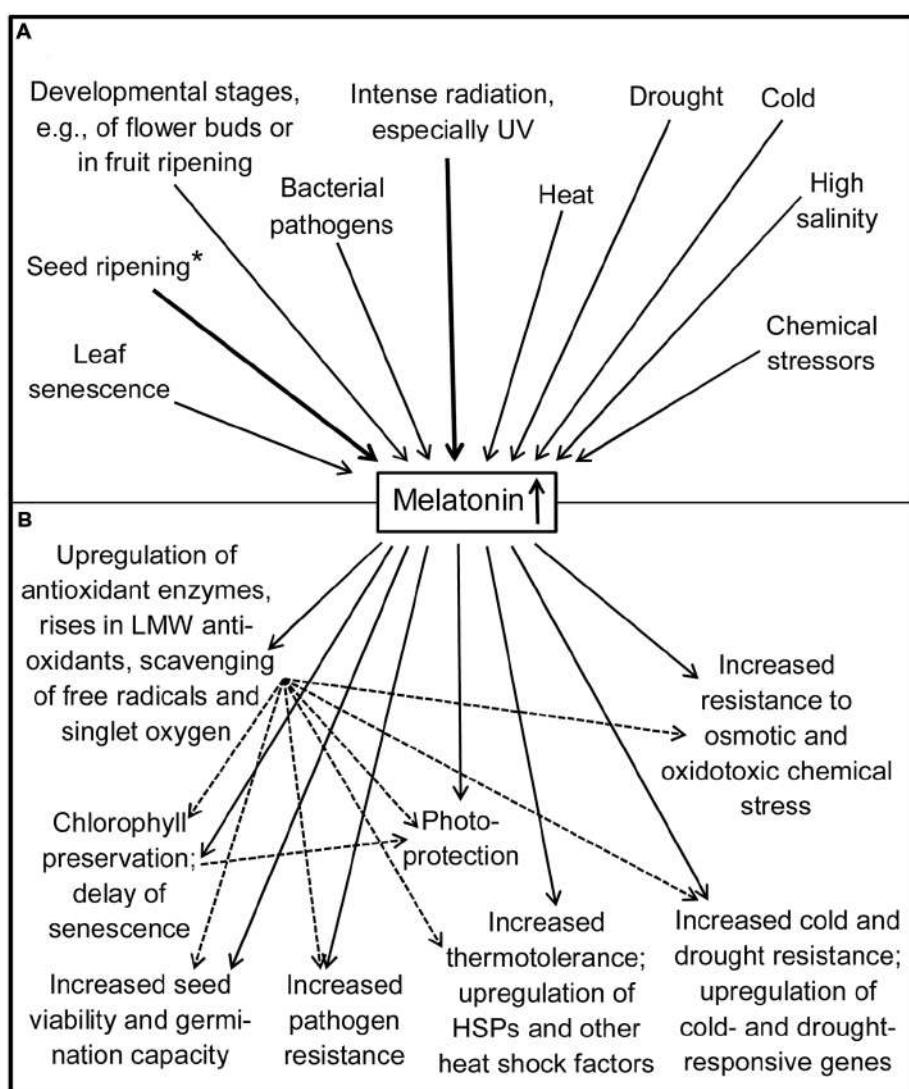
The complex of stress resistance, healing, and senescence has been fully or partially reviewed in recent publications (Arnao and Hernández-Ruiz, 2014, 2015; Hardeland, 2015; Zhang et al., 2015) or extensively addressed (Shi et al., 2015f). Instead of repeating in detail the findings summarized there, only their essence shall be briefly outlined, followed by a focus on the most recent results. A remarkable observation has been that the various forms of environmental or biotic influences that may be interpreted as stress typically lead to increases in melatonin (**Figure 2**). This includes extreme temperatures – both cold stress and heat stress –, intense radiation, drought, high salinity, and chemical stressors such as hydrogen peroxide, ZnSO<sub>4</sub> or herbicides (for details see Arnao and Hernández-Ruiz, 2009b, 2013, 2014, 2015; Arnao, 2014; Hardeland, 2015; Zhang et al., 2015), and also exposure to a bacterial pathogen (Qian et al., 2015; Shi et al., 2015a). The rises in melatonin, caused by entirely different factors, may be indicative of its involvement in a fundamental stress resistance mechanism. Most of these observations had been made in various plant species, dicots and monocots. Increases of melatonin have been particularly described as a consequence of UV radiation, which will be discussed in the following section on photoprotection.

With regard to the complex of stress responses, as induced by various abiotic or biotic factors, of wound healing and

leaf senescence, there is a considerable overlap of actions by several pertinent phytohormones, such as ethylene, abscisic, salicylic, and jasmonic acids/methyl jasmonate (Jibran et al., 2013; Khan et al., 2014), and also in their nexus with melatonin (Hardeland, 2015). This view is in line with the observation that melatonin supports the resistance to different forms of stress caused by factors as divergent as drought, cold, heat, osmotic stress, oxidative stress (Chan and Shi, 2015; Shi et al., 2015b) and also by bacterial pathogens (Shi et al., 2015a). Despite these similarities, the factors influenced by melatonin can be different according to the type of challenge. Nevertheless, joint pathways and response types do exist, especially in the cases of cold and drought stress. Moreover, it has to be noted that many forms of stress are associated with increased oxidative damage. Therefore, the antioxidant actions of melatonin appear to be a common theme of stress management in plants, a conclusion that has been similarly applied to many animals.

Among the most recent developments in this field of plant responses, a considerably increased number of studies has been conducted in *Arabidopsis*. With regard to an improved cold tolerance by melatonin, the overlap with drought resistance has become obvious by the induction of transcription factors involved in either of these responses, in particular, *CAMTA1*, *CBF1*, *CBF2a*, *CBF2b*, *CBF3a*, and *CBF3b* (Bajwa et al., 2014). Moreover, the cold-responsive genes *COR15a*, *RD22*, and *KIN1* (Bajwa et al., 2014; Shi et al., 2015c) as well as the oxidant-induced zinc-finger transcription factors *ZAT10* and *ZAT12* were upregulated by melatonin. The connection between cold tolerance and avoidance of oxidative damage was further confirmed and focused on another zinc-finger protein, *ZAT6* (Shi and Chan, 2014). Knockdown of the *ZAT6* gene reduced the melatonin-mediated cold resistance, whereas *ZAT6* overexpression enhanced it. Additionally, melatonin was shown to upregulate *ZAT6* as well as *CBF1*, *CBF2*, and *CBF3*.

In the case of heat stress, other signaling pathways are, expectably, involved. In *Arabidopsis*, melatonin was recently shown to upregulate class A1 heat shock factors (HSFA1s). HSFA1-inducible genes such as *HSFA2s*, *HSA32* (heat stress-associated 32), *HSP90*, and *HSP101* are assumed to participate in the increased thermotolerance conveyed by melatonin (Shi et al., 2015e). With regard to other forms of stress, drought resistance was shown to be enhanced in *Arabidopsis* by overexpression of an ASMT transgene from *M. zumi* and, thus, by enhanced melatonin synthesis (Zuo et al., 2014). Another aspect of drought tolerance was investigated in *M. hupehensis* (Li et al., 2015). In this study, the antioxidant capacity of melatonin was demonstrated by decreases of H<sub>2</sub>O<sub>2</sub> levels, in conjunction with upregulations of catalase and peroxidases. Some minor increases in ascorbate peroxidase (APX) were also reported, which were, however, more pronounced in *M. prunifolia*, a species with higher natural drought tolerance. Additionally, the protective effects of melatonin were associated with increased stomatal aperture and moderate improvements of photosynthesis. In *Glycine max*, melatonin enhanced both drought resistance and salt tolerance, in conjunction with a reversal of salt-induced reductions in the expression of numerous genes (Wei et al., 2015). Increased salt tolerance was also recently demonstrated in



**FIGURE 2 | Increases in melatonin (A) and their consequences for protection and stress tolerance (B).** Bold arrows: particularly strong accumulations of melatonin. Dotted arrows: secondary effects via antioxidative protection. \*Melatonin may be more strongly taken up than synthesized on-site. HSP, heat shock protein; LMW, low-molecular weight. Some actions may be species-specific or conditional.

*O. sativa* (Liang et al., 2015) and in seedlings of *Citrus aurantium* (Kostopoulou et al., 2015). In the latter case, combinations of melatonin and ascorbic acid were tested, which increased the levels of several low molecular-weight antioxidants and activities of antioxidant enzymes. As in this study, many of the recent investigations revealed a causal relationship between exogenous stress factors and induction of oxidative stress and damage, which were mitigated by melatonin (cf. Figure 2). This was especially observed in dicots such as *Arabidopsis* (Shi and Chan, 2014) and *Malus* (Li et al., 2015), and monocots such as *Cynodon* (Shi et al., 2015b,f) and *Oryza* (Liang et al., 2015). For further details reviewed earlier see Tan et al. (2012). In *Cynodon*, melatonin was shown to reduce reactive oxygen species and lipid peroxidation (Shi et al., 2015b). In *Oryza*, an  $H_2O_2$ -overproducing mutant, *noe1*, was also tested, in which melatonin protected against

accelerated leaf death (Liang et al., 2015). This finding may be also seen in the context of normal leaf senescence, since melatonin reduced chlorophyll degradation and suppressed senescence-associated genes. Protection by melatonin against losses of chlorophyll and delay of senescence appear to be a common theme in plants (for further details and mechanistic aspects see: Hardestrand, 2015). Among the most recent investigations, the effects of melatonin on gene expression during leaf senescence, as revealed by a proteomic approach, should be mentioned (Wang et al., 2014a), and also a study in *Arabidopsis* on the relationship to *IAA17* (Shi et al., 2015d). While *IAA17* overexpression caused advanced senescence, melatonin downregulated this gene as well as the levels of *SEN4* (*senescence 4*) and *SAG12* (*senescence-associated gene 12*) mRNAs, thereby decelerating senescence.

The reduction of oxidative stress by melatonin has been recently investigated in the context of phytotoxic agents. In *Arabidopsis* treated with paraquat (=methyl viologen), melatonin upregulated APX and catalase, however, without substantially altering the production of superoxide and H<sub>2</sub>O<sub>2</sub> under the influence of the oxidotoxin. Instead, melatonin promoted autophagy, which may be interpreted as a survival strategy to eliminate the oxidatively damaged organelles (Wang et al., 2015). In *Solanum lycopersicum*, cadmium toxicity was reduced by melatonin (Hasan et al., 2015). This was not restricted to the upregulation of antioxidant enzymes, but also included metal sequestration by phytochelatins and elimination of cadmium from the cytosol by transfer to the cell wall and to the vacuole.

Particular attention has been recently paid to actions of melatonin under conditions of biotic stress. This has been mainly studied in *Arabidopsis*, occasionally also in *Nicotiana*, using the pathovar tomato DC3000 of *Pseudomonas syringae*. First, melatonin was shown to upregulate pathogen-related, salicylic acid- and ethylene-dependent genes, effects that were suppressed in mutants defective in salicylic acid and ethylene signaling, such as *npr1*, *ein2*, and *mpk6* (Lee et al., 2014a). Next, SNAT knockouts did not only exhibit reduced levels of melatonin, but also of salicylic acid, along with a higher susceptibility to the pathogen (Lee et al., 2015). Infection with this pathogen caused rises in both melatonin and nitric oxide, while melatonin itself increased NO and salicylic acid-related genes, in conjunction with a reduced susceptibility (Shi et al., 2015a). Further studies revealed a relationship of melatonin to sugars that may also contribute to the thickness and resistance of cell walls (Zhao et al., 2015b). More detailed analyses (Qian et al., 2015) showed that melatonin enhanced the levels of various sugars, including glucose, fructose, sucrose, and melibiose, as well as that of glycerol, effects that were also elicited by the infection. Administration of fructose, glucose, sucrose, or glycerol increased pathogen resistance in wildtype plants, but not so in the salicylic acid-deficient NahG strain and in two NO-deficient mutants, *noa1* and *nia1nia2*. Moreover, the sugars and glycerol caused rises in NO but not in melatonin. In conclusion, melatonin seems to be involved in innate plant immunity as a factor that acts upstream of salicylic acid and NO, while rises in sugars and glycerol stimulate NO formation. With regard to the reduced resistance in SNAT knockouts, a full stimulation of immunity likely requires upregulation of melatonin synthesis.

## PHOTOREACTIONS AND PHOTOPROTECTION

In the context of increased levels of melatonin and protection conveyed by this compound, most publications on exposure to high light intensities and especially UV have discussed this as just one of various forms of stress. However, there are several peculiarities that seem to exceed a more generalized stress response. First, the extent of UV-induced rises in melatonin is, in many cases, remarkably large. Alpine or Mediterranean species or varieties, which are exposed to high natural light

and UV intensities, were shown to contain considerably higher melatonin levels than the same or related species from other habitats (Conti et al., 2002; Caniato et al., 2003; Hardeland et al., 2007b). Conti et al. (2002) published data of nine Alpine or Mediterranean plants with levels of 10–43 µg melatonin/g tissue. In fruits of an Egyptian *Fragaria* cultivar, high vis/UV light exposure was reported to increase melatonin (Badria, 2002). In leaves of the pontederiaceous *E. crassipes*, melatonin levels were considerably higher (up to 300 ng/g fresh weight) in plants directly collected from a pond (maximal diurnal irradiation 10,000–15,000 µW/cm<sup>2</sup>) than those (ca. 3 ng/g) from laboratory conditions (400–450 µW/cm<sup>2</sup>; Tan et al., 2007). Presumably, this increase has included effects by UV, but the contribution of a temperature cycle in the natural environment cannot be ruled out. An aspect that speaks for a role of radiation and, perhaps, also for a photoprotective function of melatonin concerns the observed increase of melatonin in the course of the photophase, with a maximum around the transition to the scotophase. Notably, the diurnal change in melatonin concentration was accompanied by a corresponding rhythm in AFMK formation (Tan et al., 2007). As mentioned at the end of the section on metabolism, a contribution of indoleamine 2,3-dioxygenase to this rhythm cannot be excluded. However, melatonin is known to undergo several photocatalytically mediated reactions including dioxygenation (Behrmann et al., 1997; Hardeland et al., 2007b). Light-exposed extracts from phototroph organisms, such as the dinoflagellate *L. polyedrum* and the pheophycean *Pterygophora californica*, converted melatonin efficiently to AFMK, even in the presence of the hydroxyl radical scavenger DMSO (Hardeland et al., 1995; Behrmann et al., 1997). Among known light-induced reactants, melatonin is, in particular, oxidized by singlet oxygen to AFMK (de Almeida et al., 2003; Schaefer and Hardeland, 2009). The photoproduct AFMK is relatively inert against singlet oxygen and can, therefore, accumulate under these conditions. However, its secondary, deformylated metabolite, N<sup>1</sup>-acetyl-5-methoxykynuramine (AMK), which may be formed in plants enzymatically or by interactions with free radicals, is, among low molecular weight metabolites, one of the most efficient singlet oxygen quenchers detected, more potent than melatonin or histidine (Schaefer and Hardeland, 2009), and additionally an effective scavenger of various reactive oxygen and nitrogen species (Ressmeyer et al., 2003; Guenther et al., 2005; Hardeland et al., 2007a).

A presumed role of melatonin and, perhaps, its metabolites in photoprotection of plants would also be in line with the observed protection against chlorophyll degradation (Arnao and Hernández-Ruiz, 2009a; Tan et al., 2012; Wang et al., 2012, 2013), although this has certainly also to be seen under additional aspects of chlorophyll a/b binding protein (CAB) expression, delayed upregulation of pheophorbide a oxygenase (Wang et al., 2013) as well as stress- and senescence-related signaling.

However, the assumed photoprotection of plants should not be generalized. In *Glycyrhiza uralensis*, exposure to visible or UV-B light caused rises in melatonin, but mainly in the roots (Afreen et al., 2006), a response that should rather be interpreted as a stress response. Moreover, high light intensities do not

generally favor an elevation of melatonin. In *Oryza sativa*, a heat-induced increase was antagonized by light (Byeon and Back, 2014).

A further aspect, which merits more future attention, concerns the concentration dependence of protection. Very high melatonin levels in leaves, as sometimes observed in strongly UV-exposed plants (Conti et al., 2002; Caniato et al., 2003), may allow nonenzymatic elimination of free radicals and singlet oxygen at substantial rates, in addition to the anyway effective antioxidative protection system based on ascorbate, glutathione, APX, glutathione peroxidase (GPX) and other compounds. Moreover, two properties of melatonin should be considered, namely, a nonadditive synergistic interaction of melatonin and other antioxidants, notably, ascorbate and glutathione (Tan et al., 2003) and melatonin's scavenger cascade, which allows elimination of up to 10 free radicals per melatonin molecule by formation of consecutively formed metabolites with scavenging properties (Rosen et al., 2006). The highly divergent levels of melatonin found in different plant species may allow, in some of them, a substantial contribution to nonenzymatic protection, whereas, in other species containing this compound in much lower concentrations, actions are restricted to signaling mechanisms. The background of this divergence may be sought in unfavorable physiological effects of elevated melatonin in certain species. Such a case was recently reported. In *Zea mays*, low doses of melatonin favored photosynthesis and nocturnal starch catabolism, whereas high doses caused the opposite, downregulated sucrose transporter expression and inhibited seedling growth (Zhao et al., 2015a). In conclusion, physiologically relevant differences in melatonin tolerance may exist between plant species.

## CONCLUSION

During the last years, considerable progress has been made in melatonin research in plants. This does not only concern the extension of knowledge on its presence in an increasing number of species and taxa, but also the identification of metabolic pathways and a remarkable amount of new information on the involvement in physiological functions. Nevertheless, there is still a strong demand for clarification of several fundamental points.

One of these is related to the enormous differences in the melatonin content of different species and organs. As already outlined in the Introduction, signaling mechanisms based on high-affinity binding sites for melatonin cannot be expected to work at strongly elevated concentrations that would permanently saturate a binding protein. However, this aspect of divergent concentrations requires a distinction between metabolically active and dormant tissues. In the case of extremely high melatonin levels in leaves, signaling mechanisms known from low-melatonin species may not be functional. Alternately, one would have to assume either different mechanisms based on low-affinity instead of high-affinity binding sites or a sequestration

of melatonin in areas of limited metabolic activity, such as the vacuole or the apoplast. The necessity of determining the distribution of melatonin within the tissue (Hardeland, 1997) might resolve some concentration-related discrepancies that appear rather enigmatic to date. An additional possibility of removing melatonin from the cytosol might consist in its uptake into oil bodies, which has been shown to occur in sunflower seedlings (Mukherjee et al., 2014). Moreover, this could be of particular importance in oily seeds. High melatonin levels in seeds of various plants are well documented (Manchester et al., 2000). A more or less dry, dormant seed is practically devoid of enzymatic antioxidative protection and has, therefore, to rely on low molecular-weight antioxidants (Balzer and Hardeland, 1996; Hardeland et al., 2007b), among which melatonin has a number of advantages. Moreover, melatonin might contribute to the maintenance of the dormant state and to the survival in dormancy (Balzer and Hardeland, 1996; Hardeland et al., 2007b), at least by reducing oxidative damage, which limits the germination potential of seeds. In fact, the preservation of seed viability by applying high concentrations of exogenous melatonin has been recently demonstrated in *Arabidopsis* (Hernández et al., 2015). In rehydrated seeds, melatonin may promote seed germination, especially under unfavorable conditions. This view is supported by recent findings in seeds of *Cucumis sativus* under high salinity (Zhang et al., 2014). Support of germination was also reported for the negatively photoblastic seeds of *Phacelia tanacetifolia*, but interpreted, in this case, as a mimicking of darkness by melatonin (Tiryaki and Keles, 2012).

Another aspect that awaits further clarification concerns the role of isoenzymes in the melatonin biosynthetic pathway, as outlined in the section on metabolism. More specifically, the contribution of different isoenzymes to the highly divergent melatonin levels detected in different species should be elucidated.

The field in which progress is most urgently required is that of signaling pathways. Although the influence of melatonin on gene expression otherwise known to be controlled by phytohormones has been amply documented by transcriptomic and proteomic analyses, primary signaling mechanisms by melatonin have remained entirely unexplored. With regard to the cytoskeletal effects, binding of melatonin to calmodulin may occur, as known from animals, but has not been directly demonstrated. However, high-affinity binding sites of melatonin and their properties are still unknown in plants. In the absence of their identification, no information can be obtained on receptor-mediated signal transduction and second messengers involved in the modulation of actions of established phytohormones and gene regulation. Progress in this field may be critical to the possible classification of melatonin as a phytohormone.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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# Melatonin: Current Status and Future Perspectives in Plant Science

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Melatonin (*N*-acetyl-5-methoxytryptamine) is a ubiquitous molecule with pleiotropic actions in different organisms. It performs many important functions in human, animals, and plants; these range from regulating circadian rhythms in animals to controlling senescence in plants. In this review, we summarize the available information regarding the presence of melatonin in different plant species, along with highlighting its biosynthesis and mechanisms of action. We also collected the available information on the effects of melatonin application on commercially important crops to improve their growth and development. Additionally, we have identified many new aspects where melatonin may have possible roles in plants, for example, its function in improving the storage life and quality of fruits and vegetables, its role in vascular reconnection during the grafting process and nutrient uptake from roots by modifying root architecture. Another potentially important aspect is the production of melatonin-rich food crops (cereals, fruits, and vegetables) through combination of conventional and modern breeding approaches, to increase plant resistance against biotic and abiotic stress, leading to improved crop yields, and the nutraceutical value of produce to solve food security issues.

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## INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) is a widely studied bio-molecule and its function has been investigated in bacteria, mammals, birds, amphibians, reptiles, fish, and plants. It is a low molecular weight molecule, ubiquitously present with pleiotropic biological activities (Hardeland et al., 2011). Melatonin was discovered in bovine pineal gland in 1958 (Lerner et al., 1958). After its discovery, for the subsequent four decades it was considered exclusively as an animal hormone, especially a neurohormone (Reiter, 1991). In 1993, melatonin was possibly identified in the Japanese morning glory (*Pharbitis nil*) but these data were not reported until 1995 (Van-Tassel et al., 1995). Also in this year, the presence of melatonin in a number of edible plants was unequivocally demonstrated (Dubbels et al., 1995; Hattori et al., 1995). However, even prior to its identification in plants, melatonin had been shown to have effects on endosperm cells of the amaryllidacean *Scadoxus multiflorus* (syn. *Haemanthus skatherinae*) and on the epidermal cells of *Allium cepa* (Jackson, 1969; Banerjee and Margulis, 1973). Currently, research on plant melatonin is in an exponential growth phase and its functions in numerous plants have been uncovered; the number of publications related to plant melatonin has rapidly increased within the last decade (Arnao and Hernández-Ruiz, 2015; Reiter et al., 2015).

In animals, melatonin has many physiological roles including regulating circadian rhythms, mood, sleep, body temperature, bone metabolism, seasonal reproduction, locomotor activity, food intake, retina physiology, and immune system regulation (Maronde and Stehle, 2007; Pandi-Perumal et al., 2008; Reiter et al., 2009; Hardeland et al., 2012; Carrillo-Vico et al., 2013; García et al., 2014; Maria and Witt-Enderby, 2014; Manchester et al., 2015; Vriend and Reiter, 2015). Irrespective of its proven functions in animals, melatonin also has a wide range of functions in plants such as the promotion of seed germination and seedling growth and influencing plant senescence [death (Arnao and Hernandez-Ruiz, 2014)]. In this review, we summarize the currently-available information related to biosynthesis of melatonin, mechanisms of action and occurrence, role and functions in higher plants. We also speculate on new potential aspects where melatonin may have possible functions in plants.

## BIOSYNTHESIS

The biosynthetic pathway of melatonin (N-acetyl-5-methoxytryptamine) is well known in vertebrates (Reiter, 1991; Falcón et al., 2009). In these species, tryptophan (an amino acid) is converted to 5-hydroxytryptophan; tryptophan 5-hydroxylase (T5H) is involved in this conversion. Thereafter, 5-hydroxytryptophan is converted to serotonin (Falcón et al., 2009). In animals, 5-hydroxytryptophan is the exclusive pathway for serotonin production in the pineal gland. In the plant St. John's Wort (*Hypericum perforatum* L.), 5-hydroxytryptophan is also involved in serotonin synthesis (Murch et al., 2000; Murch and Saxena, 2006). A recent study on rice, however, documents that the tryptamine pathway (tryptophan to tryptamine to serotonin) is more important in the production of serotonin (Park et al., 2012); this pathway has subsequently been found to be common to many plant species. Serotonin, in both plants and animals, is converted to N-acetyl serotonin catalyzed by serotonin N-acetyltransferase (SNAT), which is then methylated by hydroxyindole-O-methyltransferase (HIOMT; also known as acetyl serotonin methyl transferase, ASMT) resulting in the formation of melatonin. In plants (rice), N-acetyl serotonin is also directly produced from tryptamine and N-acetyltryptamine serves as an intermediate product; this pathway is catalyzed by SNAT and tryptophan 5-hydroxylase. Melatonin can be directly produced from serotonin with 5-methoxytryptamine serving as intermediate product in a process and catalyzed by HIOMT/ASMT and SNAT (Arnao and Hernandez-Ruiz, 2014; Byeon et al., 2014; **Figure 1**). Indole acetic acid (IAA) is also produced from tryptamine and indole-3-acetylaldehyde serves as an intermediate product (Krystyna et al., 2009; Arnao and Hernandez-Ruiz, 2014).

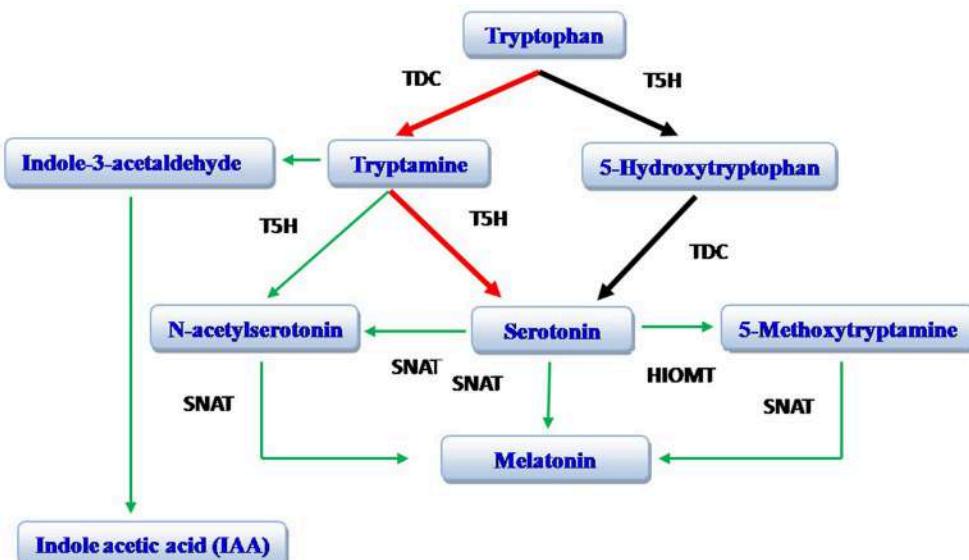
The concentration of melatonin in plants is much higher than levels in animals, and the biosynthesis of melatonin in plants also seems more complicated than in animals (Arnao and Hernandez-Ruiz, 2014). Due to more limited availability of information on the biosynthesis of melatonin and related products in plants, the definitive pathways await final definition (Byeon et al., 2015). Recently, transgenic rice has been shown to have genes for all the enzymes involved in the biosynthesis of

melatonin (Byeon et al., 2014), and it is expected that soon the biosynthetic pathway will be described in detail. As far as the degradation of melatonin is concerned, N<sup>1</sup>-acetyl-N<sup>2</sup>-formyl-5-methoxykynuramine (AFMK) has been shown to be a secondary metabolite in vascular plants due to its enzymatic or non-enzymatic conversion from melatonin (Tan et al., 2007a). Recent studies on rice document that other metabolites of melatonin include 2-hydroxymelatonin (99%) and, to a much lesser extent, 4-hydroxymelatonin (0.05%) (Byeon et al., 2015).

## MELATONIN: PRESENCE IN HIGHER PLANTS

Melatonin is found in a large number of plant species (**Table 1**). The roots, seeds, leaves, bulbs, and flowers were found to be rich sources of melatonin in most of the plant species examined. The roots of Huang-qin (*Scutellaria baicalensis*), which belongs to the family *Lamiaceae*, are an especially rich source of melatonin (7110 ng/g) (Reiter and Tan, 2002). Most of the plant species in which the presence of melatonin has been reported belong to the families *Rosaceae*, *Vitaceae*, *Poaceae*, *Apiaceae*, and *Brassicaceae*; however, the plants from some other families have also been shown to possess melatonin in large amounts. There is possibility that yet unstudied plant species may contain even higher concentrations of melatonin than have been reported. The concentration of melatonin in plants is affected by the genotype, environmental factors (photoperiod, temperature), stage of development (Zhao et al., 2012; Byeon and Back, 2014a; **Table 1**) and method of determination (Feng et al., 2014). This latter factor may be a major issue in the reports of melatonin in plants. The concentration of melatonin can vary among the different cultivars of a same species. Wang et al. (2009) quantified the level of melatonin in 58 and 25 different varieties of corn and rice, respectively, grown at same geographical location (Institute of Rice, Fujian Academy of Agricultural Sciences, Fuzhou, China), and observed huge variations in melatonin concentrations. The values of melatonin ranged from 11 to 2034 ng/g and 11 to 264 ng/g in corn and rice, respectively. These massive differences in concentrations suggest that the melatonin levels are determined, in large part, by the genotype of plants or the method used for melatonin quantitation.

Melatonin biosynthesis in rice seedlings is enhanced when they are exposed to high temperatures or are maintained under dark conditions. Melatonin concentration was increased from 2.95 to 4.9 ng/g when rice seedlings were exposed to darkness at 55°C. The increase in melatonin level was associated with the enhanced activity of both SNAT and HIOMT/ASMT, both of which are involved in the biosynthetic pathway of melatonin (Byeon and Back, 2014a). Similarly, tomato plants exposed to shade exhibited an increase in the levels of melatonin (135%) (Riga et al., 2014). In sweet cherry, the endogenous levels of melatonin exhibited two obvious peaks, one at 05:00 h and a second at 14:00 h (Zhao et al., 2012); the first peak was attributed to darkness while the second peak may have been related to high temperature or high light intensity (stressful conditions). The enzyme kinetic analyses show that SNAT and HIOMT/ASMT



**FIGURE 1 | Biosynthesis of melatonin.** The red arrows identify the preferred pathway in plants while the black arrows identify the major pathway in animals. TDC, tryptophan decarboxylase; T5H, tryptophan 5-hydroxylase; SNAT, serotonin N-acetyltransferase; HOMT, hydroxyindole-O-methyltransferase [also known as acetyl serotonin methyl transferase (ASMT)]. Modified from Arnao and Hernandez-Ruiz (2014).

enzymes exhibited thermophilic features (Byeon et al., 2014); purified recombinant SNAT and HIOMT/ASMT have shown optimal activities at 55 and 45°C, respectively (Byeon et al., 2013). Such high temperatures (45–55°C), however, are not typically experienced under natural environmental conditions.

In many edible fruits the presence of melatonin has also been reported (Table 1). In these species, endogenous levels of melatonin also vary according to cultivars studied, and with the stage of fruit development (Zhao et al., 2012; Feng et al., 2014). In sweet cherries, the first stage of fruit development is associated with low melatonin levels in both cultivars (Hongdeng and Rainier) (15 ng/g); during the second stage, these values exhibit large increases (36.6 and 124.7 ng/g, respectively) and in the third stage, the values drop to 10–20 ng/g. In most fruits, during the second stage of fruit development, cell elongation and cell expansion, and embryo and seed development occurs. So a possible role of melatonin in fruit development cannot be precluded; however, this still needs to be verified.

In a recent study it was reported that seed germination is associated with marked increases in the concentration of melatonin (2–3 fold); this suggested to the authors that the germinated seeds of the edible species may have utility as a food to raise the melatonin levels in plasma (Aguilera et al., 2015). Plants have also been genetically engineered to produce increased levels of melatonin compared to their wild types. Thus, Kang et al. (2010) observed that rice transgenic lines overexpressing human SNAT increased the biosynthesis of melatonin when compared with wild type rice plants. Also, in a recent study *oAANAT* and *oHIOMT* genes, encoding key enzymes catalyzing the last two steps in melatonin biosynthesis were introduced into the Micro-Tom tomato from the pineal gland of *Ovis aries* (sheep).

The melatonin contents of the Micro-Tom tomato transgenic lines were higher compared to their wild type, indicating the transferred animal genes were functional in the biosynthesis of melatonin in plants (Wang et al., 2014). Similarly, melatonin-rich transgenic rice plants overexpressing sheep SNAT have significantly higher levels of melatonin than in wild type rice (Park and Back, 2012). Clearly, the biosynthesis of melatonin in plant species can be altered by the introduction of genes from vertebrates; such genetically-altered plants may have utility as food because of their induced resistance against diseases, and to increase the yield, quality and nutritional value of crops.

In addition to plants listed in Table 1, Chen et al. (2003) measured melatonin levels in 64 commonly used medicinal herbs; they found concentrations of melatonin ranging from 12 to 3771 ng/g. Also, exceptionally high melatonin concentrations (227–233 µg/g) have been found in four different varieties of Pistachio (*Pistacia vera L.*) (Oladi et al., 2014); they are the highest values reported for any plant organ to date.

Melatonin in plants has been detected by several methods including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), gas chromatography-mass spectrometry (GS-MS), and high-performance liquid chromatography (HPLC) with electrochemical detection (HPLC-ECD), fluorescence detection (HPLC-FD), or HPLC-MS. These methods differ in their sensitivity and specificity (Feng et al., 2014). Kolár and Machácková (2005) suggested that RIA is not a reliable method for melatonin detection in plant samples, since the measurements have not been validated by other methods. For each method, different extraction solvents were employed. Oladi et al. (2014) measured melatonin using GC-MS, while for melatonin extraction an ultrasound-assisted

**TABLE 1 | Reported levels of melatonin in plants.**

Common name	Scientific name	Family	Concentration (ng/g)	References
<b>SEEDS</b>				
Milk thistle	<i>Silybum marianum</i>	Asteraceae	2	Reiter and Tan, 2002
Poppy	<i>Popaver somniferum</i>	Papaveraceae	6	Reiter and Tan, 2002
Anise	<i>Pimpinella anisum</i>	Apiaceae	7	Reiter and Tan, 2002
Coriander	<i>Coriandrum sativum</i>	Apiaceae	7	Reiter and Tan, 2002
Celery	<i>Apium graveolens</i>	Apiaceae	7	Reiter and Tan, 2002
Flax	<i>Linum usitatissimum</i>	Linaceae	12	Reiter and Tan, 2002
Green cardamom	<i>Elettaria cardamomum</i>	Zingiberaceae	15	Reiter and Tan, 2002
Alfalfa	<i>Medicago sativa</i>	Fabaceae	16	Reiter and Tan, 2002
Fennel	<i>Foeniculum vulgare</i>	Apiaceae (Umbelliferae)	28	Reiter and Tan, 2002
Sunflower	<i>Helianthus annuus</i>	Asteraceae	29	Reiter and Tan, 2002
Fenugreek	<i>Trigonella foenum-graecum</i>	Fabaceae	43	Reiter and Tan, 2002
Wolf berry	<i>Lycium barbarum</i>	Solanaceae	103	Reiter and Tan, 2002
Black mustard	<i>Brassica nigra</i>	Brassicaceae	129	Reiter and Tan, 2002
White mustard	<i>Brassica hirta</i>	Brassicaceae	189	Reiter and Tan, 2002
Barley	<i>Hordeum vulgare L.</i>	Poaceae	0.4, 0.87	Hattori et al., 1995; Badria, 2002
Rice (different varieties)	<i>Oryza sativa japonica L.</i>	Poaceae	1, 1.50, 11–234	Hattori et al., 1995; Badria, 2002; Wang et al., 2009
Oat	<i>Avena sativa L.</i>	Poaceae	2	Hattori et al., 1995
Corn (different varieties)	<i>Zea mays L.</i>	Poaceae	2, 1.88, 11–2034	Hattori et al., 1995; Badria, 2002; Wang et al., 2009
Tall fescue	<i>Festuca arundinacea</i>	Poaceae	5	Hattori et al., 1995
Huang-qin	<i>Scutellaria baicalensis</i>	Lamiaceae	7	Manchester et al., 2000
Almond	<i>Prunus amygdalus Batsch</i>	Rosaceae	39	Manchester et al., 2000
<b>GERMINATED SEEDS</b>				
Alfalfa	<i>Medicago sativa</i>	Fabaceae	0.133	Aguilera et al., 2015
Lentil	<i>Lens culenta</i>	Fabaceae	0.217	Aguilera et al., 2015
Mung bean	<i>Vigna radiata</i>	Fabaceae	0.166	Aguilera et al., 2015
Onion	<i>Allium cepa</i>	Amaryllidaceae	0.302	Aguilera et al., 2015
Broccoli	<i>Brassica oleracea</i>	Brassicaceae	0.439	Aguilera et al., 2015
Red cabbage	<i>Brassica oleracea capitata rubra</i>	Brassicaceae	0.857	Aguilera et al., 2015
Radish	<i>Raphanus sativus japonicum, rambo, sinicum rosae</i>	Brassicaceae	0.536	Aguilera et al., 2015
<b>LEAVES</b>				
St. John's wort	<i>Hypericum perforatum</i>	Hypericaceae	1750	Murch and Saxena, 2006
Feverfew, gold	<i>Tanacetum parthenium</i>	Asteraceae	1920	Murch and Saxena, 2006
Feverfew, green	<i>Tanacetum parthenium</i>	Asteraceae	2450	Murch and Saxena, 2006
Morning glory	<i>Pharbitis choisy</i>	Convolvulaceae	0.0005	Van-Tassel et al., 2001
Chine cabbage	<i>Brassica chinensis</i>	Brassicaceae	0.10	Hattori et al., 1995
Cabbage	<i>Brassica oleracea L. var. capitata</i>	Brassicaceae	0.10, 0.30	Hattori et al., 1995; Badria, 2002
Tomato (Transgenic)	<i>Solanum lycopersicum L.</i>	Solanaceae	45	Wang et al., 2014
Lupin	<i>Lupinus albus L.</i>	Fabaceae	75.6	Arnao and Hernández-Ruiz, 2013
<b>SHOOTS</b>				
Morning glory	<i>Pharbitis choisy</i>	Convolvulaceae	0.004	Van-Tassel et al., 2001
Asparagus	<i>Asparagus officinalis L.</i>	Asparagaceae	0.01	Hattori et al., 1995
Red pigweed	<i>Chenopodium rubrum L.</i>	Chenopodiaceae	0.20	Kolar et al., 1997
<b>FLOWERS</b>				
St. John's wort	<i>Hypericum perforatum</i>	Hypericaceae	4390	Murch and Saxena, 2006

(Continued)

**TABLE 1 | Continued**

Common name	Scientific name	Family	Concentration (ng/g)	References
<b>FRUITS</b>				
Banana	<i>Musa paradisiaca</i> L.	Musaceae	0.47	Dubbels et al., 1995
Banana	<i>Musa ensete</i>	Musaceae	0.66	Badria, 2002
Banana	<i>Musa sapientum</i> L.	Musaceae	0.01	Arnao and Hernández-Ruiz, 2013
Cucumber	<i>Cucumis sativus</i> L.	Cucurbitaceae	0.03, 0.59	Hattori et al., 1995; Badria, 2002
Pineapple	<i>Ananas comosus</i> (L.) Merr.	Bromeliaceae	0.04, 0.28, 0.30	Hattori et al., 1995; Badria, 2002; Arnao and Hernández-Ruiz, 2013
Apple	<i>Malus domestica</i> (Borkh)	Rosaceae	0.05, 0.16	Hattori et al., 1995; Badria, 2002
Tomato	<i>Solanum lycopersicum</i> L.	Solanaceae	0.5, 0.30	Dubbels et al., 1995; Badria, 2002
Tomato (dry weight basis)	<i>Solanum lycopersicum</i> L.	Solanaceae	7.5–250	Riga et al., 2014
Chilies (dry weight basis)	<i>Capsicum annuum</i> L.	Solanaceae	31–93	Riga et al., 2014
Cherry	<i>Prunus cerasus</i> L.	Rosaceae	18.0	Burkhardt et al., 2001
Kiwifruit	<i>Actinidia chinensis</i>	Actinidiaceae	0.02	Hattori et al., 1995
Pomegranate	<i>Punica granatum</i> L.	Lythraceae	0.17	Badria, 2002
Barbera grape (skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.63	Iriti et al., 2006
Croatina grape (skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.87	Iriti et al., 2006
Cabernet franc grape (Skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.01	Iriti et al., 2006
Cabernet sauvignon grape (Skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.42	Iriti et al., 2006
Marzemino grape (skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.03	Iriti et al., 2006
Nebbiolo grape (skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.97	Iriti et al., 2006
Sangiovese grape (skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.33	Iriti et al., 2006
Merlot grape (skin)	<i>Vitis vinifera</i> L.	Vitaceae	0.26	Iriti et al., 2006
Sangiovese grape	<i>Vitis vinifera</i> L.	Vitaceae	1.50	Mercolini et al., 2012
Albana grape	<i>Vitis vinifera</i> L.	Vitaceae	1.20	Mercolini et al., 2012
Burlat cherry	<i>Prunus avium</i> L.	Rosaceae	0.22	Gonzalez-Gomez et al., 2009
Sweetheart cherry	<i>Prunus avium</i> L.	Rosaceae	0.06	Gonzalez-Gomez et al., 2009
Pico Negro cherry	<i>Prunus avium</i> L.	Rosaceae	0.12	Gonzalez-Gomez et al., 2009
Navalinda cherry	<i>Prunus avium</i> L.	Rosaceae	0.03	Gonzalez-Gomez et al., 2009
Van cherry	<i>Prunus avium</i> L.	Rosaceae	0.01	Gonzalez-Gomez et al., 2009
Pico Colorado cherry	<i>Prunus avium</i> L.	Rosaceae	0.05	Gonzalez-Gomez et al., 2009
Hongdeng cherry	<i>Prunus avium</i> L.	Rosaceae	35.6	Badria, 2002
Rainier cherry	<i>Prunus avium</i> L.	Rosaceae	124.7	Badria, 2002
Tart cherry (Balaton)	<i>Prunus cerasus</i>	Rosaceae	22.90	Burkhardt et al., 2001; Kirakosyan et al., 2009
Tart cherry (Montmorency)	<i>Prunus cerasus</i>	Rosaceae	15, 12.30	Burkhardt et al., 2001; Kirakosyan et al., 2009
Wild strawberry	<i>Fragaria ananassa</i> Duch.	Rosaceae	0.01	Hattori et al., 1995
Camarosa strawberry	<i>Fragaria ananassa</i> Duch.	Rosaceae	5.58	Sturtz et al., 2011
Candonga strawberry	<i>Fragaria ananassa</i> Duch.	Rosaceae	5.50	Sturtz et al., 2011
Festival strawberry	<i>Fragaria ananassa</i> Duch.	Rosaceae	11.26	Sturtz et al., 2011
Primoris strawberry	<i>Fragaria ananassa</i> Duch.	Rosaceae	8.50	Sturtz et al., 2011
Orange	<i>Citrus sinensis</i> Osbeck.	Rutaceae	0.15	Johns et al., 2013
Mango	<i>Mangifera indica</i> L.	Anacardiaceae	0.70	Johns et al., 2013
Papaya	<i>Carica papaya</i> L.	Caricaceae	0.24	Johns et al., 2013
Walnut	<i>Juglans regia</i> L.	Juglandaceae	3.5	Reiter et al., 2005
<b>COLEOPTILES</b>				
Canary grass	<i>Phalaris canariensis</i> L.	Poaceae	26.7	Hernández-Ruiz et al., 2005
Wheat	<i>Triticum aestivum</i> L.	Poaceae	124.7	Hernández-Ruiz et al., 2005

(Continued)

**TABLE 1 | Continued**

Common name	Scientific name	Family	Concentration (ng/g)	References
Barley	<i>Hordeum vulgare</i> L.	Poaceae	82.3	Hernández-Ruiz et al., 2005
Oat	<i>Avena sativa</i> L.	Poaceae	90.6	Hernández-Ruiz et al., 2005
<b>ROOTS</b>				
Beet	<i>Beta vulgaris</i> L.	Amaranthaceae	0.01	Dubbels et al., 1995
Carrot	<i>Daucus carota</i>	Apiaceae	0.06, 0.49	Hattori et al., 1995; Badria, 2002
Ginger	<i>Zingiber officinale</i> (Roscoe)	Zingiberaceae	0.6, 1.42	Hattori et al., 1995; Badria, 2002
Red radish	<i>Raphanus sativus</i> L.	Brassicaceae	0.6	Hattori et al., 1995
Radish	<i>Raphanus sativus</i> L.	Brassicaceae	0.76	Badria, 2002
Turnip	<i>Brassica campestris</i> L.	Brassicaceae	0.7, 0.50	Hattori et al., 1995; Badria, 2002
Lupin	<i>Lupinus albus</i> L.	Fabaceae	55.6	Arnao and Hernández-Ruiz, 2013
Huang-qin	<i>Scutellaria baicalensis</i>	Lamiaceae	7110	Reiter and Tan, 2002
<b>BULBS</b>				
Onion	<i>Allium cepa</i> L.	Amaryllidaceae	0.03, 0.29	Hattori et al., 1995; Badria, 2002
Garlic	<i>Allium sativum</i> L.	Amaryllidaceae	0.58	Badria, 2002

The plants having more than one value for melatonin concentration are reported from different sources, and references are given in sequence.

solid-liquid extraction method was used. They found that the type of solvent, volume of solvent, temperature, sonication time and pH influenced extraction efficacy. Under optimized conditions they found highest ever reported melatonin levels from Pistachio (*Pistacia vera* L.) compared to any other plant in which melatonin was estimated. So the detection method might be the source of variation in melatonin concentrations among plant species. This issue needs to be addressed.

## ROLE IN PLANTS

Melatonin has proven to be ubiquitously synthesized in plant organs (Park et al., 2012; Byeon et al., 2013; Byeon and Back, 2014a,b; Byeon et al., 2014; Wang et al., 2014). Pleiotropic roles ranging from enhancing germination to delaying senescence of plants have been reported (Kolář and Machácková, 2005; Arnao and Hernández-Ruiz, 2006; Krystyna et al., 2009; Tan et al., 2012; Chan and Shi, 2015; Wei et al., 2015). While melatonin's role have highlighted the modulation of circadian rhythms in mammals (Bonnati-Carrion et al., 2014; Hardeland, 2015; Vriend and Reiter, 2015), this function has not been thoroughly examined in plants (Kolar et al., 1997). Thus, this subject is not discussed below; rather the data summarized below primarily consider the functions of melatonin in enhancing growth and preserving the integrity of plants under stressful conditions.

## Propagation

*In vitro* germplasm storage via cryopreservation is an effective tool to ensure conservation of tree species, but plant cells and tissues are exposed to multiple stresses including osmotic injury, desiccation and low temperature injury during the cryopreservation process; this contributes to problems during the regrowth of cryopreserved materials (Uchendu et al., 2013). Supplementing both preculture and regrowth media with melatonin (0.1–0.5 μM melatonin for 24 h) significantly

enhanced regrowth of frozen shoots compared with the untreated shoots (Uchendu et al., 2013). Similarly, 0.1 μM melatonin as pre-cryopreservation treatment to callus of *Rhodiola crenulata* (endangered plant species) also improved their recovery (Zhao et al., 2011).

Seed treatment with 100 μM melatonin for 12 h significantly improved the percentage germination of cucumber seeds (Zhang et al., 2013). Low concentrations of melatonin (1 μM) enhanced the germination rate of cucumber under salinity stress by regulating the biosynthesis and catabolism of abscisic acid (ABA) and gibberellic acid (GA<sub>4</sub>) (Zhang et al., 2014). Cuttings are also used as a means of propagation for many commercially important horticultural crops. The exogenous application of melatonin to roots of grape cuttings improved their growth by enhancing water stress tolerance. It increased the activity of antioxidant enzymes and the activities of non-enzymatic antioxidants; melatonin treatment also kept the internal lamellar system of chloroplasts well preserved and reduced ultrastructural destruction caused by drought stress (Jiang et al., 2014). A 2–3 fold rise in seed germination rate is common when they are treated with melatonin (Aguilera et al., 2015). While melatonin has been shown consistently to elevate the germination rate of seeds, the mechanisms of this stimulatory action remain to be identified.

## Growth and Development

Several studies have noted that melatonin regulates these physiological functions of plants; melatonin generally improves the growth of roots, shoots and explants (Murch et al., 2001; Hernández-Ruiz et al., 2005). The initial report of the direct involvement of melatonin in stimulating plant growth was reported in 2005 by Hernández-Ruiz et al. (2005); they observed that melatonin extended the coleoptiles (10–55%) of canary grass, wheat, barley and oat (monocots). Later, it was found that 0.5–1 μM application of melatonin enhanced the initial

seminal root length, growth and root biomass of transgenic rice plants (Park and Back, 2012). Melatonin is now known to alter many plant characteristics including germination (Zhang et al., 2014), seedling growth, alteration of flowering time, grain yields, and senescence (Wang et al., 2013a,b; Byeon and Back, 2014b). Somewhat unexpectedly, Byeon and Back (2014b) found that melatonin increased early seedling growth, but delayed flowering and reduced grain yield in transgenic rice over expressing sheep SNAT.

In animals, melatonin has also been reported to have anti-aging actions by delaying senescence (Acuna-Castroviejo et al., 2011; Rosales-Corral et al., 2012; Hardeland, 2013; Reiter et al., 2014). Long term soil application of 100  $\mu\text{M}$  melatonin also altered the metabolic status and delayed protein degradation in the apple plant (*Malus hupehensis* Rehd.), increased the chlorophyll content, the photosynthetic rates, and photosynthetic end products (sucrose, sorbitol, and starch) compared to control plants (Wang et al., 2013b). These changes were all associated with better protein preservation capacity. Consistent with this, Wang et al. (2013a), reported that long term application of 100  $\mu\text{M}$  melatonin to “Hanfu” apple (*Malus domestica* Borkh.) delayed drought-induced leaf senescence by reducing oxidative stress and suppressing the up-regulation of *senescence-associated gene 12* (*SAG-12*) and *pheophorbide a oxygenase* (*PAO*). Exogenous melatonin application also delayed natural leaf senescence in *Arabidopsis* (Shi et al., 2015a). Post-harvest losses of fruits and vegetables are very high (20–40%); according to estimates of the Food and Agricultural Organization (FAO), 32% of the all food produced in 2009 was wasted or lost (Lipinski et al., 2013). Considering the delay of aging and senescence due to pre and post-application of melatonin, it is possible that melatonin may play an important role in extending the shelf life of fruits and vegetables, and prove helpful in “on tree storage” (fruit crops), both of which may decrease post-harvest losses of fresh horticultural commodities.

Zhao et al. (2012) observed that in sweet cherry the concentrations of melatonin are comparatively lower in the first and third stages of fruit development while being much higher during the second stage of fruit development. During the second stage, cell elongation, cell expansion and embryo and seed development occurs, so the possible involvement of melatonin in these processes is clearly likely; however, additional investigations are required to precisely define the mechanisms by which melatonin influences development of fruits and vegetable crops. A recent study provides direct evidence that seed coating with melatonin significantly increased the leaf area, plant height, pods per plant, seeds per plant, and fatty acid contents of soybean plants (Wei et al., 2015). This study suggests new avenues to enhance crop yields. Seeds coated with melatonin could be potentially used for a large number of commercially important agronomic and horticultural crops. This has the potential to revolutionize the seed industry.

## Stress Tolerance

### Salinity

Salinity is a major environmental factor that limits crop growth and productivity; it leads to huge economic losses worldwide

(Allakhverdiev et al., 2000). Salinity not only induces water deficit caused by osmotic stress, it also disturbs key biochemical process (photosynthesis, protein synthesis, energy, and lipid metabolism) in plant cells (Allakhverdiev et al., 2000; Li et al., 2012). Plants use various strategies to cope with these stressors; these involve the exclusion of selective ions, ion compartmentalization, synthesis of compatible solutes, alterations in the photosynthetic pathway, changes in membrane structure, induction of antioxidant enzymes, stimulation of phytohormones and regulation of gene expression (Parida and Das, 2005). Recently, Arnao and Hernandez-Ruiz (2014) reviewed the auxin-independent effects of melatonin as a plant growth regulator in various plant species. Exogenous application of melatonin (0.1  $\mu\text{M}$ ) significantly alleviated the growth inhibition caused by elevated salinity; this enabled the plants to maintain their photosynthetic capacity. The application of melatonin also decreased the oxidative damage caused by ROS by directly scavenging  $\text{H}_2\text{O}_2$  and enhancing the activities of antioxidant enzymes including ascorbate peroxidase, catalase, and peroxidase (Li et al., 2012). Salinity exerts its negative impact irrespective of growth stage of the plants, and its effects range from seed germination to plant senescence, and occur throughout the life cycle. Seed germination and plant growth is severely affected by saline stress (Ungar, 1996; Parida and Das, 2005; Li et al., 2012). In every case melatonin proved its importance by ameliorating the effects caused by salt stress and improved germination and plant growth. In cucumber (*Cucumis sativus* L.), pre-sowing seed treatment with melatonin (1  $\mu\text{M}$ ) enhanced the rate of germination and subsequent growth under 150 mM NaCl stress; this increase was accompanied by approximately a 5-fold elevation in antioxidant enzyme activities (superoxide dismutase, catalase, peroxidase; Zhang et al., 2014). Melatonin has also been found to be involved in the biosynthesis and catabolism of gibberellin (GA) and abscisic acids (ABA), respectively; it was shown to up-regulate ABA catabolism genes and down-regulate ABA biosynthesis genes resulting to a rapid reduction in ABA. At the same time, it positively up-regulated GA biosynthesis genes during the early stage of germination, which leads to better germination and better plant growth during the initial stages (Zhang et al., 2014). Melatonin application enhanced tolerance to salt and drought stress in soybean, and up-regulated the expression of genes that were inhibited by salt stress (Wei et al., 2015).

A recent study conducted using bermudagrass revealed that exogenous melatonin application conferred abiotic stress tolerance and it was observed that 3933 transcripts (2631 were up-regulated and 1572 were down-regulated) were differentially expressed compared to non-treated plants (Shi et al., 2015b). The genes involved in nitrogen metabolism, major carbohydrate metabolism, tricarboxylic acid (TCA)/org transformation, transport, hormone metabolism, metal handling, redox, and secondary metabolism were over expressed, clearly showing the involvement of melatonin in influencing metabolic activity.

### Cold

Low temperature stress leads to significant damage to agricultural crops; low temperature alters plant physiology, biochemistry

and molecular biology (Bajwa et al., 2014). Many scientists are working on the development of cold tolerant commercially-important crop cultivars. Recently, melatonin was shown to significantly alleviate cold stress in a number of plants. Melatonin treated (10–30  $\mu$ M) *Arabidopsis thaliana* plants produced higher fresh weight, root length and plant height compared to untreated plants (Bajwa et al., 2014). Like other plants, low temperature damages wheat plants by reducing leaf area, leaf water content, photosynthetic pigment content, and the accumulation of ROS caused lipid peroxidation of membranes. The application of melatonin (1 mM for 12 h) to wheat seedlings increased the activity of the antioxidant enzymes, superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase leading to improved plant growth by reducing oxidative damage (Turk et al., 2014). More recently, it has been found that the exogenous application of melatonin increased salt, drought and cold resistance in bermudagrass (*Cynodon dactylon* L. Pers.). In this study, melatonin activated not only several antioxidants but also induced higher concentration of 54 secondary metabolites including amino acids, organic acids, sugars, and sugar alcohols (Shi et al., 2015b).

### Heat Stress

Extremes temperatures affect membrane fluidity and enzyme activities (Zhang et al., 2015) leading to alterations in growth and development patterns and yield losses. In plants under stressful conditions, the genes responsible for melatonin biosynthesis are typically activated resulting in higher levels of melatonin. As an example, under high temperature conditions the level of melatonin is increased in rice (Byeon and Back, 2014a) suggesting a role of melatonin in defense against heat stress. In green micro-algae *Ulva* sp. rise in temperature increases melatonin levels, confirming its ability to improve heat tolerance (Tal et al., 2011). Application of melatonin has the potential to reverse the inhibitory effect of light and high temperature on photosensitive and thermosensitive *Phacelia tanacetifolia* Benth seeds (Tiryaki and Keles, 2012). Melatonin application increased germination percentage of heat stressed *Arabidopsis thaliana* seeds up to 60% compared to control; this effect was likely due to powerful antioxidant activity of melatonin (Hernández et al., 2015). Similarly in another recent study, Shi et al. (2015b) reported that application of melatonin activated stress responsive genes in Bermuda grass. *C-REPEAT BINDING FACTORS/DEHYDRATION-responsive ELEMENT-BINDING PROTEIN (CBF/DREB)* genes and target genes, heat shock transcription factors (TFs), zinc finger TFs, *WRKY*, *MYB*, *bHLH* genes, and hormone-related genes exhibited a 16-fold over expression compared to levels in control plants.

### Drought, Ultraviolet Radiations, Heavy Metals, and Chemicals Stress

Melatonin has also proven its protective role against drought, ultraviolet radiation, heavy metals and chemicals stress. Transgenic Micro-Tom tomato plants overexpressing the homologous ovine *AANAT* and *HIOMT* genes exhibited loss of apical dominance and enhanced drought tolerance (Wang et al., 2014). Plant species sensitive to ozone damage have lower

levels of melatonin compared to ozone resistant species (Dubbels et al., 1995). Similarly, Alpine and Mediterranean plant species growing in high UV-exposed natural habitats have higher levels of melatonin compared to their counterparts growing under low UV exposure areas (Simopoulos et al., 2005). Afreen et al. (2006) observed higher concentrations of melatonin in roots of *Glycyrrhiza uralensis* when exposed to UV-B radiation; they proposed elevated melatonin levels were protective against plants the augmented oxidative damage. Subsequently, Zhang et al. (2012) confirmed the protective role of melatonin against UV-B. When exposed to UV-B radiation, DNA damage was reduced in transgenic *Nicotiana sylvestris* plants expressing melatonin synthesis genes. Melatonin is also useful to save plants from heavy metals stress, as presowing seed treatment of red cabbage seed (*Brassica oleracea rubrum*) eliminated the toxic effects of copper ions (0.5 and 1 mM) during germination and early seedling growth (Posmyk et al., 2008). Similarly, Arnao and Hernández-Ruiz (2009) observed that application of zinc sulfate (1 mM) increased the concentration of melatonin up to 6-fold in barley (*Hordeum vulgare* L.) roots, suggesting the protective role of melatonin against chemical and other abiotic stressors. Melatonin has also been reported to provide protection against butafenacil (a singlet oxygen-generating herbicide), in the study in question, melatonin-rich transgenic rice plants exhibited lower levels of malondialdehyde and hydrogen peroxide. These plants also exhibited elevated superoxide dismutase and catalase activities compared to control plants (Park et al., 2013).

### Disease Resistance/Control

Plant diseases cause major production and economic losses in agriculture worldwide, and both the public and private sectors are working to control the plant diseases through various strategies ranging from forecast and diagnosis of diseases to the production of disease resistant cultivars. In addition to many other positive functions in plants, exogenous application of melatonin (0.05–0.5 mM) improved resistance against one of the most severe diseases, *Marssonina* apple blotch (fungal diseases caused by *Diplocarpon Malo*); this involved modulating the activities of antioxidant enzymes and plant defense related enzymes (Yin et al., 2013). Also, Ishihara et al. (2008) observed that the activation of tryptophan pathway leads to establishment of effective physical defenses by enriching serotonin in rice leaves; serotonin suppresses the growth of fungal hyphae in leaf tissues. Although the authors did not mention the possible involvement of melatonin, this possibility cannot be ignored since serotonin is the precursor of melatonin in the biosynthetic pathway (Reiter, 1991; Falcón et al., 2009; Park et al., 2012; Arnao and Hernandez-Ruiz, 2014). In a recent study, the application of 10  $\mu$ M melatonin on to *Arabidopsis* induced pathogenesis-related genes which further supports the idea that melatonin may be a defense signaling molecule in plants against pathogens (Lee et al., 2014). After compiling a recent review, the authors (Vielma et al., 2014) concluded that melatonin is an important therapeutic alternative to fight against bacterial, viral and parasitic infections in vertebrates (human, mammals, equine) as has been observed in plants. Certainly, the possibility that melatonin may help in controlling plant diseases (fungal, bacterial, viral, viroides)

should not be overlooked and requires further investigation. Similarly, the potential role of melatonin in defense against insect attacks should be considered, as it has been reported that dopamine (a catecholamine) functions as an antiherbivore defense in temperate green alga *Ulvari aobscura* (Van Alstyne et al., 2006). Other secondary metabolites have also been isolated from plants which serve as juvenile hormone antagonists against insects and can be used to kill the insects at the larval stage (Lee et al., 2015). Thus, melatonin (an indoleamine) might have a role in defense against insect attack, and could prove to be a potential means to control or reduce insect feeding on commercial crops, as insects cause huge losses (billions of dollars) and substantially reduce crop yields (Boyer et al., 2012).

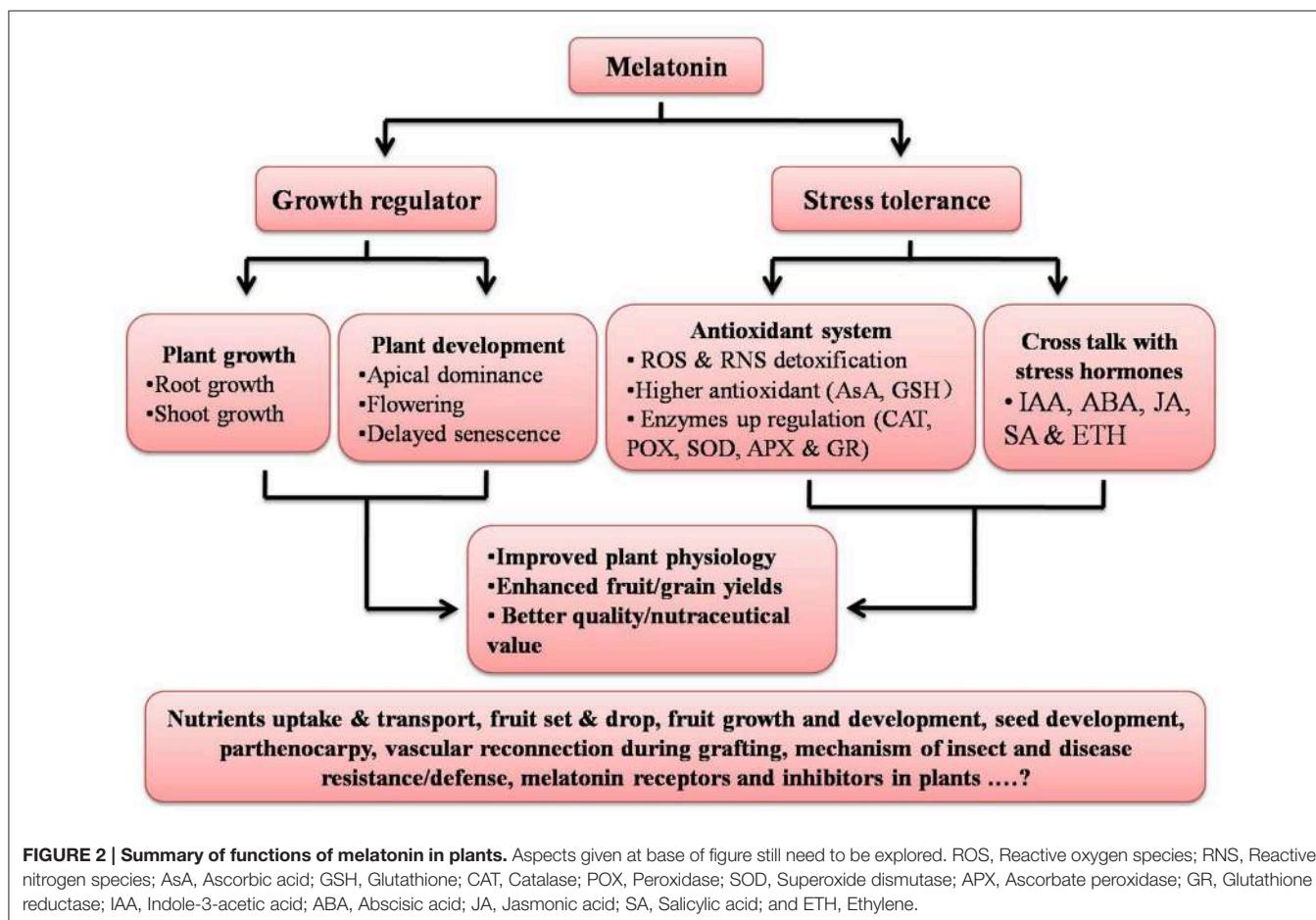
### Phytoremediation

The water hyacinth grown under bright sunlight (10,000–15,000  $\mu\text{W}/\text{cm}^2$ ) produces extremely high concentrations of melatonin and  $\text{N}^1\text{-acetyl-N}^2\text{-formyl-5-methoxykynuramine}$  (AMFK) as compared to plants grown in artificial light (400–450  $\mu\text{W}/\text{cm}^2$ ) (Tan et al., 2007a). On the basis of these findings and others, the authors proposed that the presence of high concentrations of these molecules save these pollutant-resistant plants from the harsh environmental contaminants. They suggested that plants containing up-regulated melatonin and AMFK levels could be

used for soil phytoremediation (Tan et al., 2007a). They further supported this when they found that soil application (5  $\mu\text{M}$ ) of melatonin improved the copper tolerance of pea (*Pisum sativum* L.) plants (Tan et al., 2007b). Thus, melatonin was found to be effective in preventing the death of pea plants grown in soil contaminated with copper. Moreover, melatonin itself is environmentally friendly. Other studies suggest the use of biotechnology and genetic engineering techniques to increase the phytoremediative potential of already existing plants used for this purpose (Dietz and Schnoor, 2001; Cherian and Oliveira, 2005; Lal and Srivastava, 2010; Behera, 2014). Thus, an integrated approach toward phytoremediation may lead to the desired results.

### MECHANISMS OF ACTION

The mechanisms of action of melatonin is not clearly understood in plants; however, it modifies plant growth and development by acting as an antioxidant, membrane stabilizer, and by up and down regulating gene expression. Some of melatonin actions in plants may be receptor-mediated while others are receptor-independent. Recently, Arnao and Hernandez-Ruiz (2014) suggested that melatonin performs some of its functions in plants by actions similar to those of indole-3-acetic acid



(IAA). However, in *Gonyaulax polyedra* (dinoflagellate) the first observable reaction to melatonin is a 90-fold increase in bioluminescence coupled with the release of H<sup>+</sup> into the cytoplasm (Hardeland, 1993). In plants, this aspect is a missing link in understanding the biological functions of melatonin and it requires the attention of plant scientists.

## FUTURE PERSPECTIVES

Some successful examples of transgenic plants having introduced genes from vertebrates have shown substantially elevated concentrations of melatonin. In the future, additional transgenic crops will likely be produced, and the altered biosynthesis of melatonin in these plants may be used as a tool to induce resistance against biotic and abiotic stresses leading to increased crop yields. Also, considering the importance and therapeutic value of melatonin for humans, the pharmaceutical industry should capitalize on the potential human benefits of related pharmaceutical preparations for humans, animals and plants. Presumably, in the future we will see fruits and vegetables with higher levels of melatonin produced by the combination of conventional and modern breeding approaches. Few reports are available related to the possible role of melatonin in helping to control diseases and insects; this area of research should be aggressively explored with a definition of the specific defense mechanisms. This information could lead to the use of melatonin on a commercial scale. Melatonin enhances the phytoremediative capacity of plants, but further studies are required. Whether melatonin improves the phytoremediative capacity of the hyperaccumulator plant species would be important to document, and if so mechanism involved would require definition.

It has been widely reported that application of melatonin promotes root growth but the roles of melatonin in nutrient uptake still needs to be investigated; to date no reports have been published clarifying the interactions between melatonin and nutrient uptake and transport. Similarly, very limited information is available on the response of foliar applications of melatonin to its absorption and modification of plant growth and development.

Although melatonin is ubiquitously distributed in plants, it is not known whether all plant organs synthesize this indoleamine. Its mechanism of transport throughout the plant also must be explored.

As many scientists have observed the auxin-like activity of melatonin along with its pleiotropic functions (in animals and

plants). Further detailed investigations on the possible role of melatonin in *in vitro* plant propagation, propagation through cutting, grafting and vascular reunion, flower development, enhancing male to female ratio in vegetables (cucurbits), improvement of fruit setting, fruit development, parthenocarpy, fruit drop (a major issue in commercially important fruit crops like citrus, mango, guava, etc.), role in breaking seed and tuber dormancy, fruit quality (size, color, nutraceutical value), seed development, fruit ripening and senescence (to improve post-harvest life/shelf life of fruits, vegetables, and cut flowers) needs clarification. Root treatment of melatonin may help to improve the success ratio and initial root development and growth of crops which require nursery transplanting (rice, tomato, chilies, cabbage, cauliflower, eggplant etc.). The role of melatonin in grafted plants also should be investigated; do different scion stock combinations affect the concentration of melatonin in roots and shoots alternatively?

Recently, scientists have documented an active role of auxin in grafting and vascular connection establishment (Melnik et al., 2015); since melatonin has been reported to act like an auxin, its involvement with auxin in terms of the vascular reconnection should to be examined. While the physiological and biochemical roles of melatonin in plants are in part clarified, there is not a single study related to melatonin specific synthesis or action inhibitors or the presence or absence of melatonin receptors in plants; this is an area worthy of investigation (Figure 2). Keeping in mind the physiological, biochemical, and genetic and epigenetic actions of melatonin in multiple organisms, it seems melatonin may prove to be an important molecule to influence especially field crops, and may prove helpful in increasing crops yields and the nutraceutical value helping to address the food security issues around the world.

## AUTHOR CONTRIBUTIONS

MAN, YH, WA, MN, and SH wrote the manuscript, ZB and RR revised, and finally approved the manuscript for publication.

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# Fundamental Issues of Melatonin-Mediated Stress Signaling in Plants

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As a widely known hormone in animals, melatonin (*N*-acetyl-5-methoxytryptamine) has been more and more popular research topic in various aspects of plants. To summarize the these recent advances, this review focuses on the regulatory effects of melatonin in plant response to multiple abiotic stresses including salt, drought, cold, heat and oxidative stresses and biotic stress such as pathogen infection. We highlight the changes of endogenous melatonin levels under stress conditions, and the extensive metabolome, transcriptome, and proteome reprogramming by exogenous melatonin application. Moreover, melatonin-mediated stress signaling and underlying mechanism in plants are extensively discussed. Much more is needed to further study in detail the mechanisms of melatonin-mediated stress signaling in plants.

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## INTRODUCTION

*N*-acetyl-5-methoxytryptamine (melatonin) was first identified in the pineal gland of cow (Lerner et al., 1958, 1959). Later on melatonin was also discovered in plants (Dubbels et al., 1995; Hattori et al., 1995). Thereafter, melatonin has been identified in almost all plant species, although with different concentrations, including model plants (*Arabidopsis*, rice, tobacco), fruits (banana, cucumber, apple, beestrawberry), and so on (Arnao and Hernández-Ruiz, 2014, 2015; Reiter et al., 2001, 2014, 2015; Van Tassel et al., 2001; Simopoulos et al., 2005; Tan et al., 2007, 2012, 2014; Shi and Chan, 2014; Shi et al., 2015b,d,e,a,c,f). In the meantime, melatonin biosynthetic and metabolic pathways in plants have been revealed (Kang et al., 2010; Tan et al., 2012, 2014; Arnao and Hernández-Ruiz, 2014, 2015; Wang L. et al., 2014; Wang P. et al., 2014; Zuo et al., 2014; Reiter et al., 2015; Hardeland, 2016). Melatonin biosynthesis begins from tryptophan through four sequential enzyme reactions, involving tryptophan decarboxylase (TDC), arylalkylamine *N*-acetyltransferase (AANAT)/serotonin *N*-acetyltransferase (SNAT), tryptamine 5-hydroxylase (T5H), *N*-acetylserotonin methyltransferase (ASMT)/hydroxyindole-O-methyltransferase (HIOMT) (Tan et al., 2016; Wei et al., 2016). Thereafter, melatonin is converted to 2-hydroxymelatonin by melatonin 2-hydroxylase (M2H) (Byeon et al., 2015).

Based on previous studies using exogenous melatonin treatment or transgenic plants with higher or lower melatonin levels, some more general comprehension has been achieved as to the involvement of the compound in seed germination, root development, fruit ripening, senescence, yield, circadian rhythm, stress responses (Kolář and Macháčkova, 2005; Posmyk et al., 2008, 2009a,b; Li et al., 2012, 2015; Wang et al., 2012, 2013, 2015; Park et al., 2013; Yin et al., 2013; Zhang et al., 2013; Zhao et al., 2013; Bajwa et al., 2014; Lee et al., 2014, 2015; Zhang H. J. et al., 2014; Zhang N. et al., 2014; Liang et al., 2015; Byeon and Back, 2016).

Considering the new advances in recent 5 years (Tan et al., 2012, 2014, 2015; Lee et al., 2014, 2015; Kaur et al., 2015; Reiter et al., 2015), we focus on the regulatory effects of melatonin in plant responses to multiple abiotic stress factors and plant-pathogen interactions (**Table 1**).

## MELATONIN-MEDIATED STRESS RESPONSES

Secondary messengers including calcium and hydrogen peroxide ( $H_2O_2$ ) play essential roles in plant stress responses by linking upstream receptors and activating downstream signal transduction (Shi et al., 2015b,d,e,a,c,f; Zhang et al., 2015). It has been shown that nearly all stresses including salt, drought, cold, heat, zinc sulfate,  $H_2O_2$ , anaerobic, pH, pathogen, and senescence can cause a rapid and massive up-regulation of melatonin production in various plants (Tan et al., 2012, 2014; Reiter et al., 2015; Shi et al., 2015b,d,e,a,c,f), indicating the possible role of melatonin as an important messenger in plant stress responses.

Most of previous studies focused on the effect of melatonin on reactive oxygen species (ROS) metabolism, as well as the alleviation of stress-induced ROS production and the activation of antioxidants in melatonin-conferred stress resistance in plants (Zhang et al., 2015). In recent years, more and more studies

have extended our understanding on the molecular mechanisms of melatonin-mediated stress responses in plants. Based on previous studies, plant transcription factors play important roles in plant stress responses, by directly regulating the transcription of stress-responsive genes and through acting in cross-talk between multiple signaling pathways (Reiter et al., 2014, 2015; Shi et al., 2015b,d,e,a,c,f). In *Arabidopsis*, we have found that four transcription factors including *Arabidopsis thaliana* Zinc Finger protein 6 (ZAT6) (Shi and Chan, 2014), Auxin Resistant 3 (AXR3)/Indole-3-Acetic Acid inducible 17 (IAA17) (Shi et al., 2015d), class A1 Heat Shock Factors (HSFA1s) (Shi et al., 2015e), and C-repeat-Binding Factors (CBFs)/Drought Response Element Binding 1 factors (DREB1s) (Shi et al., 2015c), are involved in melatonin-mediated signaling. Briefly, AtZAT6-activated CBF pathway is essential for melatonin-mediated freezing stress response (Shi and Chan, 2014); AtIAA17-activated senescence-related *Senescence 4 (SEN4)* and *Senescence-Associated Gene 12 (SAG12)* transcripts may contribute to the process of natural leaf senescence (Shi et al., 2015d); HSFA1s-activated transcripts of *HSFA2*, *Heat-Stress-Associated 32 (HSA32)*, *Heat Shock Protein 90 (HSP90)*, and *HSP101* may contribute to melatonin-mediated thermotolerance (Shi et al., 2015e); AtCBFs-mediated signaling pathway and sugar accumulation may partially be involved in melatonin-mediated stress response (Shi et al., 2015c). Moreover, the diurnal

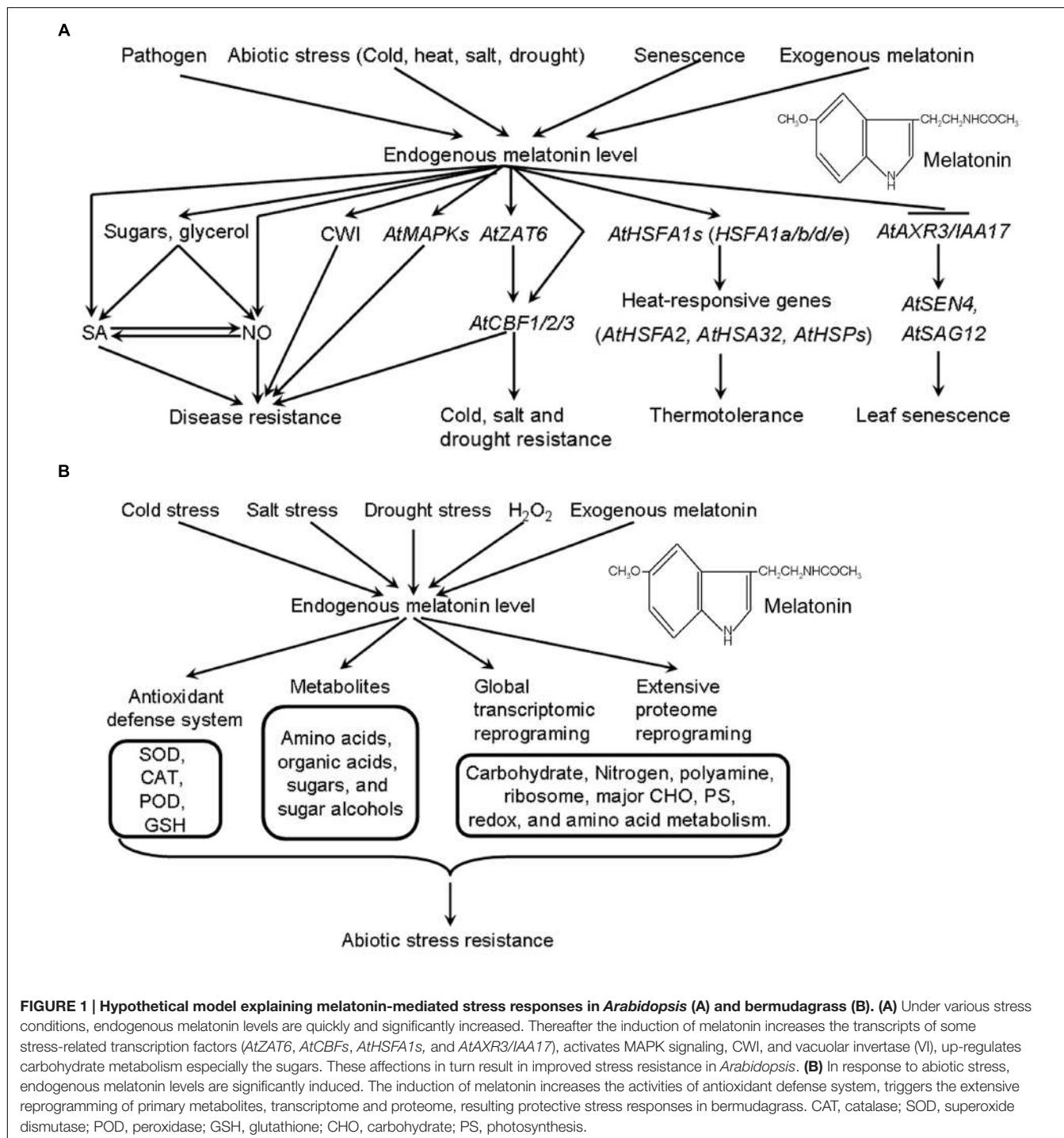
**TABLE 1 | The functions of melatonin in plant stress responses.**

Plant species	Stress responses	Melatonin treatment or transgenic plants	References
<i>Arabidopsis</i>	Cold stress	Melatonin treatment	Bajwa et al., 2014; Shi and Chan, 2014
<i>Arabidopsis</i>	Disease resistance against <i>Pseudomonas syringae</i> pv. tomato	Melatonin treatment and transgenic plants	Lee et al., 2014, 2015; Lee and Back, 2016; Qian et al., 2015; Shi et al., 2015a,c, 2016; Zhao et al., 2015a
<i>Arabidopsis</i>	Leaf senescence	Melatonin treatment	Shi et al., 2015d
<i>Arabidopsis</i>	Thermotolerance	Melatonin treatment	Shi et al., 2015e
<i>Arabidopsis</i>	Salt and drought stresses	Melatonin treatment	Shi et al., 2015c
<i>Arabidopsis</i>	Oxidative stress	Melatonin treatment	Weeda et al., 2014; Wang et al., 2015
Bermudagrass	Salt, drought and cold stresses	Melatonin treatment	Shi et al., 2015b
Bermudagrass	Oxidative stress	Melatonin treatment	Shi et al., 2015f
<i>Nicotiana benthamiana</i>	Disease resistance against <i>Pseudomonas syringae</i> pv. Tomato	Melatonin treatment	Lee et al., 2014
<i>Lupinus albus</i>	Disease resistance to fungal infection ( <i>Penicillium</i> spp.)	Melatonin treatment	Arnao and Hernández-Ruiz, 2015
Rice	Salt and cold stresses	Transgenic plants	Kang et al., 2010; Byeon and Back, 2016
Rice	Herbicide-induced oxidative stress	Transgenic plants	Park et al., 2013
Rice	Cadmium stress	Transgenic plants	Byeon et al., 2015
Rice	Leaf senescence and salt stress	Melatonin treatment	Liang et al., 2015
<i>Malus</i>	Disease resistance to Marssonina apple blotch	Melatonin treatment	Yin et al., 2013
<i>Malus</i>	Salt stress	Melatonin treatment	Li et al., 2012
<i>Malus</i>	Drought stress	Melatonin treatment	Li et al., 2015
<i>Malus</i>	Senescence	Melatonin treatment	Wang et al., 2012, 2013; Wang P. et al., 2014
Cucumber	Chilling stress	Melatonin treatment	Posmyk et al., 2009a
Cucumber	Salt stress	Melatonin treatment	Zhang H. J. et al., 2014
Cucumber	Drought stress	Melatonin treatment	Zhang N. et al., 2014
Red cabbage	Copper ion	Melatonin treatment	Posmyk et al., 2008, 2009b
Tomato	Drought stress	Transgenic plants	Wang L. et al., 2014

changes of *AtCBF/DREB1s* expression may be regulated by the corresponding change of endogenous melatonin level and be involved in diurnal cycle of plant immunity (Shi et al., 2016). Thus, these transcription factors may play important roles in melatonin-mediated stress responses in plants.

Salicylic acid (SA) and NO are required small molecules for plant disease resistance, and SA-deficient plants (*NahG* overexpressing plants) and NO deficient mutants (*noa1* and

*nia1nia2*) show increased sensitivity to bacterial pathogen. Moreover, both of SA and NO confer enhanced disease resistance against bacterial pathogen in *Arabidopsis*, and the cooperation between them plays important roles in plant innate immunity (Shi et al., 2012). Recently, we also found that melatonin treatment increases the accumulation of sugars and glycerol, and the elevated sugars and glycerol thereafter increase the endogenous NO level, which confers an enhanced innate



**FIGURE 1 | Hypothetical model explaining melatonin-mediated stress responses in *Arabidopsis* (A) and bermudagrass (B).** **(A)** Under various stress conditions, endogenous melatonin levels are quickly and significantly increased. Thereafter the induction of melatonin increases the transcripts of some stress-related transcription factors (*AtZAT6*, *AtCBFs*, *AtHSFA1s*, and *AtAXR3/IAA17*), activates MAPK signaling, CWI, and vacuolar invertase (VI), up-regulates carbohydrate metabolism especially the sugars. These affections in turn result in improved stress resistance in *Arabidopsis*. **(B)** In response to abiotic stress, endogenous melatonin levels are significantly induced. The induction of melatonin increases the activities of antioxidant defense system, triggers the extensive reprogramming of primary metabolites, transcriptome and proteome, resulting protective stress responses in bermudagrass. CAT, catalase; SOD, superoxide dismutase; POD, peroxidase; GSH, glutathione; CHO, carbohydrate; PS, photosynthesis.

immunity against bacterial pathogens via a SA and NO-dependent pathway in *Arabidopsis* (Qian et al., 2015; Shi et al., 2015a,f). Consistently, Yin et al. (2013) showed that melatonin improves *Malus* resistance to *Marssonina apple blotch*, and Lee et al. (2014, 2015) found that melatonin confers disease resistance against pathogen attack in *Arabidopsis* and tobacco, which may be related with endogenous SA level. Zhao et al. (2015a) found that exogenous melatonin regulates carbohydrate metabolism, increases cell wall invertase (CWI), increases production of sucrose, glucose, fructose, cellulose, xylose and galactose, and cellose deposition during pathogen infection. They also found that melatonin-mediated sugar metabolism, especially its metabolites exert significant promotional and inhibitory effects, for instance on the growth of maize seedling, as was demonstrated by treatment with different doses of exogenous melatonin (Zhao et al., 2015b). Together with previous studies suggesting that sugars are functional, well compatible solutes for osmotic adaptation in response to abiotic stress, being also involved in the protection against bacterial pathogens (Thibaud et al., 2004; Shi et al., 2015a,f; Tsutsui et al., 2015), the above studies highlight the important roles of sugar metabolism in complex plant stress responses. Recently, Lee and Back (2016) found that the mitogen-activated protein kinase (MAPK) signaling through MAPK kinase (MKK) 4/5/7/9-MPK3/6 cascades are also required for melatonin-mediated innate immunity in plants.

Based on these results, a hypothetical model explaining melatonin-mediated signaling in *Arabidopsis* is proposed (**Figure 1A**). Under various stress conditions, endogenous melatonin levels are quickly and significantly increased. As a consequence, the induction of melatonin increases the transcripts of some stress-related transcription factors (*AtZAT6*, *AtCBFs*, *AtHSFA1s*, and *AtAXR3/IAA17*) and the underlying downstream genes, activates MAPK signaling, CWI and vacuolar invertase (VI), up-regulates carbohydrate metabolism especially the sugars. These induced affects in turn result in improved stress resistance in *Arabidopsis*.

With the development of omics, several studies indicated that melatonin triggers extensive reprogramming of primary metabolites, transcriptome, and proteome in plants, further confirming its involvement in plant signal transduction. Weeda et al. (2014), Liang et al. (2015), and Shi et al. (2015b) identified 1308 differentially expressed genes (DEGs) (566 up-regulated genes and 742 down-regulated genes), 3933 DEGs (2361 up-regulated genes and 1572 down-regulated genes) and 457 DEGs (191 up-regulated genes and 266 down-regulated genes) by exogenous melatonin treatment in *Arabidopsis*, bermudagrass and rice, respectively. Wang P. et al. (2014) and Shi et al. (2015f) identified 309 and 63 differentially expressed proteins (DEPs) after exogenous melatonin treatment in apple and bermudagrass, respectively. MapMan and gene ontology (GO) analyses found that that several pathways were enhanced by melatonin treatment in bermudagrass, including nitrogen-metabolism, polyamine metabolism, major carbohydrate (CHO) metabolism, hormone metabolism, metal handling, photosynthesis (PS), redox status, and amino acid metabolism. Notably, all these transcriptome and proteome studies identified a large number of transcription

factors as DEGs or DEPs, the functional identification of these DEGs or DEPs may provide more valuable clues into melatonin-mediated signaling. Additionally, both Wang P. et al. (2014) and Shi et al. (2015f) indicated the possible role of melatonin in epigenetic modification in plants. Based on our previous studies (Shi et al., 2015b,f), we also propose a hypothetical model explaining melatonin-mediated stress responses in bermudagrass (**Figure 1B**). In response to abiotic stress, endogenous melatonin levels are significantly induced. The induction of melatonin activates antioxidant defense system, triggers the extensive reprogramming of primary metabolites, transcriptome, and proteome, resulting protective stress responses in bermudagrass. The “omics” approaches can give some clues about the effect of melatonin on plants, focusing on the extensive reprogramming of gene transcripts, protein expression and metabolites, as well as the relationship among them. This is just the beginning to reveal melatonin signaling in plants, many questions need to be investigated, including the crosstalk between melatonin and other phytohormones, the interaction between melatonin and primary or secondary metabolism.

## CONCLUSION AND PERSPECTIVES

The objective of this review is to update the research on melatonin-mediated stress signaling, and to encourage plant researchers to dissect further molecular mechanism and signaling pathway. Although melatonin has continuously drawn the attentions of plant biologists and some advances have been made in recent years, melatonin-mediated complex signaling pathways are largely unknown. Since melatonin shares the common substrate (tryptophan) with IAA, the cross-talk between melatonin and auxin signaling pathways needs to be further investigated (Arnao and Hernández-Ruiz, 2014, 2015). Moreover, unlike for animals (Jackers et al., 2008; Yu et al., 2014), no specific melatonin-associated phenotype and no melatonin receptor have been characterized in plants. Thus, these open questions still prevent a full understanding of melatonin signaling in plants (Reiter et al., 2015; Zhang et al., 2015). Therefore, the identification of melatonin receptor or sensor and the establishment of molecular link between melatonin sensing and the regulators for plant stress responses will be an important next step.

Moreover, several fundamental issues need to be resolved. How is endogenous melatonin production regulated? How to perceive and transfer melatonin signaling in plant cells? What are the major or limiting steps in melatonin signaling transduction in plants? Which genes are specifically regulated by melatonin and underlying signaling pathways? Together with the development of more new techniques, further studies will shed more light on the global involvement of melatonin in plants and underlying signaling pathway.

## AUTHOR CONTRIBUTIONS

HS initiated this project, wrote and revised the manuscript, KC, and YW wrote the manuscript, CH provided suggestions and

revised the manuscript. All authors approved the manuscript and the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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# Corrigendum: Melatonin: Current Status and Future Perspectives in Plant Science

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## BIOSYNTHESIS

The readers are informed that the text given in the biosynthesis section of originally published article (doi: 10.3389/fpls.2015.01230) at line number 20–30 of page number two is not definitive and has very limited scientific evidence, so it should not be considered.

Some steps illustrated in originally published Figure 1, like the conversion of tryptamine to Indo-3-acetaldehyde and indole acetic acid (IAA), and direct conversion of serotonin to melatonin by SNAT are not definitive, as they have very limited scientific evidence. So these steps should not be considered the part of originally published **Figure 1**.

## AUTHOR CONTRIBUTIONS

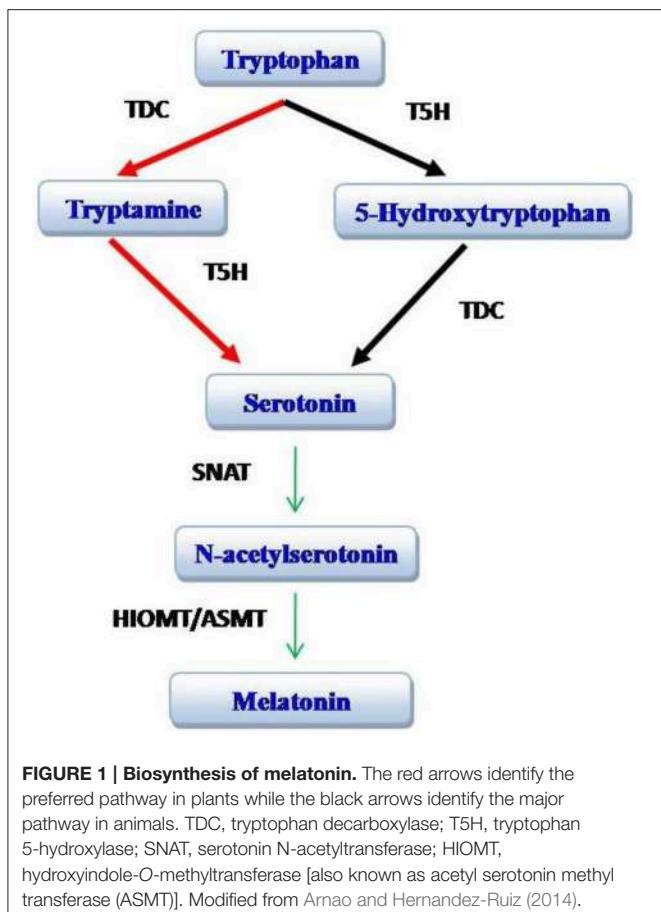
All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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# Exogenous Melatonin Mitigates Photoinhibition by Accelerating Non-photochemical Quenching in Tomato Seedlings Exposed to Moderate Light during Chilling

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Melatonin plays an important role in tolerance to multiple stresses in plants. Recent studies have shown that melatonin relieves photoinhibition in plants under cold stress; however, the mechanisms are not fully understood. Non-photochemical quenching (NPQ) is a key process thermally dissipating excess light energy that plants employ as a protective mechanism to prevent the over reduction of photosystem II. Here, we report the effects of exogenous melatonin on NPQ and mitigation of photoinhibition in tomato seedlings exposed to moderate light during chilling. In response to moderate light during chilling, the maximum quantum yield ( $F_v/F_m$ ) and the effective photochemical efficiency ( $F'v/F'm$ ) of PSII were both substantially reduced, showing severe photoinhibition in tomato seedlings, whereas exogenous application of melatonin effectively alleviated the photoinhibition. Further experiment showed that melatonin accelerated the induction of NPQ in response to moderate light and maintained higher level of NPQ upon longer exposure to light during chilling. Consistent with the increased NPQ was the elevated de-epoxidation state of xanthophyll pigments in melatonin-pretreated seedlings exposed to light during chilling. Enzyme activity assay showed that violaxanthin de-epoxidase (VDE), which catalyzes the de-epoxidation reaction in the xanthophyll cycle, was activated by light and the activity was further enhanced by application of melatonin. Further analysis revealed that melatonin induced the expression of VDE gene in tomato seedlings under moderate light and chilling conditions. Ascorbic acid is an essential cofactor of VDE and the level of it was found to be increased in melatonin-pretreated seedlings. Feeding tomato seedlings with dithiothreitol, an inhibitor of VDE, blocked the effects of melatonin on the de-epoxidation state of xanthophyll pigments and the induction of NPQ. Collectively, these results suggest that exogenous melatonin mitigates photoinhibition by accelerating NPQ through the stimulation of VDE activity and the enhancement of de-epoxidation state of xanthophyll pigments.

**Keywords:** chilling, melatonin, non-photochemical quenching, photoinhibition, *Solanum lycopersicum*, violaxanthin de-epoxidase, xanthophyll cycle

## INTRODUCTION

Plants are largely dependent on the efficient conversion of absorbed light energy to chemical energy to sustain growth and development. However, under high light, light absorption generally exceeds photochemical demands in plants, inevitably leading to energy imbalance (Björkman and Demmig-Adams, 1994; Kaňa and Govindjee, 2016; Zhao et al., 2017). The resulting energy imbalance can be exacerbated by environmental stresses, such as drought, high or low temperatures, and salinity (Takahashi and Murata, 2008). The excess light energy can ultimately result in the generation of destructive singlet oxygen and other reactive oxygen species (ROS) (Niyogi, 1999), which pose severe oxidative damage to photosynthetic apparatus (Melis, 1999; Yin et al., 2010). Not surprisingly, in the long-term evolution, plants have developed multiple mechanisms to balance excess light absorption with photochemical utilization in order to protect photosystems against photodamages (Horton et al., 1996; Niyogi et al., 2001). One important mechanism is to thermally dissipate excess absorbed light energy in the light-harvesting antenna complexes of photosystem II (PSII), which confers protection of PSII against inactivation and potential damages by excess light energy. This process of thermally dissipation is referred to as non-photochemical quenching (NPQ). NPQ involves energy-dependent quenching ( $q_E$ ), quenching associated with state transition ( $q_T$ ) and photoinhibition ( $q_I$ ), among which  $q_E$  acts predominantly to dissipate excess excitation energy absorbed in the PSII antenna as heat and thus plays an important role in alleviation of PSII photoinhibition (Wright and Crofts, 1970; Briantais et al., 1979; Horton et al., 1996; Nilkens et al., 2010).

The  $q_E$  component of NPQ relies on the light-mediated de-epoxidation of violaxanthin to zeaxanthin in the xanthophyll cycle, which requires acidification of thylakoid lumen (Niyogi et al., 1998; Munekage et al., 2001). In the xanthophyll cycle, the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin is catalyzed by violaxanthin de-epoxidase (VDE). VDE is a 43 kD protein encoded by the nuclear gene *VDE/NPQ1* and its activation requires acidification of thylakoid lumen as a result of light-driven electron movement through the photosynthetic electron transport chain (Briantais et al., 1979; Pfundel and Dilley, 1993; Hager and Holocher, 1994). VDE activity is also influenced by ascorbic acid, which is an essential cofactor of VDE (Bratt et al., 1995; Smirnoff, 1996, 2000a,b). Suppression of dehydroascorbate reductase (DHAR) expression, which is responsible for the generation of ascorbic acid, results in reductions in xanthophyll pigments, reduced NPQ and increased photoinhibition (Chen and Gallie, 2008). While VDE activity is light-dependent, VDE transcript expression in *Arabidopsis* is suppressed by light and induced by drought under light (North et al., 2005). Moreover, in most cases transcriptional regulation of VDE gene is not correlated to protein level and activity (Bugos et al., 1999).

Melatonin (*N*-acetyl-5-methoxytryptamine) is an important hormone involved a number of biological processes in animals. Recently, melatonin has also been demonstrated to play important roles in plants. As an indoleamine, melatonin

functions as an auxin-like hormone regulating root development in plants (Murch et al., 2001; Zhang et al., 2014). Melatonin is also involved in the delay of leaf senescence (Wang et al., 2013; Shi et al., 2015b). Moreover, melatonin mitigates oxidative stress by directly scavenging ROS or indirectly improving antioxidant potential (Arnao and Hernández-Ruiz, 2015; Reiter et al., 2015). Studies have also shown that melatonin confers tolerance to a variety of abiotic and biotic stresses in plants, including cold, heat, salinity, drought, heavy metal toxicity, and pathogens (Li et al., 2012, 2016; Bajwa et al., 2014; Shi et al., 2014, 2015a; Xu et al., 2016). Recent studies demonstrate that melatonin alleviates damages to photosystems induced by cold and salinity through enhancement of antioxidant capacity and regulation of electron transport chain (Fan et al., 2015; Szafranska et al., 2016; Zhao et al., 2016; Zhou et al., 2016). In unicellular organisms, melatonin may also play important roles. In a study on cultured *Symbiodinium*, melatonin was found to stimulate xanthophyll cycle activity and increase NPQ levels as a protective mechanism against excess solar energy (Roopin et al., 2013). In the last decade, significant progress has been made in deciphering the function of melatonin in stress responses in plants; however, the role of melatonin in the alleviation of photoinhibition is only partially understood and merits further investigation.

Tomato (*Solanum lycopersicum* L.) is an important horticultural crop worldwide; however, it is highly sensitive to low temperatures because of its tropical origin. Low temperatures, particularly under light, adversely affect all aspects of tomato plants including photosynthesis, and cause severe reductions in tomato yields (Park et al., 2004; Zushi et al., 2012; Ding et al., 2016, 2017). Thus exploring melatonin-mediated alleviation of photoinhibition in tomato is of both theoretical and practical significance. The objectives of this work were to investigate the role of melatonin in regulating NPQ in tomato seedlings exposed to moderate light during chilling and thus to explore the role of melatonin in relieving photoinhibition.

## MATERIALS AND METHODS

### Plant Materials, Growth Conditions, and Treatment

Tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seeds were sterilized and germinated at 25°C in the dark on filter paper in Petri dishes. Germinated seeds were then planted in 12 cm × 12 cm plastic pots containing peat and vermiculite (3/1, v/v) and maintained in a growth room with the following conditions: 380 ppm of CO<sub>2</sub>, photon flux density of 400 μmol m<sup>-2</sup> s<sup>-1</sup>, day/night temperature of 25/20°C, relative humidity of 60% and a photoperiod of 14 h.

After the third leaf emerged, tomato seedlings were sprayed one time a day either with 100 μM melatonin (Sigma-Aldrich, St. Louis, MO, USA) solution or with distilled water for 3 days, which gave rise to two groups of seedlings. Then seedlings in each group were randomly divided into two subgroups. At the end of light cycle at 20:00 on day 3, one subgroup of each group was subjected to cold stress (4°C) in the dark and the rest of subgroups were kept under 25°C in the dark, then next morning at 6:00, all groups

were exposed to light, resulting in four different subgroups: (1) Control: seedlings grown under 25°C first in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 6:00 next morning; (2) Control + MT: seedlings pretreated with melatonin and grown under 25°C first in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 6:00 next morning; (3) Chilling: seedlings exposed to 4°C first in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 6:00 next morning; (4) Chilling + MT: seedlings pretreated with melatonin exposed to 4°C first in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 6:00 next morning. For each treatment, there were a total of 45 tomato seedlings and each of three replicates consisted of 15 tomato seedlings.

Leaf samples from four subgroups were collected at 0, 5, 10, 30, and 60 min following exposure to light next morning and then immediately placed in liquid nitrogen. Then, samples were stored at -80°C for further analysis. Chlorophyll fluorescence was recorded at 20 s intervals for the initial 180 s and then every 30 min for 6 h following illumination.

## Measurement of Chlorophyll Fluorescence

Chlorophyll fluorescence was measured with a portable fluorometer (PAM-2000, Walz, Germany). The effective photochemical efficiency ( $F'v/F'm$ ) and the maximum quantum efficiency ( $Fv/Fm$ ) of PSII were measured in light-adapted seedlings and dark-adapted seedlings, respectively. The initial chlorophyll fluorescence yield ( $F_0$ ) was determined under low-modulated light, followed by a pulse of saturating white light to obtain maximum fluorescence yield ( $F_m$ ) in seedlings in the dark. The steady-state fluorescence levels ( $F_s$ ) and the maximum fluorescence levels ( $F_m'$ ) were monitored at different time points during light exposure. NPQ was estimated from the Stern-Volmer equation as:  $(F_m - F_m')/F_m$ . The specific procedures were followed as described by Chen and Gallie (2012).

## Analyses of Pigments in the Xanthophyll Cycle

Analyses of pigments in the xanthophyll cycle were performed as described by Thayer and Björkman (1990). Leaf samples were homogenized in 100% cold acetone and pigments extracts were filtered, then xanthophyll pigments were separated and quantified by HPLC.

## Determination of Transcript Abundance by Quantitative Real-Time PCR

Total RNA was extracted from seedling leaves and was used for cDNA synthesis by PrimeScript® reverse transcriptase following standard protocols. Quantitative real-time PCR was performed using SYBR® Premix Ex TaqTM (TaKaRa) according to manufacturer's instructions. Each real-time PCR reaction was performed in 25  $\mu\text{l}$  final volume on iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) under the following program: 1 cycle of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The primers for tomato VDE were AGTGCAGGATAGAGCTTGCG (Forward)

and CGGGAGACTGCACACTCATT (Reverse). The primers for tomato DHAR were CTTCGAGCGAGAGTCGTTCC (Forward) and TAAAGCTGCACTCGTCGA (Reverse).

## Isolation of Chloroplasts

Chloroplasts were isolated as described in a previous study (Robinson et al., 1983). Ten grams of seedling leaves were extracted with buffer containing 330 mM sorbitol, 30 mM Mes, 2 mM ascorbate and 0.1% BSA. The crude extract was filtered and centrifuged at 1200  $\times g$  for 3 min. The resulting pellets were resuspended in buffer containing 330 mM sorbitol, 30 mM Hepes, and 0.2% BSA. The suspension was mixed with 80% percoll and 40% percoll, and was centrifuged at 1200  $\times g$  for 1 min. The intact chloroplasts were isolated between 80% percoll and 40% percoll.

## VDE Activity Assay

Violaxanthin de-epoxidase activity was measured as previously described (Bugos et al., 1999; Chen and Gallie, 2012). Briefly, VDE activity was assayed in a reaction mixture containing 10  $\mu\text{L}$  of 1  $\mu\text{M}$  violaxanthin, 25  $\mu\text{L}$  of 300  $\mu\text{M}$  monogalactosyldiacylglycerol in methanol, 550  $\mu\text{L}$  of 0.2 M sodium citrate (pH 5.1), and 50  $\mu\text{L}$  of VDE extract. The reaction mixture was thoroughly mixed and incubated at 30°C for 5 min. The reaction was started by adding 6  $\mu\text{L}$  of 3 M sodium ascorbate. After 10 min, the reaction was stopped by the addition of 1 N NaOH. The mixture was centrifuged at 20,000  $\times g$  for 2 min and the resulting pellets containing the lipids and pigments were analyzed by HPLC.

## Dehydroascorbate Reductase (DHAR) Activity Assay

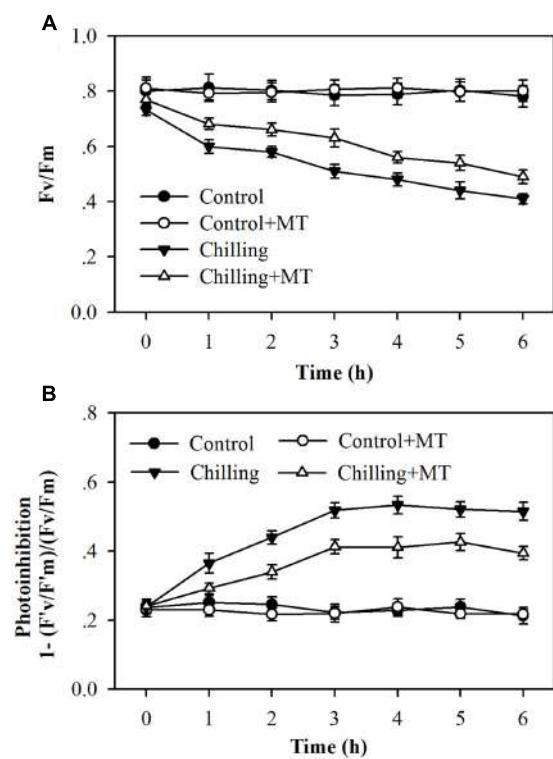
Dehydroascorbate reductase activity was analyzed essentially following Dalton et al. (1986). Crude enzyme extract was obtained by homogenizing a total volume of 3 mL of chloroplast suspension with 25 mM cold Hepes buffer (pH 7.8) containing 0.2 mM EDTA and 2% PVP. Following centrifugation at 4°C at 13,000  $\times g$  for 10 min, the supernatant was used to measure DHAR activity. One hundred  $\mu\text{L}$  enzyme extract was added to the reaction mixture containing 100 mM Hepes (pH 7.0), 1 mM EDTA, and 2.5 mM reduced glutathione. The reaction was initiated by adding 0.2 mM dehydroascorbate to reaction mixture and the increase in absorbance at 265 nm was measured as ascorbic acid was formed.

## Determination of Ascorbic Acid

A volume of 600  $\mu\text{L}$  chloroplast suspension was homogenized in 1.2 mL of 6% (v/v) cold HClO<sub>4</sub> and centrifuged at 4°C for 10 min at 10,000  $\times g$ . The supernatant was used to determine the level of ascorbic acid as previously described (Logan et al., 1998). Ascorbic acid was assayed by determining the absorbance difference of the supernatant at 265 nm in 200 mM sodium acetate buffer (pH 5.6) before and after 15-min incubation with 1.5 units of ascorbate oxidase.

## Dithiothreitol (DTT) Feeding

Dithiothreitol (DTT) feeding experiment was carried out in tomato seedlings pretreated with or without melatonin under



**FIGURE 1 | Changes in photoinhibition in the leaves of tomato seedlings pretreated with melatonin (MT) following exposure to light during chilling. (A)** The maximum quantum yield ( $F_v/F_m$ ) of PSII; **(B)** Photoinhibition of PSII. Leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seedlings at the three-leaf stage were pretreated with  $100 \mu\text{mol}$  melatonin (MT) one time a day for 3 days. At the end of light cycle at 18:00 on day 3, seedlings were exposed to chilling ( $4^\circ\text{C}$ ) for 10 h in the dark, then in the light ( $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) next morning for another 6 h. Data were collected at 0, 1, 2, 3, 4, 5, and 6 h following light exposure. The values presented are mean  $\pm$  SD ( $n = 6$ ).

chilling stress. Tomato seedlings were infiltrated with either 5 mM DTT or with water via petiole 3 h before they were exposed to light.

## Statistical Analysis

All experiments in the present study were repeated at least three times, and the values presented are mean  $\pm$  SD. Duncan's multiple range test was performed to compare the difference among treatments. Different letters in figures indicate significant differences at  $P < 0.05$ .

## RESULTS

### Melatonin Relieves Photoinhibition in Tomato Seedlings Exposed to Moderate Light during Chilling

Tomato plants have been demonstrated to undergo severe photoinhibition under high light or low light in combination with low temperatures (Zhang and Scheller, 2004; Han et al.,

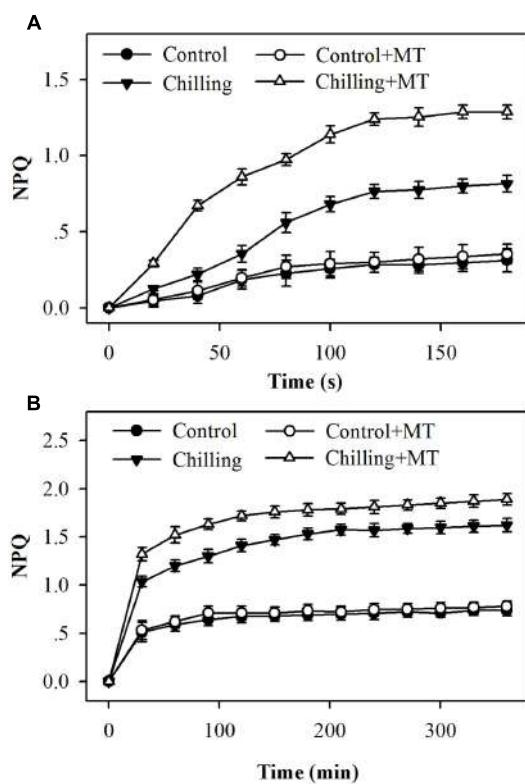
2010; Huang et al., 2010). To investigate the effects of exogenous melatonin on photoinhibition in tomato seedlings exposed to moderate light during chilling, we measured the effective photochemical efficiency ( $F'_v/F'_m$ ) and the maximum quantum yield ( $F_v/F_m$ ) of PSII. The photoinhibition was estimated by calculation of  $1 - (F'_v/F'_m)/(F_v/F_m)$ . It was found that chilling ( $4^\circ\text{C}$ ) in the dark for 10 h did not cause significant reductions in  $F_v/F_m$  and  $F'_v/F'_m$  (at time 0), however, chilling in the light ( $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) dramatically decreased  $F_v/F_m$  and  $F'_v/F'_m$  in tomato seedlings (Figure 1A; Supplementary Figure S1). It is notable that higher  $F'_v/F'_m$  and  $F_v/F_m$  were observed in melatonin-pretreated seedlings than in non-melatonin-treated ones under chilling and moderate light conditions, showing reduced photoinhibition in melatonin-treated seedlings (Figure 1B). These results indicate that exogenous application of melatonin alleviates photoinhibition in tomato seedlings exposed to chilling and light.

### Melatonin Accelerates Non-photochemical Quenching in Tomato Seedlings Exposed to Moderate Light during Chilling

We examined whether NPQ contributed to reduced photoinhibition observed in the first experiment and whether melatonin treatment affected NPQ in tomato seedlings under chilling and light conditions. The assessment of NPQ showed that in response to light during chilling, NPQ was induced rapidly within as short as 20 s, and seedlings pretreated with melatonin exhibited a faster and higher induction of NPQ than seedlings without melatonin application (Figure 2A). Following 20 s of exposure to light during chilling, melatonin-pretreated seedlings showed a 53% increase in NPQ in comparison with non-melatonin-treated seedlings (Figure 2A). Over a course of 6 h, the levels of NPQ in melatonin-treated seedlings remained significantly higher than those in non-melatonin-treated seedlings under chilling and light conditions (Figure 2B). These results indicate that melatonin increases the initial induction and final level of NPQ under moderate light during chilling.

### Melatonin Promotes De-epoxidation of Xanthophyll

In order to investigate the possible mechanism of melatonin-mediated increase in NPQ under chilling and light conditions, we determined the effects of melatonin treatment on the xanthophyll cycle, which has been proved to contribute substantially to NPQ (Holt et al., 2004). The de-epoxidation state of the xanthophyll pigments was examined in tomato seedlings following exposure to light under chilling stress. Violaxanthin predominated in fully dark-adapted seedlings whereas antheraxanthin and zeaxanthin were generated rapidly in response to light during chilling. Higher levels of antheraxanthin and zeaxanthin were observed in melatonin-pretreated seedlings than in non-melatonin-pretreated ones (Figure 3) under moderate light in combination with chilling. In order to determine whether the rapid induction of NPQ in melatonin-treated seedlings were due to the increased de-epoxidation of violaxanthin, we measured



**FIGURE 2 |** Kinetics of NPQ induction following exposure to light for (A) the initial 180 s or (B) 360 min in the leaves of tomato seedlings pretreated with melatonin (MT) during chilling. Leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seedlings at the three-leaf stage were pretreated with 100  $\mu\text{mol}$  melatonin (MT) one time a day for 3 days. At the end of light cycle at 18:00 on day 3, seedlings were exposed to chilling (4°C) for 10 h in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) next morning for another 6 h. Data were collected at 20 s intervals for the initial 180 s and then every 30 min for 6 h following light exposure. The values presented are mean  $\pm$  SD ( $n = 6$ ).

the extent of de-epoxidation in seedlings pretreated either with or without melatonin following exposure to light under chilling condition. Following exposure to 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light during chilling, a significant increase in de-epoxidation of violaxanthin to zeaxanthin was observed in melatonin-treated seedlings within 5 min, with additional de-epoxidation occurring upon longer exposure to light (Figure 4). In contrast, the rate of de-epoxidation was lower in seedlings without melatonin application, resulting in a lower de-epoxidation state. These results indicate that exogenous melatonin promoted de-epoxidation activity in tomato seedlings under moderate light during chilling, consistent with the rapid initial induction of NPQ.

### Melatonin Induces VDE Expression and Increases VDE Activity

The conversion of violaxanthin to zeaxanthin and antheraxanthin in the xanthophyll cycle depends on light-activated VDE. The increase in de-epoxidation activity observed in melatonin-treated tomato seedlings could result from an

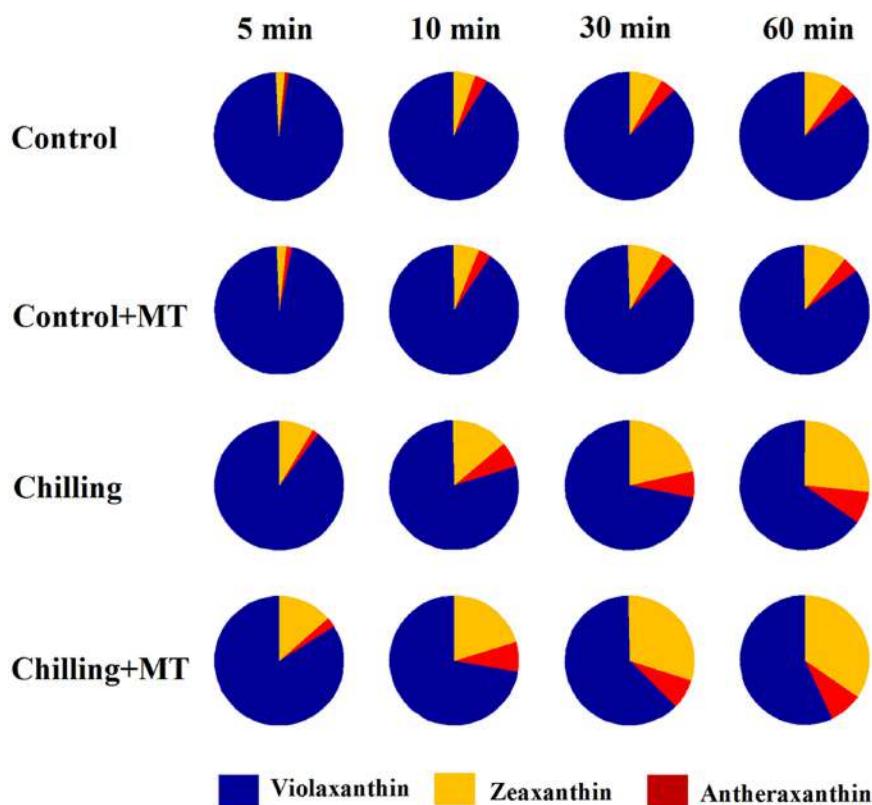
increase in the expression of *VDE* mRNA or (and) activation of VDE activity. Therefore, to further investigate the impacts of melatonin on the de-epoxidation of violaxanthin in the xanthophyll cycle, we measured *VDE* transcript abundance and VDE activity in tomato seedlings exposed to light in combination with chilling. Higher *VDE* expression was observed in melatonin-treated seedlings than in non-melatonin-treated ones under chilling and light conditions (Figure 5A). Moreover, exogenous application of melatonin led to the highest transcript level 10 min following illumination during chilling (Figure 5A). VDE activation requires the light-mediated acidification of the thylakoid lumen where VDE resides. The results showed that VDE activity was significantly increased by moderate light during chilling and the increase was much greater in melatonin-treated seedlings than in non-melatonin-treated seedlings and control seedlings (Figure 5B). The highest VDE activity was observed 60 min following light exposure under chilling condition (Figure 5B). These results suggest that melatonin promotes *VDE* expression and stimulates VDE activity.

### Effects of Melatonin on Xanthophyll De-epoxidation are Counteracted by Feeding Dithiothreitol

Dithiothreitol is an inhibitor of VDE (Yamamoto and Komite, 1972). To further ascertain the role of melatonin in promoting de-epoxidation of violaxanthin and NPQ, tomato seedlings pretreated with or without melatonin were fed with DTT. Feeding seedlings with DTT suppressed VDE activities in all examined seedlings and eliminated the effects of melatonin on the de-epoxidation state of the xanthophyll cycle (Figures 6A,B). Furthermore, application of DTT dramatically suppressed the development of NPQ (Figure 6C). These results suggest that melatonin regulates the xanthophyll cycle and NPQ by mainly acting on VDE activity.

### Melatonin Increases DHAR Expression, DHAR Activity and the Level of Ascorbic Acid

To catalyze the de-epoxidation reaction, VDE requires ascorbic acid as a cofactor. It has been demonstrated that increased DHAR expression and ascorbic acid content mitigate photoinhibition by improving VDE activity in tobacco plants (Chen and Gallie, 2008). To determine the possible mechanism of melatonin-mediated increase in VDE activity, we measured transcript abundance of *DHAR*, DHAR activity and level of ascorbic acid in the chloroplasts of tomato seedlings subject to chilling and moderate light. It was observed that expression of *DHAR*, DHAR activity and level of ascorbic acid were increased by chilling and the increase was much greater when exogenous melatonin was applied in tomato seedlings (Figures 7A,B). Together with melatonin-mediated increase in VDE activity, these results may suggest that melatonin-mediated increase in the level of ascorbic acid contributes, at least in part, to the increased VDE activity in melatonin-treated tomato seedlings exposed to light under chilling.



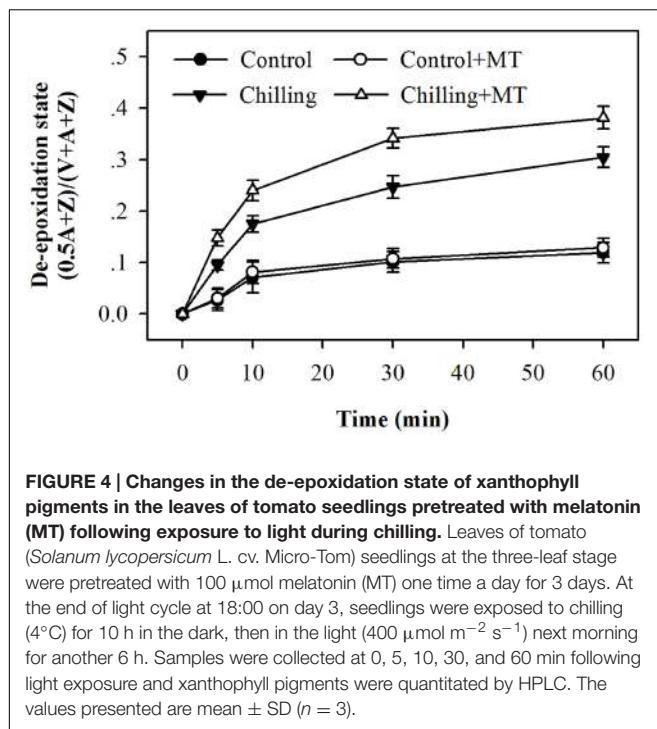
**FIGURE 3 | Alteration of violaxanthin de-epoxidation in the leaves of tomato seedlings pretreated with melatonin (MT) following exposure to light during chilling.** Leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seedlings at the three-leaf stage were pretreated with 100  $\mu\text{mol}$  melatonin (MT) one time a day for 3 days. At the end of light cycle at 18:00 on day 3, seedlings were exposed to chilling (4°C) for 10 h in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) next morning for another 6 h. Samples were collected at 0, 5, 10, 30, and 60 min following light exposure and xanthophyll pigments were quantitated by HPLC. The values presented are mean  $\pm$  SD ( $n = 3$ ).

## DISCUSSION

Non-photochemical quenching is an important physiological process employed by plants to dissipate excess absorbed light energy. NPQ is induced when absorbed light surpasses the capacity of light utilization in photochemistry, which generally occurs under conditions of high light or low light in combination with other abiotic stresses (Demmig-Adams, 1990; Huang et al., 2010). A substantial part of NPQ is attributed to the xanthophyll cycle, in which zeaxanthin is generated in a light-dependent manner (Eskling et al., 1997). In the present study, we have concluded that melatonin, an extensively studied molecule in plants, protects tomato seedlings against photoinhibition under moderate light during chilling. The evidence leading to the conclusion includes (1) application of melatonin accelerates NPQ by increasing rates of VDE; (2) melatonin-mediated increase in NPQ is a consequence of elevated VDE activity; and (3) increased VDE activity is due to melatonin-induced expression of VDE and melatonin-mediated accumulation of VDE cofactor ascorbic acid.

Melatonin plays a recognized role in the protection of plants against various abiotic stresses. There are several reports on the application of melatonin and its influence on photosynthetic

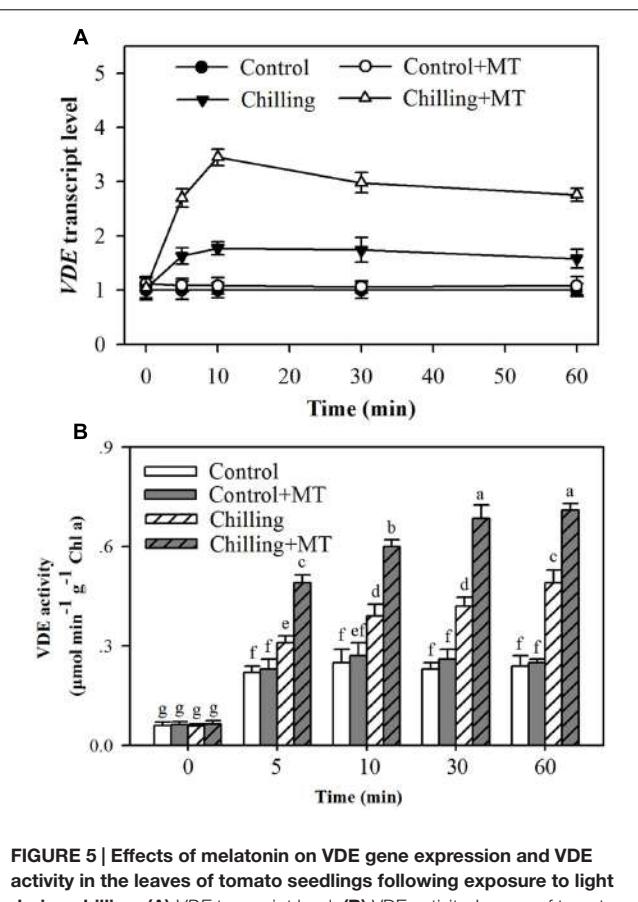
apparatus under stress conditions (Fan et al., 2015; Szafrańska et al., 2016; Zhao et al., 2016; Zhou et al., 2016). These studies have established that melatonin gives an advantage to the function of photosystems by reducing oxidative damages through scavenging of ROS or regulation of electron transport chain. However, information regarding the effects of melatonin on the xanthophyll cycle and the induced NPQ is still lacking in plants. It was observed in this study that moderate light during chilling greatly inhibited PSII, whereas melatonin application significantly alleviated this inhibition, suggesting a protective role of melatonin in amelioration of photo damage. Though the widely reported role of melatonin in promoting the capacity of scavenging ROS may contribute to the alleviated inhibition of PSII in this study, yet there might be an alternative mechanism. In order to pursue additional mechanism, we assessed the impacts of melatonin on NPQ in tomato seedlings exposed to light under chilling stress, because NPQ is indispensable to the dissipation of excess light absorbed in photosystem and thus confers protection of PSII against photoinhibition. In this study, NPQ was rapidly induced in response to moderate light during chilling in dark-adapted tomato seedlings, showing that 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  is excessive in tomato seedlings subject to chilling (Figure 2). This observation is consistent with a previous study that low



**FIGURE 4 |** Changes in the de-epoxidation state of xanthophyll pigments in the leaves of tomato seedlings pretreated with melatonin (MT) following exposure to light during chilling. Leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seedlings at the three-leaf stage were pretreated with 100  $\mu\text{mol}$  melatonin (MT) one time a day for 3 days. At the end of light cycle at 18:00 on day 3, seedlings were exposed to chilling (4°C) for 10 h in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) next morning for another 6 h. Samples were collected at 0, 5, 10, 30, and 60 min following light exposure and xanthophyll pigments were quantitated by HPLC. The values presented are mean  $\pm$  SD ( $n = 3$ ).

temperatures combined with light increase NPQ as a mechanism of dissipating excess energy as heat (Corcuera et al., 2005). The chilling-light induction of NPQ was further enhanced by the application of melatonin, supporting that melatonin is beneficial in accelerating diversion of absorbed light from photochemistry under chilling condition. However, it is unclear based on the data presented here that to what extent melatonin-mediated increases in the NPQ levels contribute to relieved photoinhibition, because melatonin is a molecule functioning at multiple levels in plants. Melatonin can serve as direct scavenger of ROS and it also promotes the expression of antioxidant enzymes and enhances the accumulation of antioxidants, thus leading to reduced level of ROS, which may be partially accountable for the alleviated photoinhibition in this study. Therefore, in future studies, it is worth comparing the role of melatonin-mediated increases in NPQ with that of melatonin-mediated reductions of ROS in the alleviation of photoinhibition.

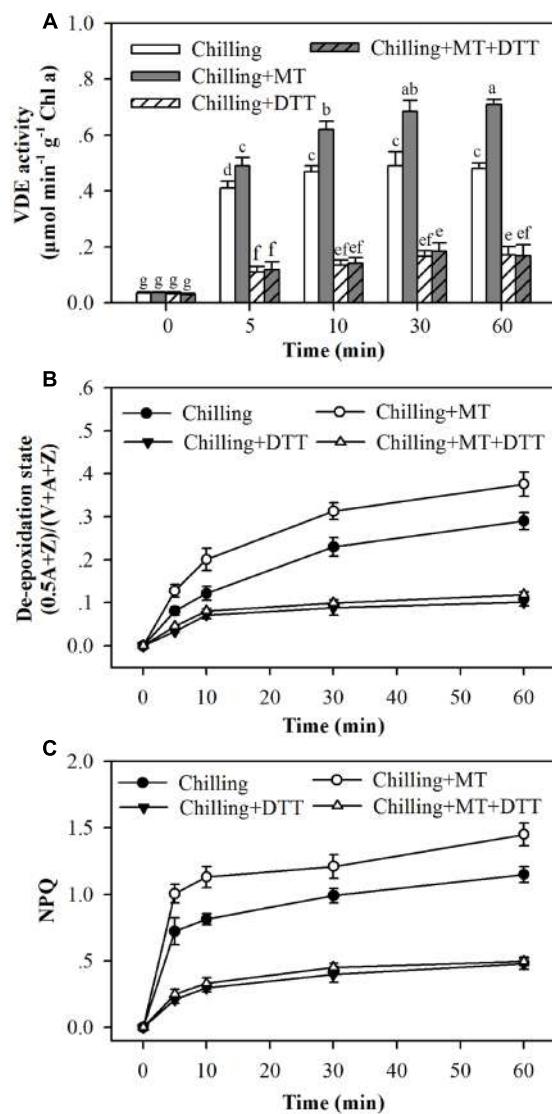
In agreement with the increased induction of NPQ by melatonin was the observed rise in de-epoxidation state of violaxanthin in the xanthophyll cycle. Melatonin significantly increased the conversion of violaxanthin to antheraxanthin and zeaxanthin after dark-adapted tomato seedlings were exposed to light during chilling. Kinetics of xanthophyll de-epoxidation in seedlings showed that melatonin accelerated the rate of de-epoxidation and maintained a high level of de-epoxidation state under moderate light and chilling condition (Figure 4). Our results further support the previously established notion that the formation of NPQ upon either excess light or low light combined with other stresses matches the changes in de-epoxidation state of xanthophyll (Johnson et al., 2008; Ware et al., 2015). The de-epoxidation of xanthophyll is



**FIGURE 5 |** Effects of melatonin on VDE gene expression and VDE activity in the leaves of tomato seedlings following exposure to light during chilling. (A) VDE transcript level; (B) VDE activity. Leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seedlings at the three-leaf stage were pretreated with 100  $\mu\text{mol}$  melatonin (MT) one time a day for 3 days. At the end of light cycle at 18:00 on day 3, seedlings were exposed to chilling (4°C) for 10 h in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) next morning for another 6 h. Samples were collected at 0, 5, 10, 30, and 60 min following light exposure. The values presented are mean  $\pm$  SD ( $n = 3$ ). Different letters indicate significant differences at  $P < 0.05$  among treatments.

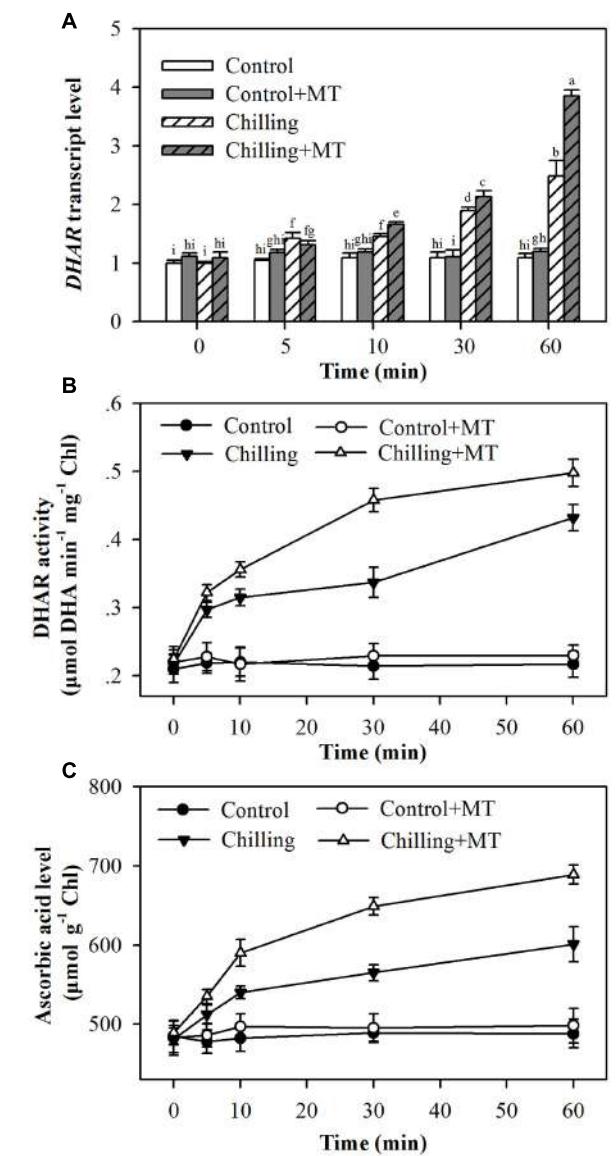
catalyzed by VDE, which is a central player in the xanthophyll cycle. A previous study has confirmed that chilling leads to reduction in VDE activity, thus resulting in lower rate of de-epoxidation and retarded formation of NPQ (Chen and Gallie, 2012). Thus, the observed increase in de-epoxidation state due to melatonin application in our study is supposed to be in line with higher activity of VDE. It was shown that VDE activity was higher in melatonin-treated seedlings than in non-melatonin-treated ones under chilling and light conditions, which substantiates that melatonin increased larger de-epoxidation state of xanthophyll and induced greater NPQ by acting on VDE activity.

Enzyme activity can be influenced by several factors, including transcript levels, protein turnover and cofactors. VDE transcript levels increased in response to light during chilling, and application of melatonin resulted in a dramatic increase in VDE transcript level. Overall, melatonin-mediated increase in transcript levels appeared consistent with the increase in VDE activity, indicating that increased VDE expression induced by



**FIGURE 6 |** Dithiothreitol blocked the effects of melatonin on (A) VDE activity, (B) the de-epoxidation state of xanthophyll pigments and (C) the induction of NPQ in the leaves of tomato seedlings following exposure to light during chilling. Leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seedlings at the three-leaf stage were pretreated with 100  $\mu\text{mol}$  melatonin (MT) one time a day for 3 days. At the end of light cycle at 18:00 on day 3, they were exposed to chilling (4°C) for 10 h in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) next morning for another 6 h. Tomato seedlings were infiltrated with either 5 mM dithiothreitol (DTT) or with water from stem 2 h before they were exposed to light. Samples were collected at 0, 5, 10, 30, and 60 min following light exposure. The values presented are mean  $\pm$  SD ( $n = 3$ ). Different letters indicate significant differences at  $P < 0.05$  among treatments.

melatonin contributes to enhanced VDE activity. Transcript level, however, did not always match VDE activity in the presented results. Peak transcript level occurred 10 min following illumination during chilling, while peak VDE activity was observed at 60 min (Figure 5A). The difference in transcript level and VDE activity may demonstrate that this enzyme does not turn over rapidly and this result is in accordance with a



**FIGURE 7 |** Effects of melatonin on (A) DHAR transcript level, (B) DHAR activity and (C) ascorbic acid level in the leaves of tomato seedlings following exposure to light during chilling. Leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) seedlings at the three-leaf stage were pretreated with 100  $\mu\text{mol}$  melatonin (MT) one time a day for 3 days. At the end of light cycle at 18:00 on day 3, seedling were exposed to chilling (4°C) for 10 h in the dark, then in the light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) next morning for another 6 h. Samples were collected at 0, 5, 10, 30, and 60 min following light exposure. The values presented are mean  $\pm$  SD ( $n = 3$ ). Different letters indicate significant differences at  $P < 0.05$  among treatments.

previous study (Bogos et al., 1999). To have catalytic activity, VDE also requires the presence of ascorbic acid, which is believed to function as a cofactor (Bratt et al., 1995); we therefore ask if melatonin-mediated increase in VDE activity is associated with the regulation of ascorbic acid generation in tomato seedlings. In this study, melatonin-pretreated seedlings accumulated more ascorbic acid than non-melatonin-pretreated ones did under

chilling stress. Moreover, melatonin application significantly promoted the expression of DHAR, which is responsible for the production of ascorbic acid in plants. It was also found that melatonin enhanced DHAR activity in tomato seedlings (**Figure 7**). In fact, it has been firmly established that melatonin is in favor of ascorbic acid production in plants under various stress conditions (Li et al., 2012; Fan et al., 2015; Shi et al., 2015a). These lines of evidence support that melatonin stimulates VDE activity, at least in part, by promoting VDE expression and accumulation of VDE cofactor ascorbic acid.

Evidence presented in this study supports that melatonin promotes NPQ by acting on VDE activity. It is thus can be speculated that inhibition of VDE activity would lead to decreased de-epoxidation state of xanthophyll and reduced levels of NPQ. Thus, in order to inhibit VDE activity, tomato seedlings were fed with DTT, a well-known VDE inhibitor. VDE was inactivated by DTT in both melatonin-treated seedlings and non-melatonin-treated ones under light and chilling conditions. In addition, the de-epoxidation of xanthophyll was inhibited no matter whether or not melatonin was applied. It was also the case for NPQ as a consequence of inhibited de-epoxidation of xanthophyll (**Figure 6**). These results showed that the effects of melatonin on NPQ were eliminated by addition of DTT, further demonstrating that melatonin-mediated regulation of NPQ is achieved through the control of de-epoxidation of xanthophyll, which is ultimately regulated by melatonin-mediated changes in VDE activity.

In summary, we have found that exogenous application of melatonin alleviated photoinhibition in tomato seedlings exposed

to moderate light during chilling. The possible mechanism is that melatonin-mediated increases in VDE transcript level and ascorbic acid level contribute to higher VDE activity in tomato seedlings exposed to light during chilling, resulting in an increase in the de-epoxidation state of xanthophyll cycle and the induction of NPQ. Relieved photoinhibition is, at least in part, attributed to higher NPQ in melatonin-pretreated tomato seedlings exposed to moderate light during chilling.

## AUTHOR CONTRIBUTIONS

FD, MW, and SZ designed the study. FD, MW, and BL performed the experiments and analyzed the data. FD wrote the manuscript. MW and SZ revised the manuscript.

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# Melatonin in Plants and Plant Culture Systems: Variability, Stability and Efficient Quantification

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Despite growing evidence of the importance of melatonin and serotonin in the plant life, there is still much debate over the stability of melatonin, with extraction and analysis methods varying greatly from lab to lab with respect to time, temperature, light levels, extraction solvents, and mechanical disruption. The variability in methodology has created conflicting results that confound the comparison of studies to determine the role of melatonin in plant physiology. We here describe a fully validated method for the quantification of melatonin, serotonin and their biosynthetic precursors: tryptophan, tryptamine and N-acetylserotonin by liquid chromatography single quadrupole mass spectrometry (LC-MS) in diverse plant species and tissues. This method can be performed on a simple and inexpensive platform, and is both rapid and simple to implement. The method has excellent reproducibility and acceptable sensitivity with percent relative standard deviation (%RSD) in all matrices between 1 and 10% and recovery values of 82–113% for all analytes. Instrument detection limits were 24.4 ng/mL, 6.10 ng/mL, 1.52 ng/mL, 6.10 ng/mL, and 95.3 pg/mL, for serotonin, tryptophan, tryptamine, N-acetylserotonin and melatonin respectively. Method detection limits were 1.62 µg/g, 0.407 µg/g, 0.101 µg/g, 0.407 µg/g, and 6.17 ng/g respectively. The optimized method was then utilized to examine the issue of variable stability of melatonin in plant tissue culture systems. Media composition (Murashige and Skoog, Driver and Kuniyuki walnut or Lloyd and McCown's woody plant medium) and light (16 h photoperiod or dark) were found to have no effect on melatonin or serotonin content. A Youden trial suggested temperature as a major factor leading to degradation of melatonin. Both melatonin and serotonin appeared to be stable across the first 10 days in media, melatonin losses reached a mean minimum degradation at 28 days of approximately 90%; serotonin reached a mean minimum value of approximately 60% at 28 days. These results suggest that melatonin and serotonin show considerable stability in plant systems and these indoleamines and related compounds can be used for investigations that span over 3 weeks.

**Keywords:** degradation, matrix effects, method validation, tissue culture, liquid chromatography-mass spectrometry, serotonin, tryptophan, tryptamine

## INTRODUCTION

Melatonin (N-acetyl-5-methoxy-tryptamine) is an indoleamine neurohormone, first identified and quantified in plants in 1995 (Dubbels et al., 1995; Hattori et al., 1995). Since then there has been an ever increasing interest in the roles and effects of melatonin in plant systems and it has since been identified as playing important roles in many plant responses including growth, reproduction, development, and stress (Erland et al., 2015; Reiter et al., 2015; Hardeland, 2016). Many of the studies providing insight into these processes rely upon some form of analytical analysis to determine endogenous levels of melatonin in response to a stimulus, treatment or mutation, while treatment often requires prolonged exposure or treatment of plants in *in vitro* culture or greenhouse studies. Validated methods are an essential requirement for accurate quantification of these compounds, and provides both the reader and author confidence in the validity and reproducibility of the data (Betz et al., 2011). Though research methods are available for serotonin and melatonin in plant tissues (Cao et al., 2006; Pape and Lüning, 2006; Garcia-Parrilla et al., 2009; Jiao et al., 2016), most do not also quantify all four of the major phytomelatonin biosynthetic precursors: serotonin, tryptophan, tryptamine and N-acetylserotonin (NAS).

There is controversy in the literature over the stability of melatonin in plants, with both analytical platform, extraction, and analysis methods varying greatly from one report to another with time, temperature, light levels, extraction solvents and mechanical disruption among others all varying widely (**Table 1**). This has in turn lead to conflicting results between labs, and has contributed to difficulty in confirming and comparing the results across various labs. This is likely, in part, due to the presence of several papers detailing the stability of melatonin from mammalian research (Cavallo and Hassan, 1995; Daya et al., 2001). Another potentially confounding factor in the field of phytomelatonin analysis, is the presence of melatonin isomers in plant products. Recent studies have hypothesized that as many as forty isomers of melatonin may exist in plants, and the presence of these compounds may explain some of this inter-lab variability (Tan et al., 2012; Vigentini et al., 2015). Though oftentimes reports define these compounds as simply “melatonin isomer,” since the initial report of melatonin isomers in wine (Rodriguez-Naranjo et al., 2011), a system of nomenclature has been proposed by Tan et al. (2012), which defines the isomers by the location of the N-acetylaminoethyl and methoxy side chains, and since then several of these theorized isomers have been identified in plant and fermented plant products, though some controversy still exists on this topic (Gomez et al., 2012,

2013; Gardana et al., 2014; Yilmaz et al., 2014; Iriti and Vigentini, 2015).

Additionally, though many reports have now examined the roles serotonin and melatonin play in plants by employing *in vitro* plant tissue culture methods, the actual quantity of melatonin and serotonin which are present in the treatment medium has not been characterized. Induction of cell division, differentiation and morphogenesis in plant cultures are highly sensitive to the relative ratios of plant growth regulators (Skoog and Miller, 1957). Both melatonin and serotonin have the potential to mimic, modulate, and modify auxin and cytokinin ratios in tissues grown *in vitro* (Erland et al., 2015). Variable stability of melatonin and serotonin may lead to a significant difference in their actual content in the medium and within the growing tissues.

This study describes the development of an efficient method for determination of melatonin and its precursors and provides evidence of stability in *in vitro* culture conditions, which may facilitate investigations of regulation of plant development as influenced by interaction of plant hormones.

## MATERIALS AND METHODS

### Sample Matrices

Eight species were utilized for validation and three sample types root, shoot (including stems and leaves) and seed for a total of 12 matrices: St. John's wort (*Hypericum perforatum*; SJW) roots and shoots, banana (*Musa* sp.) roots and shoot, African violet (*Saintpaulia* sp.) shoots, potato (*Solanum tuberosum* cv “Shepady”) shoots, sweet wormwood (*Artemisia annua*; *Artemisia*) shoots and roots, tobacco (*Nicotiana tabaccum*) shoots and roots and American elm (*Ulmus americana*) shoots and fennel (*Foeniculum vulgare*) seeds. Fennel seeds were purchased from a local supermarket in Guelph, Ontario, and all other samples were taken from *in vitro* grown cultures maintained at 26°C under a 16 h photoperiod.

### Design of Method Validation

Accuracy of the method was evaluated by adding known amounts of a given analyte to a given matrix, and the amount ascertained by the method was determined by correcting for endogenous concentrations present in unspiked matrix to determine deviation from the expected value. Precision was evaluated by calculating the relative standard deviation for all measurements for a particular matrix and analyte at each concentration. No fewer than nine determinations were made on 3 different days, with no <2 days separating each set of samples. Accuracy and precision were evaluated across the entire study to ensure method robustness across different days.

Instrument and method limit of detection and limits of quantification were determined according to accepted practices (Bliesner, 2005; AOAC International, 2013), with the limit of detection set to a signal-to-noise ratio of 3:1, and the lower limit of quantitation set to a signal-to-noise of 10:1.

**Abbreviations:** DKW, Driver and Kuniyuki walnut; ECD, electrochemical detector; ELISA, enzyme linked immunosorbent assay; ESI, electrospray ionization; FLD, fluorescence detection; LLOQ, lower limit of quantification; LN, liquid nitrogen; LOD, limit of detection; M, mechanical grinding; m/z, mass to charge ratio; MS, mass spectrometry; NAS, N-acetylserotonin; RSD, relative standard deviation; RT, room temp; SIR, single ion recording; SJW, St. John's wort; SPE, solid phase extraction; TLC, thin layer chromatography; ULOQ, upper limit of quantification; UPLC-MS, ultra performance liquid chromatography; UV, ultra violet detection; WPM, Lloyd and McCown's woody plant medium.

**TABLE 1 | Summary of methods utilized for extraction of melatonin.**

Sample	Amount of melatonin	Solvent(s)	Freezing or drying	Grinding	Vortexing/ Shaking/ Vortexing	Temperature	Light	Sonication	Dry down	SPE	Total time	Analysis type	Reference
<i>Datura metel</i> (seed & flower)	<1–250 ng/g	80% methanol LN	M			Dark	45 min	Under N2	>45 min	LC-MS		Cao et al., 2006; Murch et al., 2009	
<i>Uva</i> sp.	7–18 ng/g	100% ethanol	LN	30 min, RT	RT–100°C	Dark		100°C	>85 min	TLC-UV		Tal et al., 2011	
<i>Vitis vinifera</i> cv Merlot	100–150 µg/g	Frozen 80% methanol, 1% formic acid	M	Ice	Yes	yes			<15 min	LC-MS		Murch et al., 2010	
<i>Vitis vinifera</i>	4.91–540.12 ng/g	N2 gas	M	30 s	15°C	Dim green light	10 min		>15 min	LC-MS		Gomez et al., 2013	
Tomatoe (ripe fruit)	4.1–114.5 ng/g	Methanol	Frozen	M	Yes							Stürz et al., 2011	
Strawberry (fruit)	2.1–11.26 ng/g	Acetone			25°C							Stürz et al., 2011	
Walnut, tomato, sour cherry, green coffee	7.2–341 pg/g	Ethanol	Freeze dried	M	3 min							Kocadağlı et al., 2014	
Apple, pear, cherries, bell pepper, plum, tomato, peach, nectarine (fruit)	31.2–521.4 pg/g	Methanol, ethyl acetate	Dry	60 s	15 min	Dim light						Huang and Mazza, 2011	
Glycyrrhiza	0.2–34 µg/g	80% ethanol Chloroform		15 min	4°C	Dark			>15 min	LC-UV		Afreen et al., 2006	
Barley ( <i>Hordeum vulgare</i> )	2–80 ng/g	Methanol		M		Dark			>15 h	LC-FLD		Arnao and Hernández-Ruiz, 2009	
<i>Echinaceae purpurea</i> L.	120–300 ng/g											Jones et al., 2007	
Lupin	5–80 ng/g	Chloroform											
Lupin	16.2–18.4 ng/g	Ethy/Acetate & butylated hydroxytoluene											
Rice	0.2–0.8 ng/g	Methanol		LN	Overnight	4°C							
Rice	0.5–15 ng/g	Methanol		LN									
<i>Chara australis</i>	2.9–4.5 µg/g	80% Methanol 1% formic acid	Frozen	M			Red light					Lazar et al., 2013	
Milk thistle, poppy, anise, coriander, celery, flax, green cardamom, alfalfa, fennel, sunflower, almond, fenugreek, wolf berry, black mustard, white mustard (seeds)	2–189 ng/g	Cold ethanol	Fresh	M	4°C				>10 min	LC-ECD		Manchester et al., 2000	

(Continued)

**TABLE 1 | Continued**

Sample	Amount of melatonin	Solvent(s)	Freezing or drying	Grinding or Vortexing	Temperature	Light	Sonication	Dry down	SPE	Total time	Analysis type	Reference	
Sunflower	4.6–18.7 µg/g	1 M Tris-HCl, 0.4 M perchloric acid, 0.1% EDTA, 0.05% Na <sub>2</sub> S <sub>2</sub> O <sub>5</sub> , 10M ascorbic acid	Fresh	LN	1 h	4°C		15 min	yes	>75 min	LC-UV	Mukherjee et al., 2014	
St. John's wort	33–549 nmol/g	1 M Tris-HCl; 0.4 M perchloric acid, 0.05% sodium metabisulfite, 0.1% EDTA. Methanol	M	RT	Yes					>30 min	LC-ECD-UV	Murch et al., 2000, 2001	
Tomatoe	5–200 ng/g	50 mM phosphate buffer, pH 7.4, chloroform	M	M	5 min		Dim light	20 min	N2	yes	>55 min	LC-FLD	Sun et al., 2015
Water hyacinth	2.5–20 ng/g	Methanol									>50 min	LC-MS	Tan et al., 2007
Tomatoe	11–30 ng/g	Methanol	Frozen	LN			35 min, 45°C		N2		>65 min	LC-FLD	Wang et al., 2013
<i>Nicotiana sylvestris</i>	13.2–50.4 µg/g	Methanol	Dried	M			Dim light	45 min	N2	yes	>55 min	LC-UV	Zhang et al., 2011
Sweet cherry ( <i>Prunus avium</i> L. cv Hongdeng)	10–35 ng/g	Methanol	Frozen	LN				35 min, 45°C	N2	yes	>35 min	LC-FLD	Zhao et al., 2013
<i>Arabidopsis thaliana</i>	80–120 ng/g	50% methanol	Fresh	M	15 s		Dim green	20 min, 15°C			>50 min	LC-MS	Herrández et al., 2015
Bermudagrass	50–600 pg/g	89% acetone, 10% methanol	Fresh	LN, M						yes	>15 min	ELISA	Shi et al., 2015
<i>Pyropia yezensis</i>	0.15–0.25 ng/g	Chloroform	Frozen	M	Overnight	4°C							
Tomatoe	2–39.4 ng/g	89% acetone, 10%	Frozen	LN	30 min, RT								
		methanol, 2.5 mM trichloroacetic acid											

ECD—electrochemical detection; ELISA—enzyme linked immunosorbent assay; FLD—fluorescence detection; LC—liquid chromatography; LN—grinding in liquid nitrogen; M—mechanical grinding; MS—mass spectrometry; RT—room temperature; SPE—solid phase extraction; TLC—thin layer chromatography; UV—ultra violet detection.

## Sample Preparation

For sample preparation prior to analysis samples (~150 mg) were ground in liquid nitrogen then suspended in 0.5 mL of extraction solvent which was comprised of 50% methanol (MS Grade, Fisher Scientific, Canada; MeOH) and 4% acetic acid (glacial, Fisher Scientific, Canada) in Milli-Q water. Extraction solvent was chosen after a literature review (**Table 1**), and methanol was specifically chosen as it can be directly injected onto a reverse phase chromatography system, as employed in this study, without the need for additional dry down or clean-up steps as required for strong organic solvents such as chloroform or ethyl acetate. Samples were then sonicated (3510R-DTH, Branson, USA) for 15 min on ice and spun down (2 min, 13000 rpm) and, supernatant removed. Supernatant was then filtered through a 0.45 µM centrifuge filter (Millipore; 1 min, 13 0000 rpm) and the flow through was diluted ten times in 10 mM pH 9, adjusted with ammonium hydroxide (Sigma Aldrich, Canada). Prior to analysis samples were either left unspiked or spiked with a high or low concentration of mixed standard containing either 0.5 µg/mL or 5 µg/mL melatonin, serotonin, tryptamine, tryptophan, and NAS, for a total of three sample groups. All standards were analytical grade and purchased from Sigma Aldrich, Canada.

## Detection and Quantification

For quantification of samples by liquid chromatography-mass spectrometry, 3 µL of sample was injected onto a Waters Acquity BEH Column (2.1 × 50 mm, i.d. 2.1 mm, 1.7 µm) on a Waters Acquity Classic ultra-performance liquid chromatography (UPLC) system (binary UPLC, Waters, Canada) with single quadrupole mass spectrometer (MS) detection (Waters, QDa performance model, Waters, Canada). Samples were run on a gradient with A—10 mM ammonium acetate pH 9, adjusted with ammonium hydroxide; B—100% MeOH with initial conditions of 95% A 5% B increased to 5% A 95% B over 4.5 min using an Empower curve of 8. Column temperature was 40°C and flow rate was 0.5 mL/min. Compounds were monitored in positive mode in single ion recording (SIR) mode and quantified used standard curves (see **Table 2** for MS parameters). In all cases probe temperature was 500°C with a gain of 5.

## Youden Trial for Determination of Major Factors Effecting Melatonin Stability

Samples were prepared by diluting pure analytical melatonin standard in desired solvent to a concentration of 0.01 mg/mL.

**TABLE 2 |** Mass spectrometer parameters for analysis in SIR mode.

Analyte	m/z	Ionization mode	Cone voltage
Serotonin	177	ESI+	10
Tryptophan	205	ESI+	10
Tryptamine	144	ESI+	15
N-acetylserotonin	257	ESI+	5
Melatonin	233	ESI+	15

ESI—electrospray ionization; m/z—mass to charge ratio.

Sample were then exposed to levels according to runs designated in **Table 3**, following a fractional factorial Youden design (Karageorgou and Samanidou, 2014). Factors tested were temperature (4 or 40°C), sonication (0 or 30 min), light (dim green or white), oxidation (bubble through with nitrogen gas 10 s, or not), and solvent (10 or 100% methanol; MeOH). For example, in run one, all samples would be made up in 100% MeOH, prepared under dim green light (green) at 4°C, would be bubbled through with nitrogen gas then left to sit for 30 min without sonication. Organic solvent utilized was pure analytical grade MeOH (Fisher Scientific, Canada) diluted to 10 or 100% with ultra-pure water. For sonication samples not undergoing sonication were held under controlled conditions for an equivalent amount of time without sonication. All runs were conducted using either an ice bath or a heated temperature controlled water bath. All runs were repeated in triplicate and conducted one at a time to ensure all samples underwent the same duration of extraction (approximately 40 min). Samples were diluted ten times before being injected (1 µL) for analysis on a Waters Classic Acquity ultra performance liquid chromatography (UPLC) system with electrochemical detection (Coulochem III, ESA, Dionex, ThermoFisher Scientific; ECD) equipped with an ultra-analytical coulometric flow cell (ThermoFisher Scientific, USA). Separation was performed on a Waters BEH Phenyl column (2.1 × 50 mm, 1.7 µm) using an isocratic flow of 75% 100 mM sodium acetate (Sigma Aldrich, Canada) buffer with 100 mM citric acid (Sigma Aldrich, Canada), and 25% MeOH at a rate of 0.4 mL/min with a column temperature of 35°C. Detection was performed with screening voltage of 100 mV, and detection at 850 mV, 1 µA collecting 30 points per second. Melatonin eluted at 3.5 min, limit of detection was determined to be 10 ng/mL and limit of quantification was 30 ng/mL. To calculate effect of the various factors the average percent melatonin concentration remaining in high treatments was subtracted from the average percent melatonin content remaining in the low treatment level. Melatonin standard was purchased from Sigma Aldrich, Canada.

## Media Stability

Fifty millimolars Melatonin and serotonin stock solutions were prepared in 96% ethanol (Philips Canada, Scarborough,

**TABLE 3 |** Design for Youden trial, in all cases n = 3, uppercase letter indicates high level, lowercase letters indicate low level.

	Temperature (A/a)	Sonication (B/b)	Light (C/c)	Oxidation (D/d)	Solvent (E/e)
Run 1	4°C (a)	0 min (b)	Green (c)	Nitrogen (d)	100% MeOH (e)
Run 2	40°C (A)	0 min	Green	– (D)	10% MeOH (E)
Run 3	4°C	30 min (B)	Green	–	10% MeOH
Run 4	40°C	30 min	Green	Nitrogen	100% MeOH
Run 5	4°C	0 min	White (C)	Nitrogen	100% MeOH
Run 6	40°C	0 min	White	–	10% MeOH
Run 7	4°C	30 min	White	–	10% MeOH
Run 8	40°C	30 min	White	Nitrogen	100% MeOH

Ontario), just prior to media sterilization and stored at  $-20^{\circ}\text{C}$  until ready for use. For media preparation three media salts were utilized: Murashige and Skoog, Driver and Kunyuki (DKW) and Llyod and McCown's woody plant medium (WPM) with Gamborg B5 vitamins as per the manufacturers recommended concentrations and media were further supplemented with 3% sucrose (Murashige and Skoog, 1962; Gamborg et al., 1968; McCown and Lloyd, 1981; Driver and Kunyuki, 1984). Media pH was adjusted to 5.7 using 0.1 N sodium hydroxide (Fisher Scientific, Canada) and sterilized by autoclaving for 20 min at  $121^{\circ}\text{C}$  and 21 PSI. Post-autoclaving, media was cooled by incubating in a water bath at  $55^{\circ}\text{C}$ . Melatonin and serotonin were then added to media in an aseptic fashion for a final concentration of 25  $\mu\text{M}$  each. Media were then dispensed into Magenta GA-7 boxes (Caisson Labs, Utah, USA) and further divided into light ( $\sim 40 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) and dark (0  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) treatments, each replicated thrice. All boxes were sealed with 3M Micropore tape and were stored in growth rooms maintained at  $24 \pm 2^{\circ}\text{C}$  under a 16 h photoperiod provided by cool white fluorescent lamps (Philips Canada, Scarborough, ON). 500  $\mu\text{L}$  samples were collected aseptically at 0 min (immediately after addition of melatonin or serotonin stock to medium), 5 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 3 days, 10 days, 14 days, 21 days, and 28 days, and flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

To remove media salts and sugar from samples, samples were loaded onto a Waters Oasis HLB solid phase extraction (SPE) cartridge (1 cc, 30 mg, Waters, Canada), samples were then washed with 1 mL of Milli-Q water and eluted in 0.5 mL of 100% MS grade MeOH. Samples were then diluted ten times in Milli-Q water and 5  $\mu\text{L}$  was injected and analyzed following the validated UPLC-MS protocol as described above.

## Data Analysis

All data were plotted and analyzed in Microsoft Excel 360 (Microsoft, USA) for experiments performed on the UPLC-ECD system, while all data from UPLC-MS experiments were plotted and analyzed in GraphPad Prism 6. For media analysis treatment groups were compared using a paired two-way ANOVA, with  $\alpha = 0.05$ . The Youden trial was designed and analyzed as per the literature, with only five factors included (Karageorgou and Samanidou, 2014). All samples were repeated in triplicate, and all experiments were replicated twice, and data was combined.

## RESULTS

The method presented in this paper showed good specificity for all compounds due to the use of a single quadrupole system in SIR mode (Figures 1, 2), with all peaks being completely resolved from surrounding peaks and showing good signal to noise ( $>3:1$ ) in the linear range. Endogenous concentrations in all matrices are shown in Table 4.

Instrument limits of detection were 24.4 ng/mL, 6.1 ng/mL, 1.52 ng/mL, 6.1 ng/mL, and 92.5 pg/mL for serotonin, tryptophan, tryptamine, NAS and melatonin, respectively. Method detection limits were found to be 1.62  $\mu\text{g/g}$ , 0.407  $\mu\text{g/g}$ ,

0.101  $\mu\text{g/g}$ , 0.407  $\mu\text{g/g}$ , and 6.17 ng/g respectively. The linear range (lower limit of quantification; LLOQ–upper limit of quantification; ULOQ) for each analyte was 97.7 ng/mL–25  $\mu\text{g/mL}$ , 24.4 ng/mL–25  $\mu\text{g/mL}$ , 6.1 ng/mL–6.25  $\mu\text{g/mL}$ , 24.4 ng/mL–25  $\mu\text{g/mL}$  and 38.1 pg/mL–6.25  $\mu\text{g/mL}$  for serotonin, tryptophan, tryptamine, NAS, and melatonin respectively, showing a linear range of more than 4 orders of magnitude (Table 5).

Excellent reproducibility, presented as percent relative standard deviations (% RSD), was demonstrated for all five analytes in all of the eight matrices ranging from 4–8 and 1–9% in low and high spikes respectively for serotonin; 2–4 and 4–5% for tryptophan; 2–7 and 1–5% for tryptamine; 1–4% for both low and high spikes in N-acetylserotonin and 7–8 and 6–10% for melatonin (Table 6).

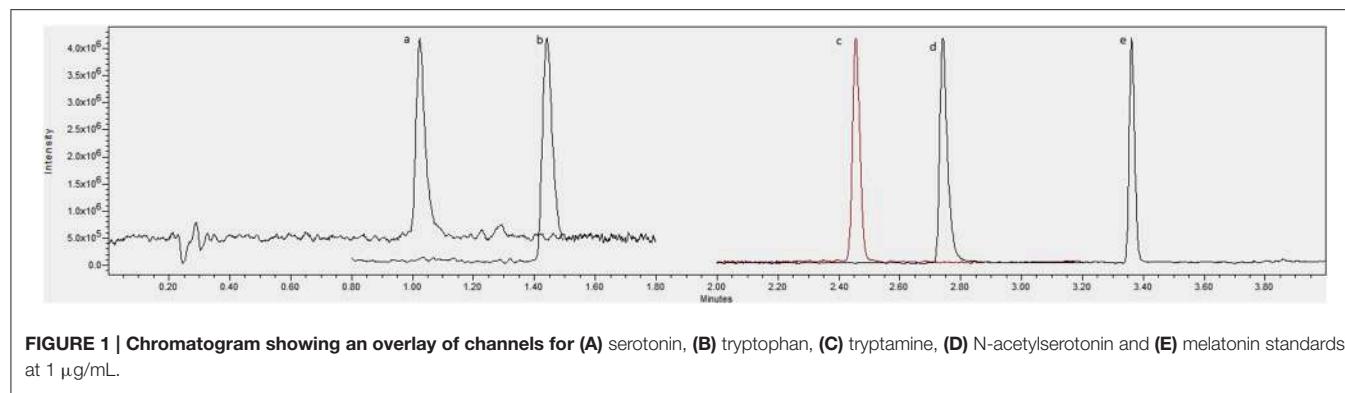
Recovery was also acceptable for all matrices and analytes with low concentration spike recoveries ranging from 85% in banana root to 110% in potato shoot for serotonin; 93% in SJW shoot to 101% in tobacco shoot for tryptophan; 90% in SJW root to 110% in *Artemisia* root for tryptamine; 93% in *Artemisia* shoot to 110% in fennel seed for NAS and 94% in fennel seed to 113% in *Artemisia* root for melatonin. At high concentration recoveries were similar with values of 97–113% for serotonin (SJW root, and banana root); 94–101% for tryptophan (SJW and *Artemisia* root); 82–104% for tryptamine (SJW and *Artemisia* root); 90–101% for NAS (fennel seed and root); and 92–105% for melatonin (SJW shoot and *Artemisia* root) (Table 6).

Youden trials are factorial designs which are generally utilized to test the robustness of a method, and determine the level of variability which can exist in a particular method before the results are effected. As such two extreme values for likely conditions which a sample may be subjected to: a high and a low value are utilized and effects can then be measured (Karageorgou and Samanidou, 2014). In this case the Youden trial was run to investigate the stability of melatonin during the extraction protocol found that light, oxygen exposure (oxidation) and solvent concentration were not significant factors contributing to melatonin degradation. Temperature had a significant impact on melatonin concentration remaining in samples, while sonication had a nominal effect (Figure 3).

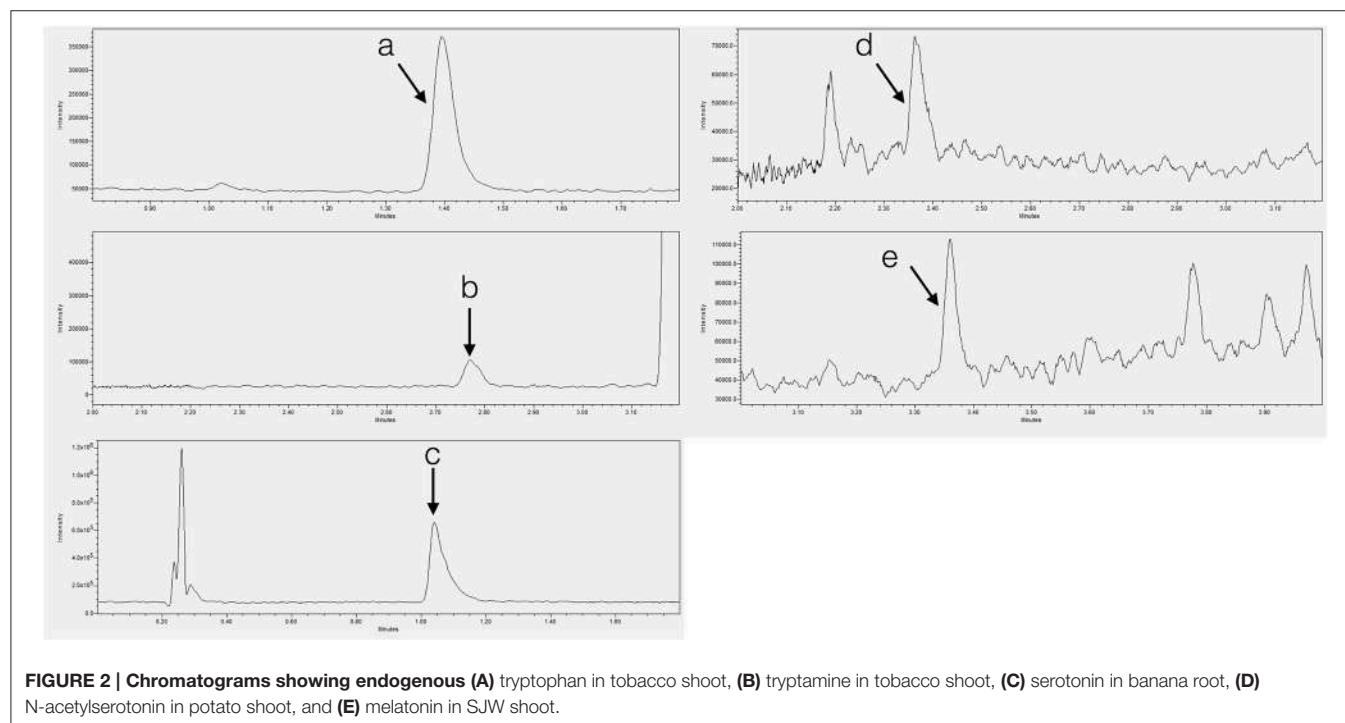
Investigation of the stability of melatonin and serotonin found that compounds remained relatively stable across the first 10 days in media with values declining up to a 10% loss for melatonin at day 28 and losses of up to 40% at 28 d for serotonin. There was no significant difference in the trends observed for any of the three media types: WPM, DKW, and Murashige and Skoog and no difference between culture boxes stored in the light or in complete darkness of the 28 d period (Figure 4).

## DISCUSSION

Melatonin is increasingly being recognized as an important regulator of plant growth, development and adaptation (Erland et al., 2015). As such there is a rapidly growing body of knowledge examining the roles melatonin plays in plants, many of which employ controlled environment systems and in particular *in vitro* culture systems, many of which are helping to solidify the



**FIGURE 1 | Chromatogram showing an overlay of channels for (A) serotonin, (B) tryptophan, (C) tryptamine, (D) N-acetylserotonin and (E) melatonin standards at 1  $\mu\text{g/mL}$ .**



**FIGURE 2 | Chromatograms showing endogenous (A) tryptophan in tobacco shoot, (B) tryptamine in tobacco shoot, (C) serotonin in banana root, (D) N-acetylserotonin in potato shoot, and (E) melatonin in SJW shoot.**

role of melatonin in plant processes. As both the receptors and mechanisms underlying the functions of melatonin in plants while an active area of research are still poorly understood, *in vitro* systems offer a valuable platform for their investigation (Lee and Back, 2016; Sanchez-Barcelo et al., 2016; Shi et al., 2016; Wei et al., 2016). One such important strategy, which has helped to confirm for example the importance of calcium signaling in melatonin responses in several species, is the inclusion of inhibitors in plant medium (Murch et al., 2001; Jones et al., 2007; Ramakrishna et al., 2009, 2011). The *in vitro* culture system also offers a unique opportunity compared to greenhouse and field trials, in that treatment conditions can be closely monitored and allow for treatment of many plants, and all cultures are maintained in aseptic conditions. This of particular value with respect to the indoleamines, due to their ubiquitous production across kingdoms, therefore any microbial contamination could confound important results.

As the interest in melatonin continues to rise, many labs require not only practical and effective platforms via which to study the physiological effects of melatonin but also assays by which to determine the actual quantities of melatonin in a particular sample. Though melatonin has now been examined in many systems, including in culture, there still remains inconsistency in the literature as to the quantities of melatonin in individual plants and the methods by which to extract it. This is well illustrated in **Table 1** which summarizes some of the many extraction protocols which have been utilized. Times to complete extraction range from <15 min to over 16 h, and in most cases specific times to complete the entire extraction process are not specifically mentioned, with the reader left to presume extraction times based on the time for individual steps such as shaking, sonication or drying. The relevance of this inconsistency in extraction conditions, is also reflected in several reports on the stability of melatonin. Though several reports

**TABLE 4 | Concentrations of tryptophan (Trp), tryptamine (Trm), serotonin (Ser), N-acetylserotonin (NAS) and melatonin (Mel) in tissues studied in this validation.**

Species	Tissue	Mean concentration in tissue (standard error)				
		Trp ( $\mu\text{g/g FW}$ )	Trm ( $\mu\text{g/g FW}$ )	Ser ( $\mu\text{g/g FW}$ )	NAS ( $\text{ng/g FW}$ )	Mel ( $\text{ng/g FW}$ )
SJW	Shoot	164.8 (25.4)	1.51 (0.31)	nd	nd	32.5 (1.70)
	Root	15.2 (2.8)	0.599 (0.057)	nd	nd	9.72 (1.12)
African violet	Shoot	58.9 (18.2)	0.508 (0.085)	nd	nd	nd
Banana	Shoot	91.2 (12.3)	0.627 (0.063)	7.17 (1.68)	nd	16.81 (2.2)
	Root	20.2 (5.0)	0.486 (0.008)	34.17 (6.36)	nd	nd
Elm	Shoot	16.7 (1.7)	nd	nd	nd	nd
Tobacco	Shoot	16.3 (2.2)	0.721 (0.069)	nd	nd	nd
	Root	4.5 (1.9)	nd	nd	77.1 (9.9)	nd
Potato	Shoot	39.1 (5.3)	0.719 (0.17)	nd	330 (86.4)	40.05 (1.85)
Artemisia	Shoot	29.9 (5.9)	nd	nd	nd	nd
	Root	15.1 (1.1)	nd	nd	nd	nd
Fennel	Seed	24.43 (2.73)	0.0733 (0.0126)	nd	nd	33.30 (13.0)

**TABLE 5 | Summary of retention time, and instrument and method limits of detection and quantification for all analytes investigated.**

Analyte	Retention time (min)	Instrument LOD ( $\text{ng/mL}$ )	Method LOD ( $\mu\text{g/g}$ )	Instrument LLOQ ( $\text{ng/mL}$ )	Method LLOQ ( $\mu\text{g/g}$ )	Instrument ULOQ ( $\mu\text{g/mL}$ )	Method ULOQ ( $\text{mg/g}$ )
Serotonin	1.057	24.4	1.62	97.7	6.51	25	1.67
Tryptophan	1.045	6.10	0.407	24.4	1.62	25	1.67
Tryptamine	2.752	1.52	0.101	6.1	0.407	6.25	0.42
N-acetylserotonin	2.480	6.10	0.407	24.4	1.62	25	1.67
Melatonin	3.369	0.093	0.00617	0.38	0.0254	6.25	0.42

LOD—limit of detection, LLOQ—lower limit of quantification, RT—retention time, ULOQ—upper limit of quantification.

have found melatonin to be stable in aqueous solution over long periods of time regardless of pH (e.g., 5–12) and storage temperatures (e.g., 4 vs.  $-70^{\circ}\text{C}$ ), still others have suggested that melatonin may undergo degradation under particular conditions such as light exposure (Cavallo and Hassan, 1995; Daya et al., 2001; Maharaj and Dukie, 2002). The majority of these studies have, as noted, been performed in aqueous solution and with a view toward mammalian systems. As plant systems have highly complex phytochemical environments, this level of complexity may suggest that further investigation is required specifically addressing the unique challenges presented by the plant system. Though the issue of the presence of melatonin isomers in plants has been raised by several reports (Tan et al., 2012), and represents an important field of study, the total melatonin content, or the sum of all melatonin isomers present in a sample, is the most commonly reported value across studies, and represents sufficient information for many basic plant physiological studies. Additionally, by considering only the total content, this allows for many more labs, beyond those with the specialized equipment and analytical background required for isomer determination, to investigate melatonin content in plants.

In view of these inconsistencies, this study presents two noteworthy achievements. First it presents a simple, accessible and easy to implement protocol for the analysis of not just melatonin or serotonin in plant tissues, but also their three

precursors: tryptophan, tryptamine, and NAS, and secondly it presents an in-depth investigation of the levels of melatonin and serotonin which persist in plant tissue culture medium under variable lighting conditions and with varying media composition.

Despite previously published literature suggesting that light is a major factor leading to the degradation of melatonin, this study found that heat and to a lesser extent sonication had the greatest effect on the stability of melatonin, as sonication produces heat, even when sonication is performed in an ice bath, these two factors are interrelated emphasizing the impact of temperature on the stability of melatonin (Figure 3) (Maharaj and Dukie, 2002).

The method provided in this report is robust, reproducible and relatively simple, eliminating many factors which provide opportunities for loss of analyte, which is of particular importance, given concerns about the stability of melatonin in previous reports. Additionally, as interest in the mechanism behind melatonin's action in plants continues to grow, there is a need for easy to implement methods which provide reproducible results for not just melatonin, but its biosynthetic precursors as well. Accurate quantification of these compounds will allow for more in-depth studies into this important phytochemical signaling molecule.

This study has employed this method to address a significant question in melatonin investigations in plants. There are now

**TABLE 6 | Recovery data for serotonin, tryptophan, tryptamine, N-acetylserotonin and melatonin.**

Species	Tissue	Low	Low	High	High
		Recovery <sup>a</sup> Average	Recovery <sup>a</sup> %RSD	Recovery <sup>b</sup> Average	Recovery <sup>b</sup> %RSD
<b>SEROTONIN</b>					
SJW	Shoot	103%	5%	100%	1%
SJW	Root	101%	6%	97%	2%
African Violet	Shoot	101%	5%	100%	3%
Banana	Shoot	102%	7%	111%	8%
Banana	Root	85%	8%	113%	9%
Elm	Shoot	100%	7%	102%	5%
Tobacco	Shoot	100%	7%	98%	1%
Tobacco	Root	100%	5%	101%	1%
Potato	Shoot	110%	5%	116%	2%
Artemisia	Shoot	104%	5%	107%	1%
Artemisia	Root	107%	4%	106%	1%
Fennel	Seed	101%	1%	101%	1%
<b>TRYPTOPHAN</b>					
SJW	Shoot	93%	3%	98%	5%
SJW	Root	94%	3%	94%	5%
African Violet	Shoot	93%	4%	96%	5%
Banana	Shoot	90%	4%	97%	5%
Banana	Root	95%	4%	96%	5%
Elm	Shoot	94%	2%	96%	5%
Tobacco	Shoot	101%	5%	97%	5%
Tobacco	Root	96%	3%	95%	5%
Potato	Shoot	97%	2%	100%	4%
Artemisia	Shoot	96%	2%	98%	4%
Artemisia	Root	99%	2%	101%	4%
Fennel	Seed	99%	2%	102%	2%
<b>TRYPTAMINE</b>					
SJW	Shoot	99%	2%	101%	2%
SJW	Root	90%	3%	98%	1%
African Violet	Shoot	92%	2%	96%	1%
Banana	Shoot	94%	2%	94%	2%
Banana	Root	106%	7%	100%	2%
Elm	Shoot	92%	3%	103%	2%
Tobacco	Shoot	98%	3%	104%	2%
Tobacco	Root	98%	5%	104%	2%
Potato	Shoot	106%	2%	105%	2%
Artemisia	Shoot	86%	7%	82%	5%
Artemisia	Root	110%	7%	104%	2%
Fennel	Seed	110%	3%	99%	2%
<b>N-ACETYL SEROTONIN</b>					
SJW	Shoot	97%	4%	96%	3%
SJW	Root	97%	1%	99%	2%
African Violet	Shoot	100%	1%	95%	1%
Banana	Shoot	104%	2%	98%	1%
Banana	Root	104%	2%	99%	2%
Elm	Shoot	99%	1%	100%	2%
Tobacco	Shoot	99%	2%	97%	3%
Tobacco	Root	102%	2%	99%	2%

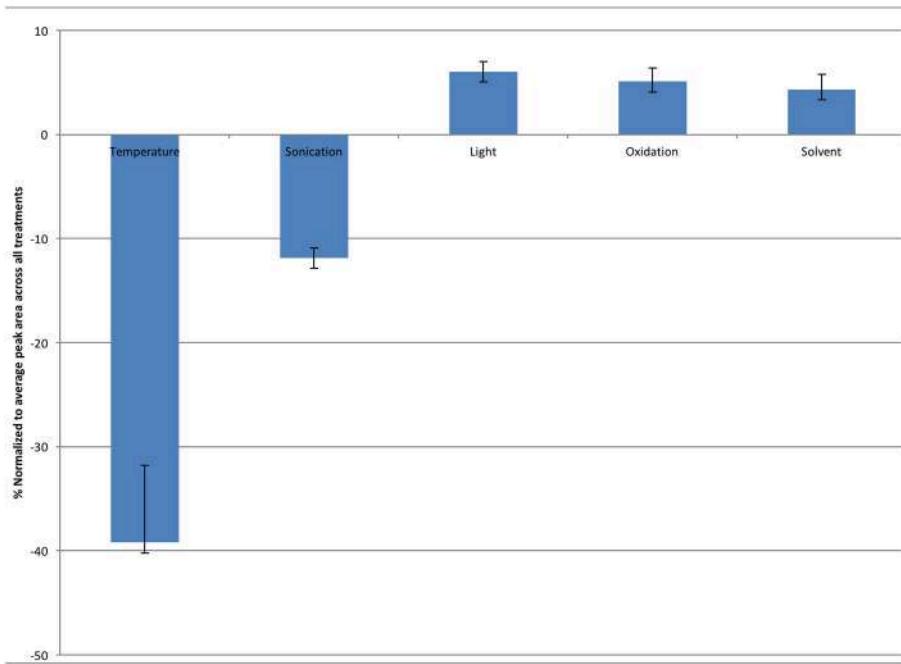
(Continued)

**TABLE 6 | Continued**

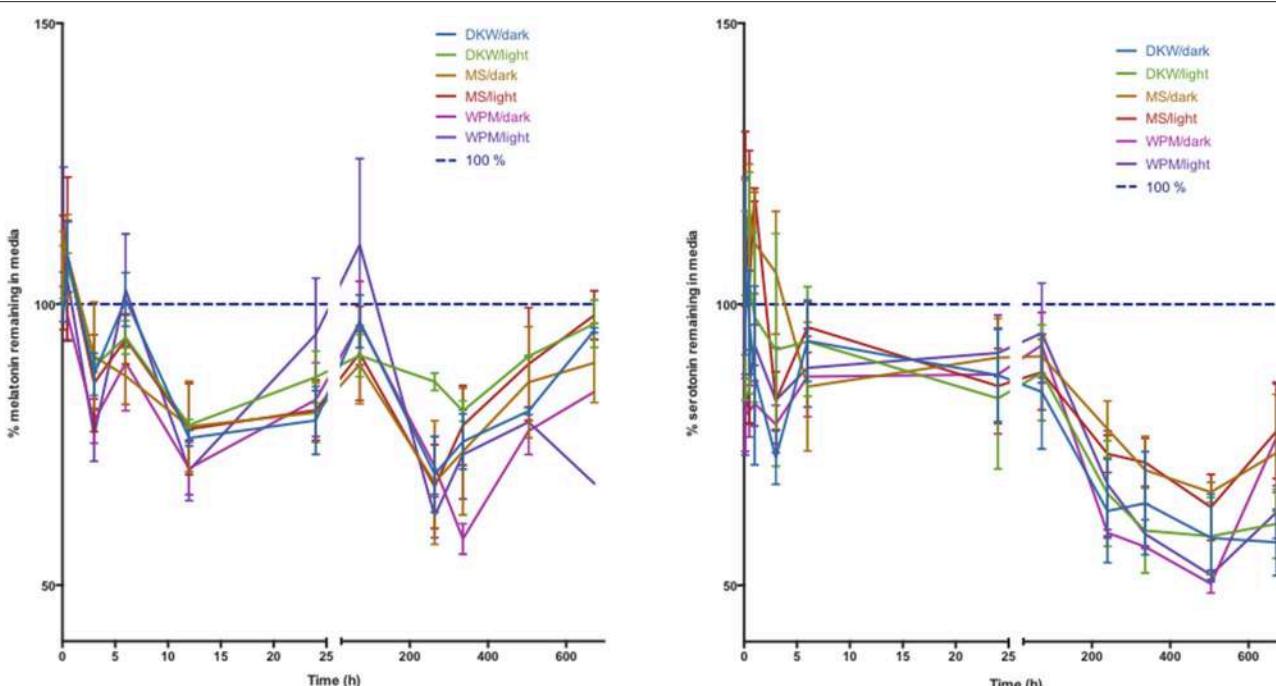
Species	Tissue	Low	Low	High	High
		Recovery <sup>a</sup> Average	%RSD	Recovery <sup>b</sup> Average	%RSD
<b>MELATONIN</b>					
Potato	Shoot	98%	1%	97%	2%
Artemisia	Shoot	93%	2%	94%	1%
Artemisia	Root	95%	3%	101%	4%
Fennel	Seed	110%	3%	90%	2%

<sup>a</sup>low spike concentration was 0.5 µg/mL.<sup>b</sup>high spike concentration was 5 µg/mL.

many reports on the role melatonin plays in plant development, reproduction and survival of biotic and abiotic stresses (Erland et al., 2015; Reiter et al., 2015; Hardeland, 2016). Many investigations employ treatment of plants with melatonin in a liquid dose, or in systems such as plant tissue culture in liquid or solid medium. In particular, *in vitro* culture experiments involve the storage of plants under light conditions and relatively warm temperatures for extended periods of time with data collection often happening after days or weeks. The actual levels of melatonin or serotonin in this medium, however, have never been determined. Due to previous reports of the instability of melatonin under light and a negative effect of temperature as reported in this manuscript, a study was undertaken to investigate the actual exposure concentrations for melatonin in *in vitro* culture systems. This is particularly salient as significant or immediate decreases in indoleamine content could lead to much lower exposure concentrations than are reported and present an artificially inflated active concentration. Surprisingly, both serotonin and melatonin were found to be stable in three common media types tested for 10 days of culture. This time-frame is important as developmental patterns are often established early-on in *in vitro* cultured plants and tissues. Many experiments, however, use 21 and 28 days as convenient points for data collection as plant organs are generally sufficiently developed by this time to allow for accurate measurement. It was therefore important to determine if the concentrations in medium are sustained over the entire experiment. Though variability is present in media data, possibly due to box to box differential degradation across culture vessels, and variability introduced during sample collection, these fluctuations do not significantly change the observed trend. Serotonin showed up to



**FIGURE 3 | Effect of varying extraction conditions on the stability of melatonin in solution, expressed as the difference between amount remaining at the high and low levels of factors.** Error bars represent standard error,  $n = 3$ .



**FIGURE 4 | Stability of melatonin (left) and serotonin (right) in three types of plant tissue culture medium.** DKW—Driver Kuniyuki walnut; MS—Murashige and Skoog; WPM—woody plant medium. Dark indicates 24 h darkness, light indicates a 16 h photoperiod. Initial media concentration of melatonin and serotonin was 25  $\mu$ M.

a 40% loss in concentration after 28 days, surprisingly, melatonin only showed a 10% decrease in concentration.

Many plant growth experiments use different growth medium compositions varying sources of nitrogen, iron, and other important nutrients to support robust and normal plant growth. It was hypothesized that these different macro- and micronutrient compositions may have an effect on stability, if losses were due to a chemical interaction with media constituents (Murashige and Skoog, 1962; McCown and Lloyd, 1981; Driver and Kuniyuki, 1984). The results shown in **Figure 2**, however, show that there was not any difference in the degradation trend between three commonly used media types. Furthermore, it was theorized that light exposure would have a significant impact on media concentrations of melatonin and serotonin. Again, however, it was found that light levels (16 h photoperiod vs. complete dark) had no significant effect on concentrations, suggesting that photooxidation or photo-degradation is not a significant factor in the design of experiments. This is particularly important as many reports have utilized diverse culture vessels ranging from clear glass test tubes to black plastic pots and employ different lighting conditions ranging from ambient light in a greenhouse to strictly controlled light spectra (Erland et al., 2015). These results eliminate vessel color, wavelength or light conditions as an important variable in these experiments and provide further confidence moving forwards.

The data presented in this study are relevant as they address an important but previously un-investigated variable in the design of plant physiology experiments in plant culture systems. Additionally, they validate many previous studies which have been published investigating the important roles of melatonin across diverse plant species and in response to changing conditions (Erland et al., 2015; Reiter et al., 2015; Hardeland, 2016).

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- In summary, a validated method which allows for accurate, sensitive, and reproducible quantification of melatonin, and its biosynthetic precursors: serotonin, tryptophan, NAS, and tryptamine, was determined. This method was found to be robust in the analysis of these compounds across diverse plant species and tissue types. Additionally, measurement of melatonin and serotonin via this method in plant tissue culture medium found that neither light nor media composition had an effect on stability of melatonin or serotonin in these systems. Both melatonin and serotonin were found to be stable in medium across 10 days and losses after 28 days only reached 10 and 40% of the initial concentration respectively. These results pave the way for future in depth experiments examining the roles of melatonin and its precursors in both basic scientific investigations of plant physiological processes, and industrial applications such as micropagation and cryo-banking.

## AUTHOR CONTRIBUTIONS

All authors (LE, AC, AJ, and PS) participated in experimental design, manuscript preparation and agree to be accountable for all aspects of the work and provided final approval of the version to be published; LE conducted experiments and performed data analysis; AC assisted in conducting experiments; PS was responsible for study conception; LE, PS, and AJ participated in interpretation of data.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Alleviation of cold damage to photosystem II and metabolisms by melatonin in Bermudagrass

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As a typical warm-season grass, Bermudagrass [*Cynodon dactylon* (L).Pers.] is widely applied in turf systems and animal husbandry. However, cold temperature is a key factor limiting resource utilization for Bermudagrass. Therefore, it is relevant to study the mechanisms by which Bermudagrass responds to cold. Melatonin is a crucial animal and plant hormone that is responsible for plant abiotic stress responses. The objective of this study was to investigate the role of melatonin in cold stress response of Bermudagrass. Wild Bermudagrass pre-treated with 100 µM melatonin was subjected to different cold stress treatments ( $-5^{\circ}\text{C}$  for 8 h with or without cold acclimation). The results showed lower malondialdehyde (MDA) and electrolyte leakage (EL) values, higher levels of chlorophyll, and greater superoxide dismutase and peroxidase activities after melatonin treatment than those in non-melatonin treatment under cold stress. Analysis of chlorophyll *a* revealed that the chlorophyll fluorescence transient (OJIP) curves were higher after treatment with melatonin than that of non-melatonin treated plants under cold stress. The values of photosynthetic fluorescence parameters increased after treatment with melatonin under cold stress. The analysis of metabolism showed alterations in 46 metabolites in cold-stressed plants after melatonin treatment. Among the measured metabolites, five sugars (arabinose, mannose, glucopyranose, maltose, and turanose) and one organic acid (propanoic acid) were significantly increased. However, valine and threonic acid contents were reduced in melatonin-treated plants. In summary, melatonin maintained cell membrane stability, increased antioxidant enzymes activities, improved the process of photosystem II, and induced alterations in Bermudagrass metabolism under cold stress.

**Keywords:** melatonin, bermudagrass, cold stress, photosystem II, metabolism

## INTRODUCTION

Bermudagrass [*Cynodon dactylon* (L).Pers.] is widely cultivated in sports fields, lawns and golf courses and used in animal husbandry. As a typical warm-season grass, the optimal temperature for growth ranges from  $26.7$  to  $35^{\circ}\text{C}$ . When the temperature is below  $15^{\circ}\text{C}$ , the plants stop growing. Hence, the utilization of Bermudagrass is limited by low temperature and the shoots wither in late autumn and winter. Thus, cold is considered as a key factor limiting widespread use in Bermudagrass.

Cold stress can induce membrane damage to plants. Malonaldehyde (MDA) content and relative electrolyte leakage (EL) values were significantly increased after low temperature treatment (Zhang et al., 2006; Hou et al., 2010). Cold induces excessive production or inefficient deactivation of reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^-$ ), and superoxide anion ( $O_2^-$ ), thereby causing injury to plants (Monk et al., 1989). For self-protection against oxidative stress, plants have evolved efficient antioxidant systems to scavenge ROS (Allen, 1995). The activities of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) provide efficient protective mechanisms against oxidative stress (Baek and Skinner, 2003). The activities of these enzymes increased dramatically under cold stress (Hou et al., 2010; Ao et al., 2013).

Photosynthesis is a crucial plant metabolism process, which is extremely sensitive to cold stress. This is because low temperature disrupts almost all major components of photosynthesis (Allen and Ort, 2001; Dahal et al., 2012). In maize (*Zea mays* L.) and oats (*Avena sativa* L.), the efficiency of excitation capture by PSII reaction centers and the quantum yield of electron transport were higher in tolerant genotypes than that in sensitive varieties (Fracheboud et al., 1999; Rizza et al., 2001). The performance index and the chill factor index were higher in the tolerant genotypes of soybean [*Glycine max* (L.) Merr.] under chilling stress (Strauss et al., 2006).

Cold stress causes dramatic alterations in plant metabolism. Under cold stress, enzyme activities and reaction rates are generally reduced and the metabolome activity was reconfigured (Zhu et al., 2007). Metabolites such as sucrose, fructan, and proline were demonstrated to play protective roles in plants (Chen and Murata, 2002; Stitt and Hurry, 2002). Large-scale profiling of metabolites by gas chromatography-mass spectrometry (GC-MS) has revealed extensive alterations in the plant metabolome in response to low temperature (Cook et al., 2004). The active reconfiguration of the metabolome depends on the changes of cold-responsive gene expression, which is regulated by cold signaling. Soluble sugars tetrapyrrole intermediate Mg-protoporphyrin (Mg-ProtoIX), and ROS are three metabolic signals that might be crucial for cold signaling (Zhu et al., 2007).

Melatonin is a highly conserved molecule which functions as a hormone, protective antioxidant, and a mediator of circadian rhythms in both plants and animals (Murch and Saxena, 2002; Pelagio-Flores et al., 2012; Reiter et al., 2014). Dubbels et al. (1995) firstly detected melatonin in edible plants (Dubbels et al., 1995). Botanical studies of this hormone began with the discovery of abundant melatonin in the medicinal herbs, feverfew (*Tanacetum parthenium*) and St. John's wort (*Hypericum perforatum*) (Murch et al., 1997). More than 100 examined plant species contain melatonin (Chen et al., 2009).

Melatonin has a variety of functions in plants (Chen et al., 2009; Reiter et al., 2015). Melatonin behaves as an auxin which was involved in regulating root development in St. John's wort and hypocotyls growth in lupin (*Lupinus albus* L.) (Murch et al., 2001; Hernández-Ruiz et al., 2004). Consistent with animals, melatonin concentrations change over a 24 h period, but the

highest melatonin values may occur in the day (Tan et al., 2007) or at night (Kolář et al., 1997). As a free radical scavenger, melatonin protects plants from oxidative stress in all species tested (Manchester et al., 2000; Tal et al., 2011; Arnau and Ruiz-Hernandez, 2015; Reiter et al., 2015). Melatonin was also reported to modulate leaf senescence in *Arabidopsis* (Shi et al., 2015c) and it protects plants against abiotic stresses such as salinity, drought, heat and cold (Li et al., 2012; Bajwa et al., 2014; Meng et al., 2014; Shi et al., 2015d) and biotic stress (Lee et al., 2015; Zhao et al., 2015; Shi et al., 2015a).

Although, remarkable progress has been made in investigating melatonin involvement in abiotic stress response in recent years, studies on the effect of melatonin in Bermudagrass against cold stress have been rarely investigated. Recently, proteome and transcriptome analysis for Bermudagrass after melatonin treatment under salinity, drought, cold and  $H_2O_2$  stress revealed that melatonin has protective roles in Bermudagrass response to abiotic stress (Shi et al., 2015b,e). In the present study, we employed physiological, photosynthetic, and metabolic methods to elucidate the possible mechanism of melatonin involved in the Bermudagrass response to cold stress. Our results revealed that melatonin contributes positively toward cold resistance of Bermudagrass by maintaining stability of cell membrane, and by modulating processes of photosynthesis and metabolism.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Bermudagrass [*Cynodon dactylon* (L.) Pers.] used in this study was collected from wild field of Baise City, Guangxi Province, China ( $N24^{\circ}51.397$ ,  $E 106^{\circ}33.288$ ). To prepare the plant materials, stolons of Bermudagrass were planted in the plastic pots (10 cm tall and 8 cm in diameter) that were filled with matrix (brown coal soil: silver sand = 1:1). Drainage holes were drilled at the bottom of the pots to avoid excessive water accumulation, and to ensure soil aeration. The pots that were planted with stolons were kept in a greenhouse with 12 h photoperiod, and day/night temperature was  $30/25^{\circ}\text{C}$  for around 1 month to establish the Bermudagrass plant. During Bermudagrass establishment, plants were watered with full-strength Hoagland nutrient solution well (Hoagland and Arnon, 1950) every other day, until the liquid drained freely from drain holes.

### Treatments

The established grass was transferred into the growth chamber with 12 h photoperiod and  $30/25^{\circ}\text{C}$  (day/night) temperature. Bermudagrass plants were subjected to six regimes: normal temperature (NT), cold acclimation (CA), non-cold acclimation (NA), normal temperature plus melatonin (NT+MLT), cold acclimation plus melatonin (CA+MLT), and non-cold acclimation plus melatonin (NA+MLT). For the control, plants were irrigated with pure water and maintained in the temperature of  $30/25^{\circ}\text{C}$  (day/night) until the experiment ended. For melatonin treatment, plants were pretreated with  $100 \mu\text{M}$  melatonin solution for 7 d. After pre-treatment, the

plants were subjected to cold stress. For cold stress treatment, cold acclimation (CA), and non-cold acclimation (NA) were designed. For CA treatment, Bermudagrass plants were treated with 4°C for 7 d, and then transferred to -5°C for 8 h. For NA treatment, the Bermudagrass were treated with -5°C for 8 h without pre-treatment with 4°C. The plants that were treated with freezing stress were recovered at 4°C overnight and then transferred to 30°C for 1 d. Appropriate temperature for Bermudagrass growth (30°C) was used for control. Five pots with around 50 plants each were applied for each treatment.

### Crude Enzyme Extraction

0.2 g of fresh leaves were ground into fine powder with liquid nitrogen. 4 mL of 150 mM, pH 7.0 sodium phosphate buffer (pre-cooled at 4°C) was added into the powder. Then the homogenate was transferred into 10 mL centrifuge tube, and centrifuged with 13400 g at 4°C for 20 min. The supernatant was the crude enzyme solution that to be determined.

### Determination of Malonaldehyde (MDA) Content

MDA content was determined by thiobarbituric acid (TBA) method according to previous study (Hu et al., 2012; Fan et al., 2014). A 1 mL of crude enzyme solution was added into 2 mL MDA reaction buffer that included 0.5% (v/v) thiobarbituric acid (TBA) and 20% (v/v) trichloroacetic acid. The reaction solution was heated at 95°C for 30 min in a water bath, then cooled to room temperature (25°C) and centrifuged at 12000 rpm at 25°C for 10 min. The supernatant was determined for absorbance at 532 nm and 600 nm with a spectrophotometer. MDA content was calculated with following formula:

$$\text{MDA (mol g}^{-1}\text{FW}) = [(\text{OD}532 - \text{OD}600) \times L]/(1 \times \epsilon \times \text{FW}).$$

Where L indicates the volume of the extract solution, l indicates thickness of the cuvettes,  $\epsilon$  represents the molar absorption coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>, and FW is the fresh weight of the leaf.

### Quantification of Electrolyte Leakage (EL)

To quantify relative EL, 0.1 g of fully expanded leaves were collected from the plants and washed three times with deionized water. The leaves were cut into 0.5 cm fragments and transferred into 50 mL centrifuge tube filled with 15 mL deionized water. The tube-fragments systems were shaken for 24 h at room temperature and the initial conductivity ( $EL_1$ ) was measured with a conductance meter (JENCO). Then, the leaf tissue in the tube was autoclaved at 121°C for 10 min to release the electrolytes completely. The final conductivity ( $EL_2$ ) was measured after cooling the solution at room temperature. The relative EL was calculated by the formula:

$$\text{Relative EL} = EL_1/EL_2 \times 100\%.$$

### Quantification of Melatonin

Quantification of plant melatonin was performed with enzyme-linked immunosorbent assay (ELISA) method. Briefly, 0.3 g of

leaf tissues was ground into fine powder in liquid nitrogen. Then the powder was transferred to the tube containing 5 ml of extraction solution (acetone:methanol:water = 89:10:1) and homogenized on ice for 1 h. After that the homogenate was centrifuged at 4°C for 5 min at 4500 g. The supernatant was transferred to a new tube and mixed with 0.5 ml of 1% trichloric acid. Then the mixture was centrifuged at 4°C for 10 min at 4500 g, the extract was used to determine the melatonin content with Melatonin ELISA Kit (EK-DSM; Buhlmann LaboratoriesAG, Schonenbuch, Switzerland) according to the manufacturer instruction.

### Determination of Antioxidants

To determine SOD activity, 1 mL of crude enzyme solution was mixed into 3 mL solution which include 2.2 mL sodium phosphate buffer (50 mM, pH 7.8), 0.039 mM methionine, 0.3 nM ethylene diaminetetraacetic acid (EDTA), 0.012 μM riboflavin, and 0.225 μM nitro blue tetrazolium (NBT). 3 mL reaction mixture with no crude enzyme solution was set as control. For chromogenic reaction, the mixture was illuminated under 4000 lx fluorescent lamp for 60 min. The absorbance at 560 nm was measured with a spectrophotometer. One unit of SOD activity was defined as amount of SOD required to inhibit NBT reduction by 50%.

To determine POD activity, 50 μL of crude enzyme solution was added into 2.95 mL reaction solution which include 1.85 mL, sodium acetate-acetic acid buffer (pH 5.0), 0.25 mL guaiacol (dissolved in 50% ethanol solution), and 0.075 mL H<sub>2</sub>O<sub>2</sub>. Absorbance increase per minute at 460 nm was recorded for 3 min. Increment of 1 unit of the absorbance per minute was defined as one unit POD activity.

### Quantifications of Chlorophyll Content

Leaf chlorophyll content was determined by the method that described by Hiscox and Israelstam (1979) with slight modification. In detail, 0.1 g of leaf samples was submerged into 10 mL dimethylsulfoxide that was contained in 15 mL centrifuge tubes. Then the tubes which contained the leaves were kept in the dark for 48 h. Absorbance at 645 nm and 663 nm of the extract solution were measured with a spectrophotometer. Chlorophyll content was calculated with the following formula:

$$\text{Chl - content(mg} \cdot \text{L}^{-1}\text{)} = 20.2 \times \text{OD}645 + 8.02 \times \text{OD}663.$$

OD645 and OD663 indicate the absorbance of the extract solution at 645 nm and 663 nm, respectively.

### Measurement of Chlorophyll a Fluorescence (OJIP) Kinetics

Chlorophyll fluorescence was determined with a pulse-amplitude modulation (PAM) portable chlorophyll fluorometer PAM-2500. The plants were pre-adapted in dark for 30 min before measurement to ensure sufficient closure of all PSII reaction centers and estimate the maximum fluorescence yield. The OJIP transients were detected by the measuring light of 3000 μmol photons m<sup>-2</sup> s<sup>-1</sup>. The Chla fluorescence emission induced by the strong light pulses was determined and digitized between 10 μs

and 320 ms. JIP-test was applied to analyze the OJIP curve. The measurement was conducted at room temperature. To avoid the affection by temperature jump, the determination was performed immediately when the plants were took out of the chamber.

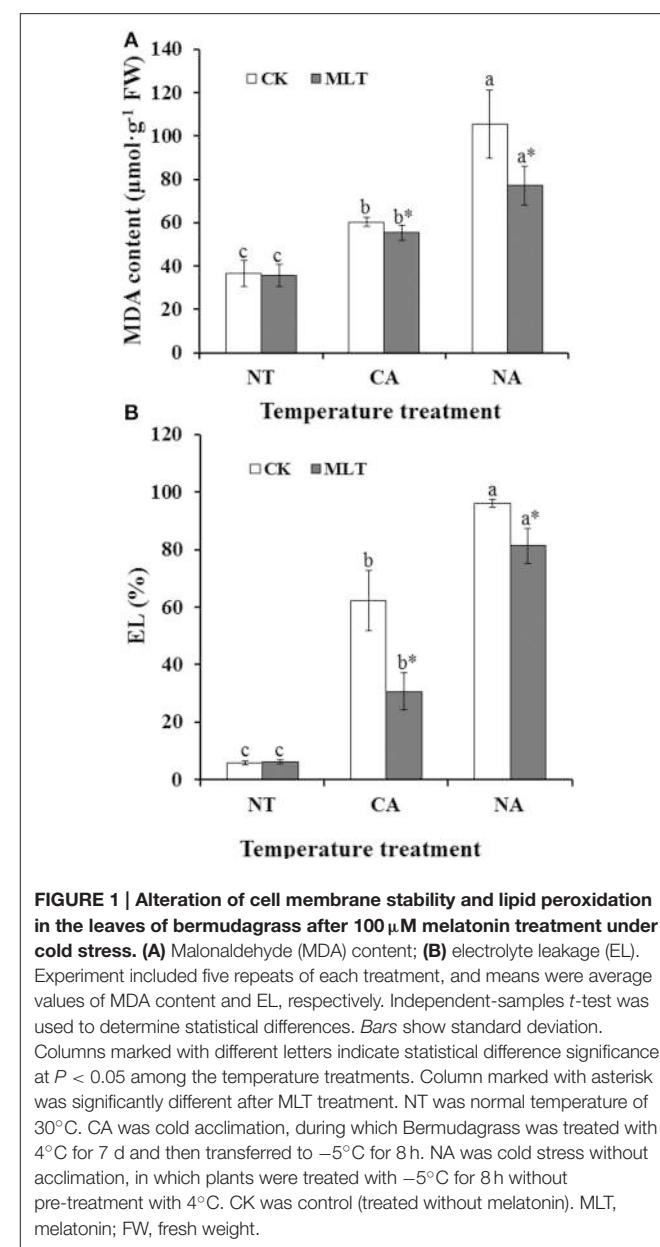
## Extraction, Derivation and Quantification of Metabolites

0.15 g of fully expanded leaves were collected from Bermudagrass plant after treatments, and frozen in liquid nitrogen immediately then stored at  $-80^{\circ}\text{C}$  until for analysis. Metabolite extraction was done according to the method described by Xie et al. (2014). The frozen leaves were grounded into fine powder with liquid nitrogen, and then the powders were transferred to 2 mL microcentrifuge tubes that containing 1.4 mL of 80% (v/v) aqueous methanol. After that, the tubes were shaken at 200 rpm for 2 h at room temperature ( $25^{\circ}\text{C}$ ) in the shaker. 50  $\mu\text{L}$  ribitol solutions (2 mg  $\text{mL}^{-1}$ ) were added as an internal standard. The mixture was incubated in a water bath at  $70^{\circ}\text{C}$  for 15 min and centrifuged at 12000 rpm for 10 min. The supernatant was transferred to new 10 mL tubes with 1.5 mL of water and 0.75 mL of chloroform was added. The mixture was vortex shocked thoroughly for 15 s and centrifuged at 13400 g for 10 min. 0.3 mL of the polar phase was transferred into 2 mL HPLC vials and dried in a centrifugal concentrator (Labogene, Denmark) overnight. The dried polar phase was derivatized with 80  $\mu\text{L}$  of 20 mg  $\text{mL}^{-1}$  methoxyamine hydrochloride in pyridine at  $30^{\circ}\text{C}$  for 2 h, and trimethylsilylated with 50  $\mu\text{L}$  *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at  $37^{\circ}\text{C}$  for 2 h. The reagents used in this study were purchased from Sigma-Aldrich Co. Ltd. (Poole, UK).

The metabolites were determined with GC-MS (Agilent 7890A/5975C, Agilent Technologies, Palo Alto, CA, USA) as described by Xie et al. (2014). For GC-MS operation, 1  $\mu\text{L}$  of derivatized sample was added into a DB-5MS capillary (30 m  $\times$  0.25 mm  $\times$  0.25 mm, Agilent J&W GC column, USA). The inlet temperature was set at  $280^{\circ}\text{C}$  and after a solvent delay for 5 min; the initial gas chromatography (GC) oven temperature was set at  $70^{\circ}\text{C}$ . After 1 min injection, the temperature of GC oven was raised 5°C per min until to  $280^{\circ}\text{C}$ , and then held at  $280^{\circ}\text{C}$  for 10 min. The injection temperature was set at  $280^{\circ}\text{C}$  and ion source temperature ( $230^{\circ}\text{C}$ ) was matched simultaneously. Helium was applied as the carrier gas, and the constant flow rate was set at 1  $\text{mL min}^{-1}$ . Mass spectra were determined at 2 scans  $\text{s}^{-1}$  with electron impact ionization (70 eV) in the full scan mode (*m/z* 30–650).

## Metabolite Data Processing and Analysis

The metabolites were identified based on the retention time with software of Agilent MSD Productivity Chemstation and associated with the commercially available compound libraries (NIST 11) (Gaithersburg, MD, USA). Relative quantification of the metabolites was estimated based on the value of ribitol which was the internal standard. The principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed on the MetaboAnalyst webpage (<http://www.metaboanalyst.ca/MetaboAnalyst/>). Log-transformed response



**FIGURE 1 | Alteration of cell membrane stability and lipid peroxidation in the leaves of bermudagrass after 100  $\mu\text{M}$  melatonin treatment under cold stress. (A)** Malonaldehyde (MDA) content; **(B)** electrolyte leakage (EL). Experiment included five repeats of each treatment, and means were average values of MDA content and EL, respectively. Independent-samples *t*-test was used to determine statistical differences. Bars show standard deviation. Columns marked with different letters indicate statistical difference significance at  $P < 0.05$  among the temperature treatments. Column marked with asterisk was significantly different after MLT treatment. NT was normal temperature of  $30^{\circ}\text{C}$ . CA was cold acclimation, during which Bermudagrass was treated with  $4^{\circ}\text{C}$  for 7 d and then transferred to  $-5^{\circ}\text{C}$  for 8 h. NA was cold stress without acclimation, in which plants were treated with  $-5^{\circ}\text{C}$  for 8 h without pre-treatment with  $4^{\circ}\text{C}$ . CK was control (treated without melatonin). MLT, melatonin; FW, fresh weight.

ratios for each identified metabolites were calculated before statistical assessment.

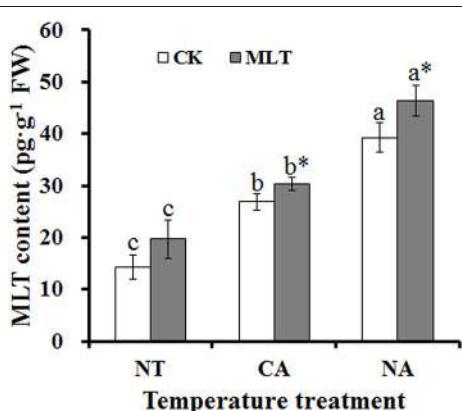
## Statistical Analysis

For metabolism analysis, each experiment was repeated for three times, and statistical analysis was performed by One-way analysis of variance (ANOVA). Means were separated with Duncan's multiple range tests at a significant level of  $P < 0.05$ . For other data, the experiment was set five repeats of each treatment, independent-samples *t*-test was used to determine statistical differences. Standard deviations (SD) were used to show the data. The means are the average of the repeats. Bars with the letters above the columns of the figures indicate significant differences ( $P < 0.05$ ).

## RESULTS

To investigate whether the exogenous melatonin played a positive role in maintaining cell membrane stability of Bermudagrass under cold stress, MDA content and EL alterations were determined. The results showed that, both MDA and EL were higher in the plants after cold treatment than those of control. Moreover, under cold stress, MDA and EL were higher in the regime of non-cold acclimation (NA) than those in regime of cold acclimation (CA) (**Figure 1**). However, in these two cold treatment regimes, MDA contents in plants treated with melatonin were 8.3% (CA regime) and 26.7% (NA regime) lower than that in the plants without melatonin treatment, respectively (**Figure 1A**). These results showed that exogenous melatonin protects the cell membrane against lipid peroxidation. Similar results were also observed regarding relative EL values. Relative EL in melatonin treatment plants were 50.8% (CA regime) and 15.3% (NA regime) lower than those in the plants without melatonin treatment, respectively (**Figure 1B**). These results suggested that melatonin participated in maintaining cell membrane stability.

To investigate how the melatonin content changed in plant under cold stress after exogenous melatonin treatment, levels of endogenous melatonin in the leaves were determined after different treatments. The results showed that melatonin content increased dramatically after cold treatment. Endogenous melatonin increased significantly after the plant was treated with exogenous melatonin in CA and NA regimes (12.8 and 18.1%,

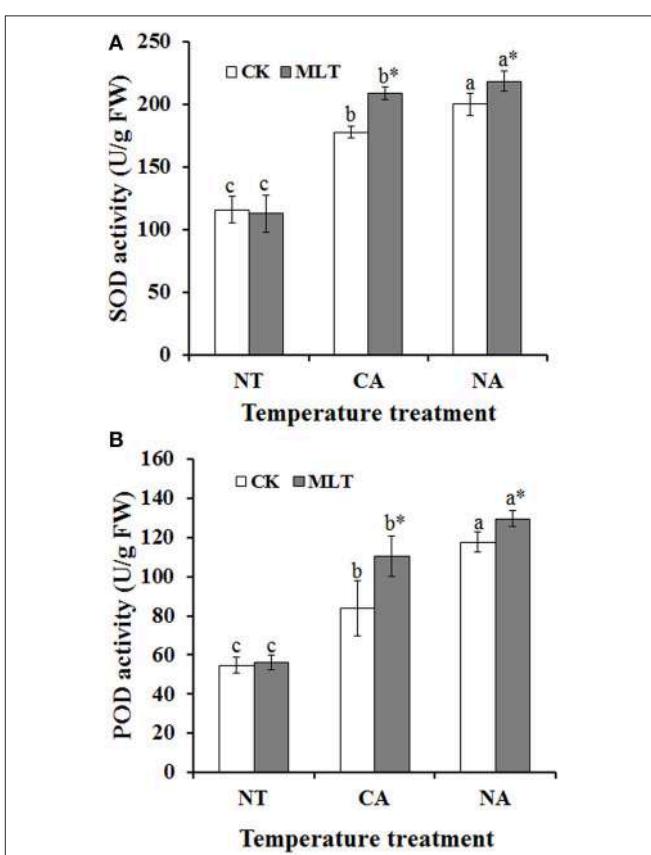


**FIGURE 2 | Alterations of endogenous melatonin content in the leaves of Bermudagrass after 100  $\mu$ M melatonin treatment under cold stress.**

Experiment included five repeats of each treatment, and means were average values of melatonin contents. Independent-samples *t*-test was used to determine statistical differences. Bars show standard deviation. Columns marked with different letters indicate statistical difference significance at  $P < 0.05$  among the temperature treatments. Column marked with asterisk was significantly different after MLT treatment. NT was normal temperature of 30°C. CA was cold acclimation, which bermudagrass were treated with 4°C for 7 d and then transferred to -5°C for 8 h. NA was cold stress without acclimation, in which plants were treated with -5°C for 8 h without pre-treatment with 4°C. CK was control (treated without melatonin). MLT, melatonin; FW, fresh weight.

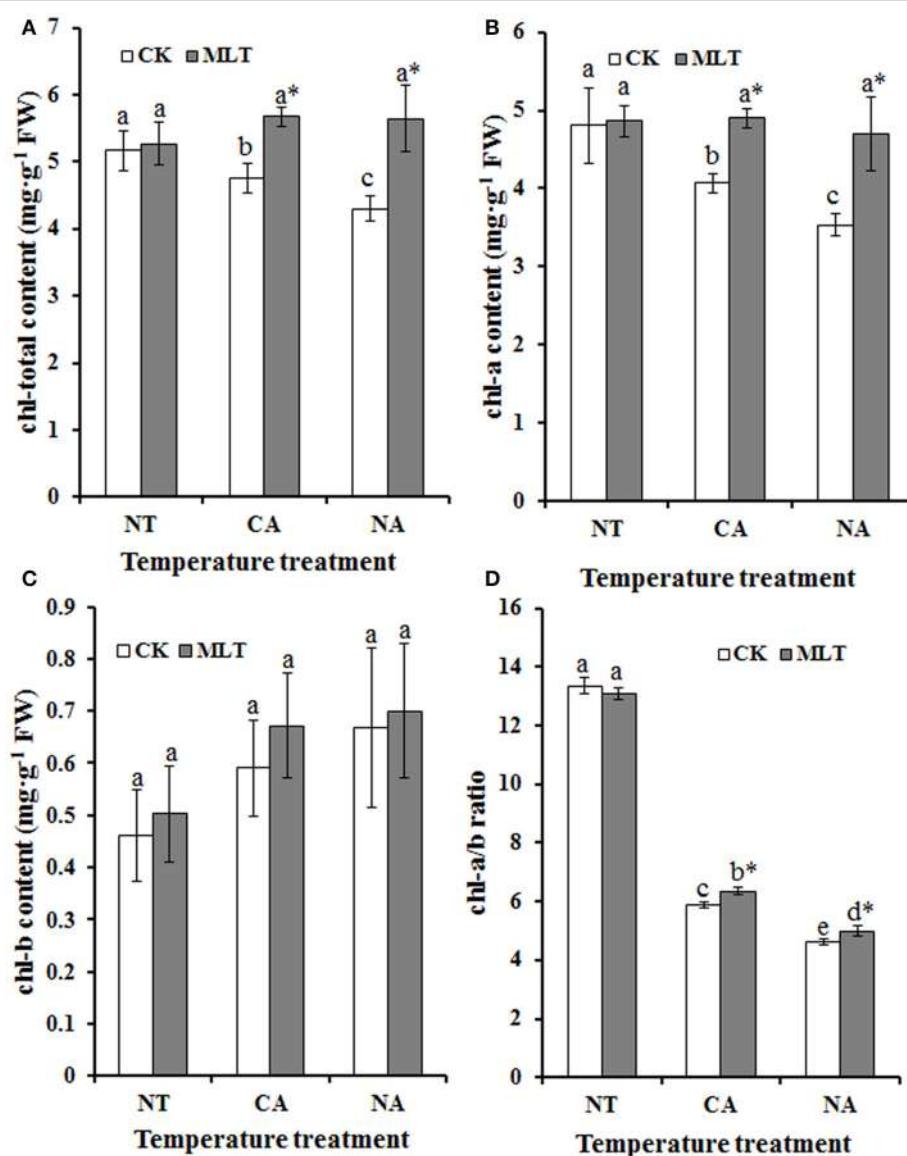
respectively) (**Figure 2**). These results suggested exogenous melatonin could affect the level of endogenous melatonin.

To investigate the effect of melatonin on antioxidant enzymes, activities of SOD and POD were determined. As shown in **Figure 2**, the antioxidant enzyme activities were increased in plants after cold treatment (NA and CA) relative to control temperature (30°C). As for melatonin treatment, there was no significant difference in melatonin pretreated plants compared with non-pretreated regimes under control conditions. However, when plants were subjected to cold stress, the SOD and POD activities increased significantly as a result of melatonin treatment. In CA and NA regimes, SOD activity was 17.3 and 9.1% higher in the melatonin-treated plants than non-melatonin treatment regimes, respectively (**Figure 3A**). Similar results were



**FIGURE 3 | Alterations of antioxidant enzyme activities in the leaves of Bermudagrass after 100  $\mu$ M melatonin treatment under cold stress. (A)** Activity of superoxide dismutase (SOD); **(B)** Activity of peroxidase (POD).

Experiment included five repeats of each treatment, and means were average values of activities of SOD and POD, respectively. Independent-samples *t*-test was used to determine statistical differences. Bars show standard deviation. Columns marked with different letters indicate statistical difference significance at  $P < 0.05$  among the temperature treatments. Column marked with asterisk was significantly different after MLT treatment. NT was normal temperature of 30°C. CA was cold acclimation, which bermudagrass were treated with 4°C for 7 d and then transferred to -5°C for 8 h. NA was cold stress without acclimation, in which plants were treated with -5°C for 8 h without pre-treatment with 4°C. CK was control (treated without melatonin). MLT, melatonin; FW, fresh weight.



**FIGURE 4 | Alteration of chlorophyll content of Bermudagrass after 100  $\mu$ M melatonin treatment under cold stress. (A)** Total chlorophyll content; **(B)** chlorophyll a content; **(C)** chlorophyll b content; **(D)** ratio of chlorophyll a to b. Experiment included five repeats of each treatment, and means were average values of the data. Independent-samples *t*-test was used to determine statistical differences. Bars show standard deviation. Columns marked with different letters indicate statistical difference significance at  $P < 0.05$  among the temperature treatments. Column marked with asterisk was significantly different after MLT treatment. NT was normal temperature of 30°C. CA was cold acclimation, which Bermudagrass were treated with 4°C for 7 d and then transferred to -5°C for 8 h. NA was cold stress without acclimation, in which plants were treated with -5°C for 8 h without pre-treatment with 4°C. CK was control (treated without melatonin). MLT, melatonin; FW, fresh weight.

also observed in POD activity. After melatonin treatment, the POD activity was 10.2% higher than non-melatonin treatment in NA regime. Additionally, the POD was as high as 1.3-fold compared with the plants without melatonin treatment in the CA regime (**Figure 3B**). These results document that melatonin plays essential roles in increasing antioxidant enzymes activities in Bermudagrass in response to cold stress.

There was chlorosis when plants were exposed to abiotic stress, and chlorophyll content was regarded as an indicator

to reveal the stress resistance of plants. In this study, total chlorophyll content was measured. As shown in **Figure 4**, total chlorophyll content was higher in plants under control conditions than the cold-treated plants, and melatonin had no effect on the plants under control conditions. But after cold treatment, the total chlorophyll content was significantly higher in plants pretreated with exogenous melatonin than the untreated regimes. In both CA and NA regimes, total chlorophyll contents were 19.2% (CA regime) and 31.2% (NA regime) higher in

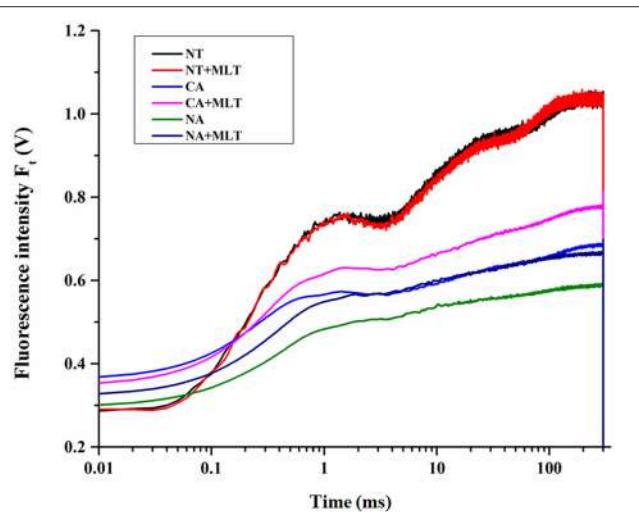
the plants treated with melatonin than those without melatonin treatment, respectively (**Figure 4A**). The ratio of chlorophyll *a* to *b* (chl-*a/b*) was higher in the plants in the CA regime than those in the NA regime (**Figure 4D**). In addition, in each regime, the melatonin-treated plants had higher chl-*a/b* compared to that non-melatonin treated plants (**Figure 4D**). These results showed that exogenous melatonin maintains chlorophyll stability of plants under cold stress, and thus likely improves cold resistance of the plants.

Since Bermudagrass (after melatonin pretreatment) had higher chlorophyll content under cold stress, we predicted that melatonin was involved in photosystem regulation. OJIP transient curves of the plants with different treatments were measured and the JIP test was analyzed. The results showed that OJIP transient curve in plants under control condition was higher than those under cold treatment. Furthermore, the plants treated with  $-5^{\circ}\text{C}$  after cold acclimation showed higher OJIP transient curve than plants treated with freezing temperatures

without cold acclimation. Moreover, in each regime under cold stress, the curves were higher in the plants given exogenous melatonin than those in plants without melatonin treatment (**Figure 5**). This confirms that cold acclimation and exogenous melatonin treatment dramatically affected the OJIP transient curves of Bermudagrass leaves under cold stress.

To further explore the effect of melatonin on PSII in Bermudagrass under cold stress, the JIP-test was applied to study the OJIP transient curves. Basic fluorescence parameters including  $F_0$ ,  $F_K$ ,  $F_J$ ,  $F_I$ ,  $F_M$ , and  $M_0$  were extracted. As the result showed, the basic parameters were higher under control condition than under cold stress except for  $F_0$  which was lower. Under cold stress,  $F_0$  was higher in CA regime than that in NA regime, but there was no significant difference in the plants with or without melatonin treatment. However, the plants that were simultaneously treated with cold acclimation and melatonin had the highest values of  $F_M$  and other parameters. Meanwhile, the plants that were neither treated with cold nor melatonin had the lowest values (**Table 1**).

JIP-test was used to analyze the basic fluorescence in order to determine the structural and functional parameters quantifying the photosynthetic behavior of the plants. The results showed significant differences between parameters under different treatments. Performance index (PI),  $\text{PI}_{\text{total}}$ , and  $\text{PI}_{\text{ABS}}$ , are important indexes to describe the overall activity of PSII. As shown in **Figure 5**, performance indexes (PI) were higher under control condition than that under cold stress. There was no significant difference between the plants with and without melatonin treatment. After cold treatment, the values of  $\text{PI}_{\text{total}}$  and  $\text{PI}_{\text{ABS}}$  were higher in the plants after cold acclimation than in the non-acclimatized plants. Both parameters were higher in melatonin-treated plants than non-melatonin treated ones under cold stress (**Figure 6**). Parameters of quantum yields and efficiencies including values of  $\varphi P_0$ ,  $\varphi R_0$ ,  $\varphi E_0$ , and  $\gamma R$  cause marked alterations in the plants with different treatments. The highest values of these four parameters were detected in the plants after melatonin treatment in the CA regime and the lowest values were found in the plants without melatonin treatment in the NA regime (**Figures 7A–D**). ABS/RC, TP0/RC, ET0/RC, and RE0/RC values, known as parameters of specific energy fluxes, also changed remarkably after different treatments. In both of CA and NA regimes, these parameters decreased in the melatonin-treated plants compared to those in non-treated regimes

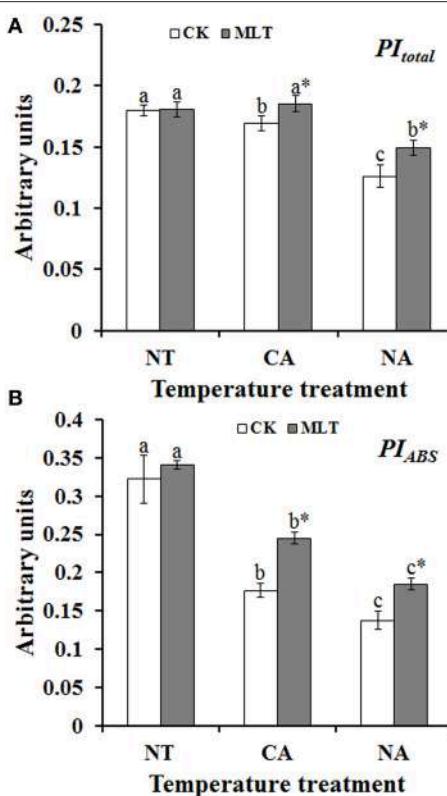


**FIGURE 5 | Alterations of chlorophyll fluorescence transients (OJIP curve) in Bermudagrass leaves after  $100 \mu\text{M}$  melatonin treatment under cold stress.** NT was normal temperature of  $30^{\circ}\text{C}$ . CA was cold acclimation, in which bermudagrass were treated with  $4^{\circ}\text{C}$  for 7 d and then transferred to  $-5^{\circ}\text{C}$  for 8 h. NA was cold stress without acclimation which plants were treated with  $-5^{\circ}\text{C}$  for 8 h without pre-treatment with  $4^{\circ}\text{C}$ . MLT, melatonin.

**TABLE 1 | Basic photosynthetic parameters extracted from the OJIP transient curves.**

Treatment	$F_0$	$F_M$	$F_K$	$F_J$	$F_I$	$M_0$
NT	$0.28 \pm 0.004\text{c}$	$1.07 \pm 0.003\text{a}$	$0.73 \pm 0.01\text{a}$	$0.76 \pm 0.02\text{a}$	$0.97 \pm 0.01\text{a}$	$2.28 \pm 0.07\text{a}$
NT+MLT	$0.28 \pm 0.006\text{c}$	$1.07 \pm 0.005\text{a}$	$0.73 \pm 0.004\text{a}$	$0.76 \pm 0.01\text{a}$	$0.97 \pm 0.012\text{a}$	$2.34 \pm 0.09\text{a}$
NA	$0.29 \pm 0.02\text{ b}$	$0.62 \pm 0.06\text{c}$	$0.40 \pm 0.02\text{c}$	$0.51 \pm 0.02\text{c}$	$0.59 \pm 0.05\text{b}$	$1.34 \pm 0.089\text{c}$
NA+MLT	$0.30 \pm 0.01\text{b}$	$0.67 \pm 0.08\text{c}^*$	$0.46 \pm 0.04\text{c}^*$	$0.54 \pm 0.04\text{c}^*$	$0.62 \pm 0.07\text{c}$	$1.62 \pm 0.071\text{bc}^*$
CA	$0.33 \pm 0.02\text{ a}$	$0.73 \pm 0.02\text{b}$	$0.50 \pm 0.01\text{b}$	$0.55 \pm 0.01\text{b}$	$0.62 \pm 0.03\text{b}$	$1.57 \pm 0.009\text{b}$
CA+MLT	$0.35 \pm 0.01\text{a}$	$0.84 \pm 0.05\text{b}^*$	$0.54 \pm 0.03\text{b}^*$	$0.64 \pm 0.03\text{b}^*$	$0.74 \pm 0.05\text{b}^*$	$1.82 \pm 0.072\text{b}^*$

NT was normal temperature of  $30^{\circ}\text{C}$ . CA was cold acclimation, which Bermudagrass were treated with  $4^{\circ}\text{C}$  for 7 d and then transferred to  $-5^{\circ}\text{C}$  for 8 h. NA was cold stress without acclimation, which plants were treated with  $-5^{\circ}\text{C}$  for 8 h without pre-treatment with  $4^{\circ}\text{C}$ . MLT was melatonin. Independent-samples t-test was used to determine statistical differences. Different letters indicate statistical difference significance at  $P < 0.05$  among the temperature treatments. Asterisk was significantly different after MLT treatment.



**FIGURE 6 | Alterations of performance index (PI) as deduced by JIP-test analysis of fluorescence transients. (A)** Alteration of PI for energy conservation from exciton to the reduction of PSI end acceptors ( $PI_{Total}$ ). **(B)** Alterations of PI for energy conservation from exciton to the reduction of intersystem electron ( $PI_{ABS}$ ). Experiment included five repeats of each treatment, and means were average values of the data. Calculations of each parameter refer to the method of Yusuf et al. (2010). Independent-samples *t*-test was used to determine statistical differences. Bars show standard deviation. Different letters indicate statistical difference significance at  $P < 0.05$  among the treatments. Column marked with asterisk was significantly different after MLT treatment. NT was normal temperature of 30°C. CA was cold acclimation, in which Bermudagrass were treated with 4°C for 7 d and then transferred to -5°C for 8 h. NA was cold stress without acclimation, which plants were treated with -5°C for 8 h without pre-treatment with 4°C. CK was control (treated without melatonin). MLT, melatonin.

(Figures 7E–H). ABS/RC, TP0/RC, ET<sub>0</sub>/RC, and RE<sub>0</sub>/RC had the highest values in the plants that were treated without melatonin or cold acclimation, and the lowest values in the melatonin and cold acclimation-treated plants (Figures 7E–H).

To investigate metabolic homeostasis induced by exogenous melatonin treatment under cold stress, GC-MS was applied to identify the metabolites. Forty-six metabolites including 9 amino acids, 14 organic acids, 16 sugars, 4 sugar alcohols, 2 alkanes, and 1 ketone were detected in all different treatments (Figure 8, Table 2). Generally, under cold stress, there were alterations in metabolite concentrations after melatonin application. This alteration was less in NA regime than that of CA regime. Dramatic changes in metabolite levels were observed in the CA regime plants, and a large proportion of them showed higher concentrations in the melatonin-treated plants than in controls

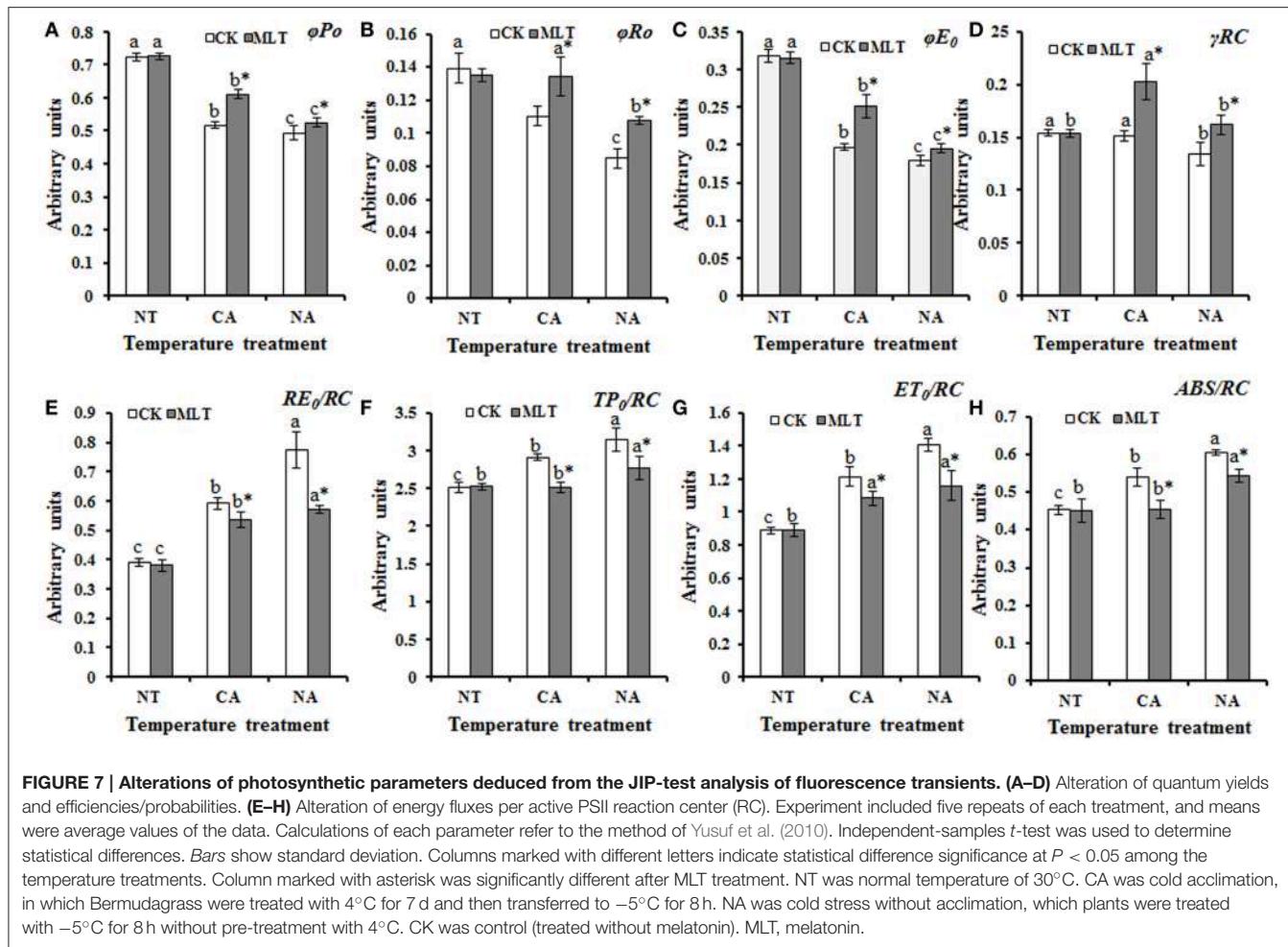
(Figure 8, Table 2). Among the various metabolites, 5 sugars (arabinose, mannose, glucopyranose, maltose, and turanose) and 1 organic acid (propanoic acid) increased significantly in melatonin-pretreated plants under both of NA and CA treatment (Table 2). PCA of the 46 metabolites separated clearly between different conditions (Supplemental Figures S1, S2). Specifically, after cold treatment, the first principal component, designated as PC1, separated the CA regime from NA regime clearly, which was represented 37% of the total variance. In the second dimension, PC2 separated the melatonin treatment from non-melatonin treatment clearly, which represented 22.2% of the total variance (Supplemental Figure S2). These results suggested that exogenous melatonin affects the principal metabolites under cold stress especially in Bermudagrass after cold acclimation.

## DISCUSSION

Cold is a key factor that limits resource utilization of Bermudagrass. Thus, finding a way of improving cold resistance of this species is important for turf industry (Chen et al., 2015). Nitric oxide and jasmonic acid were reported to improve plant cold resistance (Cheong and Choi, 2003; Cantrel et al., 2011; Fan et al., 2015). Recently, melatonin was reported to have positive functions in protecting plants against biotic and abiotic stress (Li et al., 2012; Bajwa et al., 2014; Meng et al., 2014; Lee et al., 2015; Reiter et al., 2015; Zhao et al., 2015; Shi et al., 2015b). However, the mechanisms of melatonin involvement in cold stress response in Bermudagrass are largely unknown. Here (phenotypic change, Supplemental Figure S3), physiological alterations, the process of photosystem II and changes in metabolism in pre-cold acclimated and non-cold acclimated Bermudagrass under freezing stress after melatonin treatment were investigated.

Cell membrane stability was assessed as an indicator of cellular damage induced by multiple abiotic stresses (Saneoka et al., 2004). Cell membrane systems were also the major sites of cold injury in plants (Steponkus, 1984). Lipid peroxidation and plasma membrane injury is induced by cold stress in many plants including Bermudagrass (Zhang et al., 2006). In the current study, the results showed that both of EL and MDA contents were lower in melatonin-treated plants than those in non-melatonin treated regimes under cold stress, and the values were higher in NA than CA regimes (Figure 1). These results suggested that cold acclimation remarkably improves cold resistance of Bermudagrass, and that exogenous melatonin played a positive role in maintaining cell membrane stability to protecting Bermudagrass against cold stress.

Reactive oxygen species (ROS) were formed, and hence led to oxidative damage when plants were exposed to cold stress. Recently, melatonin was reported to play roles in counteracting the effects of ROS in various stresses (Fischer et al., 2004; Posmyk and Janas, 2009; Shi et al., 2015b). In the present study, the results revealed that melatonin played a crucial role in cold resistance of Bermudagrass. Melatonin is an antioxidant that scavenges radicals directly and indirectly that exists extensively in animals and plants (Tan et al., 1993; Posmyk and Janas, 2009; Zhang and Zhang, 2014). The exogenous application of melatonin

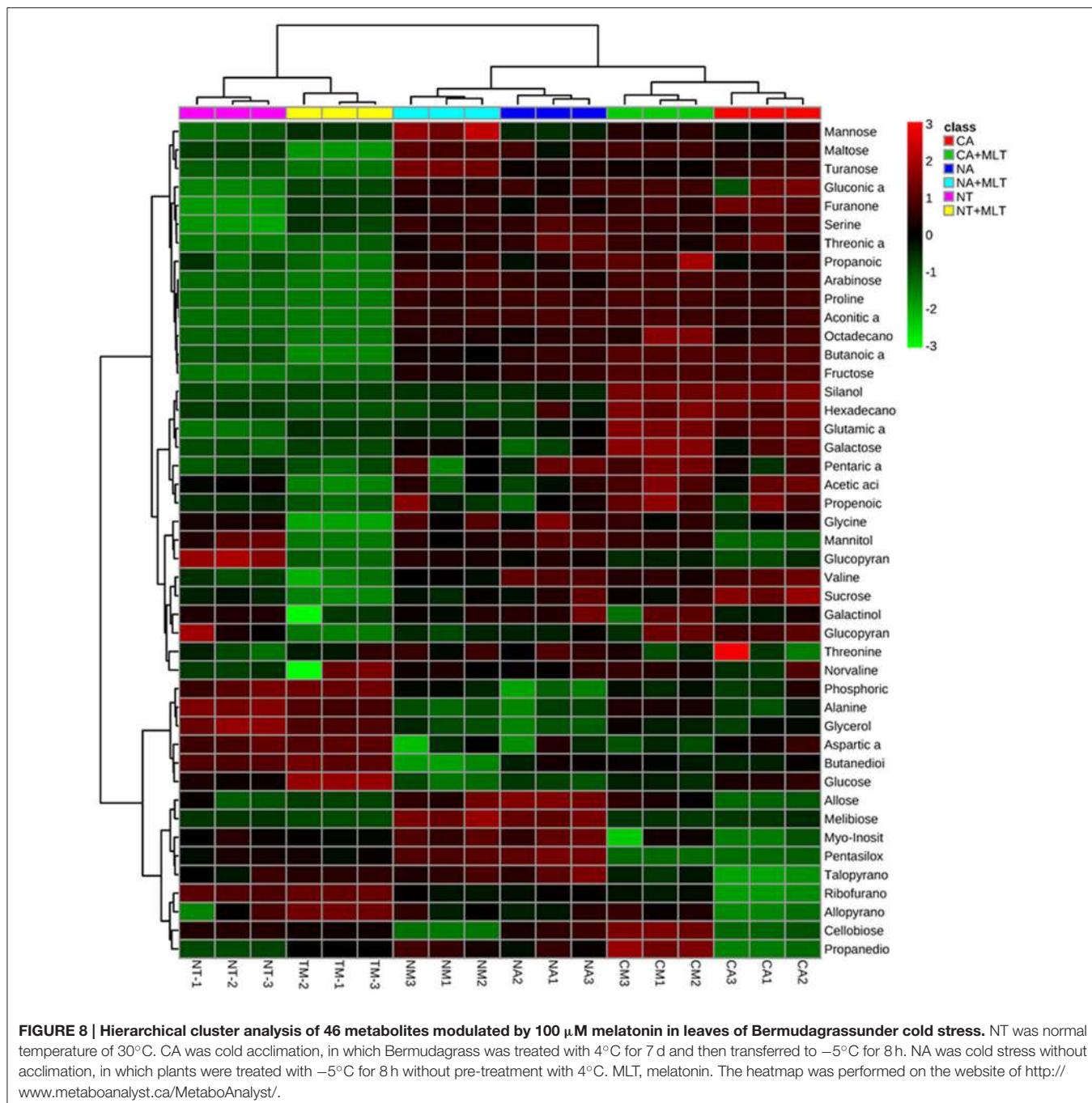


in the plants of the two regimes (CA and NA) dramatically activated antioxidant enzymes POD and SOD. Interestingly, the antioxidant enzyme activities were higher in the NA regime than that in CA regime (**Figure 3**). This might be attributed to the sudden drastic reduction of the temperature, and induction of the excessive generation of ROS in the plants. To scavenge ROS, antioxidant enzymes activities, like POD and SOD were increased in the plants. In addition, exogenous melatonin improved the activities further. It was reported that under oxidative stress, ROS generation increases antioxidant enzymes activities in plants (Yan et al., 2010; Fan et al., 2014). The results suggested that exogenously-applied melatonin stimulates antioxidant enzymes activities in Bermudagrass under cold stress, and thus enhanced plant cold resistance.

When plants were exposed to cold stress, chlorophyll was degraded thereby the leaves experienced chlorosis (Koc et al., 2010). Chlorophyll content of the leaves provides vital information about the physiological condition of the plants (Gitelson et al., 2003). In this study, chlorophyll content was higher in melatonin treated plants than that in non-melatonin treated regimes under cold stress (**Figures 4A–C**). The ratio of chlorophyll *a* to *b* (chl-*a/b*) is a valuable measurement of

the proportion of LHCII (light-harvesting complex associated with PSII) to other components that contain chlorophyll (Leong and Anderson, 1984). As the results indicated, chl-*a/b* was significantly higher in melatonin treated plants than that in control. Moreover, cold acclimation increased this ratio. As shown in the **Figure 4D**, the value of chl-*a/b* in CA regime was higher than that in NA regime. These results showed that exogenous melatonin protects chlorophyll from degradation and then improves cold resistance and photosynthetic efficiency of Bermudagrass under cold stress.

Chlorophyll *a* fluorescence has been broadly employed in studying photosystem especially under abiotic stress conditions (Chen et al., 2013; Roopin et al., 2013). To further explore the behavior of PSII of Bermudagrass under cold stress, chlorophyll *a* fluorescence analysis including OJIP curve and JIP-test were applied. Alteration of the curves implied that exogenous melatonin was crucial in Bermudagrass cold stress resistance regardless of cold acclimation or no acclimation (**Figure 5**). Abundant information was revealed by the OJIP fluorescence transient, and it was used to determine the parameters by JIP-test that quantified the energy flow through PSII at the level of reaction center (RC) (Strasser and Strasser, 1995) (**Table 3**).



For the  $F_0$  value, the minimal recorded fluorescence intensity, no significant difference was found in the plants treated with or without melatonin in the two regimes CA and NA. However, the  $F_M$  values were higher in the plants after melatonin treatment than those in non-melatonin treatment regimes. Similar changes of other basic parameters including  $F_J$ ,  $F_I$ , and  $M_0$  were observed. This suggests that exogenous melatonin was involved in cold resistance of Bermudagrass (Table 1). The performance index (PI), was a sensitive parameter of JIP-test that evaluates the photochemical activities under stress condition. It combines

three primary functional steps containing light energy absorption step, excitation energy trapping step, and conversion of excitation energy to electron transport step. This suggested photosynthetic activity through a reaction center complex of PSII into a single multi-parametric expression (Strasser et al., 1999). In the present study, the observation that  $\text{PI}_{\text{total}}$  (overall behavior of the photosynthetic activities) and  $\text{PI}_{\text{ABS}}$  (density of RC that is expressed per absorption) were higher in the melatonin-treated plants than the non-treated regimes (Figure 6), implied that exogenous melatonin plays a protective role in cold resistance

**TABLE 2 | Forty six metabolites in leaves of bermudagrass under cold stress.**

Metabolites	Treatment					
	NT	NT+MLT	NA	NA+MLT	CA	CA+MLT
<b>AMINO ACIDS</b>						
Glycine	0.329 ± 0.007b	0.327 ± 0.01c	0.364 ± 0.01a	0.422 ± 0.01a*	0.284 ± 0.06c	0.376 ± 0.01b*
Valine	0.038 ± 0.007c	0.037 ± 0.006c	0.232 ± 0.02b	0.081 ± 0.003b*	0.276 ± 0.03a	0.128 ± 0.02a*
Alanine	0.286 ± 0.01a	0.287 ± 0.01a	0.078 ± 0.002c	0.074 ± 0.004c	0.086 ± 0.002b	0.147 ± 0.005b*
Threonine	0.05 ± 0.007b	0.051 ± 0.01c	0.065 ± 0.002a	0.069 ± 0.001a*	0.045 ± 0.003c	0.115 ± 0.15b*
Proline	0.37 ± 0.05c	0.36 ± 0.08c	1.566 ± 0.24a	1.471 ± 0.29b	1.517 ± 0.45a	2.514 ± 0.48a*
Norvaline	0.045 ± 0.003c	0.046 ± 0.07c	0.073 ± 0.01a	0.071 ± 0.008b	0.06 ± 0.005b	0.081 ± 0.008a*
Serine	0.027 ± 0.004b	0.027 ± 0.01c	0.43 ± 0.03a	0.54 ± 0.03a*	0.43 ± 0.02a	0.5 ± 0.008b*
<b>ORGANIC ACIDS</b>						
Propanoic acid	0.262 ± 0.024b	0.278 ± 0.018c	0.473 ± 0.02a	0.549 ± 0.01b*	0.465 ± 0.01a	0.868 ± 0.08a*
Phosphoric acid	0.097 ± 0.029a	0.112 ± 0.005a	0.013 ± 0.0003c	0.036 ± 0.002b*	0.026 ± 0.002b	0.034 ± 0.001b*
Acetic acid	0.025 ± 0.001b	0.025 ± 0.0004b	0.024 ± 0.005b	0.024 ± 0.006b	0.033 ± 0.008a	0.036 ± 0.004a*
Propenoic acid	0.019 ± 0.001c	0.019 ± 0.001c	0.023 ± 0.003b	0.03 ± 0.008b*	0.038 ± 0.001a	0.054 ± 0.006a*
Threonic acid	0.02 ± 0.001b	0.02 ± 0.002b	0.28 ± 0.02a	0.159 ± 0.02a*	0.262 ± 0.03a	0.162 ± 0.01a*
Glutamic acid	0.114 ± 0.01c	0.117 ± 0.02c	0.42 ± 0.05b	0.455 ± 0.007b	1.89 ± 0.17a	3.042 ± 0.16a*
Octadecanoic acid	—	—	0.027 ± 0.002b	0.025 ± 0.002b	0.04 ± 0.002a	0.137 ± 0.02a*
Aspartic acid	0.08 ± 0.01a	0.086 ± 0.004a	0.036 ± 0.004c	0.037 ± 0.002b	0.059 ± 0.01b	0.033 ± 0.005b*
Butanoic acid	0.01 ± 0.001c	0.01 ± 0.0003c	0.33 ± 0.03b	0.14 ± 0.04b*	0.72 ± 0.01a	0.83 ± 0.03a*
Butanedioic acid	1.23 ± 0.07a	1.22 ± 0.22a	0.29 ± 0.11b	0.03 ± 0.007c*	0.21 ± 0.08bc	0.25 ± 0.06b
Gluconic acid	0.017 ± 0.001c	0.018 ± 0.001c	0.089 ± 0.06b	0.08 ± 0.01b	0.18 ± 0.14a	0.2 ± 0.05a
Aconitic acid	—	—	1.84 ± 0.54a	0.92 ± 0.27b*	1.08 ± 0.46b	1.62 ± 0.44a*
Pentaric acid	1.14 ± 0.09c	1.08 ± 0.06c	1.66 ± 0.35a	1.35 ± 0.4b*	1.44 ± 0.239b	1.85 ± 0.15a*
Gluconic acid	—	—	0.06 ± 0.01a	0.02 ± 0.004b*	0.03 ± 0.01b	0.12 ± 0.009a*
Hexadecanoic acid	0.06 ± 0.002b	0.04 ± 0.0001b	0.1 ± 0.06b	0.05 ± 0.007b	0.23 ± 0.04a	0.27 ± 0.04a*
Propanedioic acid	0.006 ± 0.0001c	0.009 ± 0.0003c	0.012 ± 0.005b	0.017 ± 0.005b	0.02 ± 0.006a	0.05 ± 0.01a*
<b>SUGARS</b>						
Arabinose	—	—	0.108 ± 0.04b	0.225 ± 0.04a*	0.156 ± 0.04a	0.214 ± 0.009a*
Fructose	0.023 ± 0.003c	0.023 ± 0.002c	0.887 ± 0.05b	1.483 ± 0.09b*	2.671 ± 0.09a	3.123 ± 0.09a*
Galactose	0.056 ± 0.02c	0.054 ± 0.01c	0.172 ± 0.04b	0.424 ± 0.07b*	1.48 ± 0.25a	6.1 ± 0.23a*
Glucose	0.075 ± 0.007a	0.079 ± 0.008b	0.024 ± 0.002c	0.03 ± 0.005c	0.043 ± 0.003b	0.091 ± 0.01a*
Glucopyranose	0.029 ± 0.005c	0.027 ± 0.002c	0.079 ± 0.02a	0.092 ± 0.007a*	0.039 ± 0.002b	0.05 ± 0.002b*
Sucrose	5.549 ± 0.4c	5.546 ± 0.5c	6.009 ± 0.06b	8.667 ± 0.4b*	7.424 ± 0.4a	14.44 ± 0.6a*
Maltose	0.023 ± 0.003b	0.026 ± 0.002c	0.107 ± 0.01a	0.175 ± 0.01a*	0.099 ± 0.008a	0.14 ± 0.002b*
Ribofuranose	0.12 ± 0.01a	0.12 ± 0.006a	0.025 ± 0.005b	0.02 ± 0.006b	0.02 ± 0.005b	0.019 ± 0.003b
Talopyranose	0.03 ± 0.02b	0.04 ± 0.002a	0.07 ± 0.04a	0.04 ± 0.01a*	0.02 ± 0.01b	0.01 ± 0.003b
Mannose	0.19 ± 0.02c	0.19 ± 0.01c	0.31 ± 0.02b	1.47 ± 0.5a*	0.47 ± 0.1b	0.55 ± 0.06b
Allose	0.06 ± 0.09b	0.06 ± 0.004c	1.23 ± 0.14a	0.55 ± 0.04a*	0.03 ± 0.005c	0.25 ± 0.08b
Allopyranose	0.02 ± 0.009a	0.027 ± 0.001a	0.008 ± 0.004b	0.009 ± 0.004b	0.009 ± 0.003b	0.01 ± 0.003b
Cellobiose	0.019 ± 0.0006b	0.014 ± 0.0004b	0.023 ± 0.007b	0.025 ± 0.01b	0.082 ± 0.03a	0.085 ± 0.01a
Turanose	—	—	0.018 ± 0.003b	0.12 ± 0.01a*	0.04 ± 0.008a	0.016 ± 0.001b*
Melibiose	—	—	0.079 ± 0.02a	0.081 ± 0.04a	—	—
Glucopyranoside	0.16 ± 0.05a	0.13 ± 0.002a	0.18 ± 0.08a	0.11 ± 0.03a	0.14 ± 0.02a	0.13 ± 0.06a
<b>ALCOHOLS</b>						
Glycerol	0.22 ± 0.02a	0.23 ± 0.01a	0.027 ± 0.004c	0.042 ± 0.005c*	0.075 ± 0.004b	0.09 ± 0.002b*
Myo-Inositol	0.27 ± 0.06b	0.26 ± 0.02b	0.476 ± 0.02a	0.534 ± 0.05a*	0.074 ± 0.003c	0.252 ± 0.02c*
Mannitol	0.14 ± 0.09a	0.13 ± 0.1a	0.11 ± 0.04b	0.03 ± 0.01c*	0.03 ± 0.01c	0.057 ± 0.01b*
Galactinol	0.4 ± 0.006b	0.41 ± 0.08b	0.61 ± 0.3a	0.33 ± 0.09c*	0.26 ± 0.09c	0.55 ± 0.01a*
<b>OTHERS (KETONE AND ALKANES)</b>						
Furanone	0.015 ± 0.002c	0.015 ± 0.001c	0.091 ± 0.01b	0.141 ± 0.02b*	0.162 ± 0.01a	0.436 ± 0.03a*
Pentasiloxane	0.03 ± 0.01b	0.025 ± 0.01b	0.35 ± 0.05a	0.14 ± 0.02a*	—	—
Silanol	0.3 ± 0.02c	0.37 ± 0.02c	0.57 ± 0.01b	0.56 ± 0.05b	11.8 ± 0.2a	12.4 ± 1.1a*

NT was normal temperature of 30°C. CA was cold acclimation, which Bermudagrass were treated with 4°C for 7 d and then transferred to -5°C for 8 h. NA was cold stress without acclimation, which plants were treated with -5°C for 8 h without pre-treatment with 4°C. MLT was melatonin. Statistical analysis was performed by One-way analysis of variance (ANOVA). Means were separated with Duncan's multiple range tests at a significant level of  $P < 0.05$ . Different letters indicate statistical difference significance at  $P < 0.05$  among the temperature treatments. Asterisk was significantly different after MLT treatment.

**TABLE 3 | Definitions of the photosynthetic parameters deduced by the JIP-test analysis for the analysis of Chl a fluorescence transient.**

Data extracted from the recorded Chl a fluorescence transient OJIP curve	
$F_O = F_{20\mu s}$	Minimal reliable recorded fluorescence
$F_K = F_{300\mu s}$	Fluorescence intensity at 300 $\mu$ s
$F_J = 2.97 \text{ ms}$	Fluorescence intensity at J step (2.97 ms) of OJIP curve
$F_I = 62 \text{ ms}$	Fluorescence intensity at I step (62 ms) of OJIP curve
$F_P = F_M$	Fluorescence intensity at the peak of OJIP curve
Performance indexes (partial potentials at the steps of energy bifurcations)	
$\text{PI}_{\text{ABS}}$	Performance index for energy conservation from exciton to the reduction of intersystem electron acceptors
$\text{PI}_{\text{total}}$	Performance index for energy conservation from exciton to the reduction of photosystem I end acceptors
Quantum yields and efficiencies/probabilities	
$\varphi_{P0}$	Maximum quantum yield for primary photochemistry ( $F_V/F_M$ )
$\varphi_{R0}$	Quantum yield for reduction of end electron acceptors at the PSI acceptor side (RE)
$\varphi_{E0}$	Quantum yield for electron transport (ET)
$\gamma_{RC}$	Probability that a PSII Chl molecule functions as RC
Specific energy fluxes (per $Q_A^-$ reducing PSII reaction center-RC)	
$RE_0/RC$	Electron flux reducing end electron acceptors at the PSI acceptor side, per RC
$TP_0/RC$	Trapping flux (leading to $Q_A^-$ reduction) per RC
$ET_0/RC$	Electron transport flux (further than $Q_A^-$ ) per RC
$ABS/RC$	Absorption flux (of antenna Chls) per RC

of Bermudagrass.  $\varphi_{P0}$ , the maximum quantum yield for primary photochemistry, was strongly improved by melatonin in Bermudagrass under cold stress (Figure 7A). Similar results were also detected in the values of  $\varphi_{E0}$  (quantum yield of the electron transport flux from  $Q_A$  to  $Q_B$ ),  $\varphi_{R0}$  (quantum yield for reduction of end electron acceptors at the PSI acceptor side), and  $\gamma_{RC}$  (probability that a molecule of PSII Chl functions as RC) (Figures 7B–D). This suggests that melatonin influences the quantum yield on the sides of donor and acceptor of PSII. For the analysis of functional properties of PSII, parameters of specific energy fluxes such as ABS/RC, TP<sub>0</sub>/RC, ET<sub>0</sub>/RC, and RE<sub>0</sub>/RC were analyzed. The behavior of Bermudagrass leaves was altered dramatically after the melatonin treatment, suggesting a distinct effect of exogenous melatonin on the RC (Figures 7E–H). Exogenous melatonin alleviated the negative effects of cold on RC and increased the quantity of RC under cold stress.

Alterations of photosynthesis could lead to the change of metabolites components. Previous studies reported that

metabolism dramatically changes under multiple stresses and senescence processes (Wang et al., 2014; Shi et al., 2015b). To further investigate whether melatonin modulates metabolic homeostasis, GC-MS was employed. As the results showed, almost all of the examined metabolites exhibited higher concentrations in the plants treated with melatonin than that of control (Figure 8, Table 2). Among the enhanced metabolites, carbohydrates such as fructose, galactose, glucose, and sucrose as well as proline have been reported to be crucial components for osmotic adaptation in abiotic stress response. This implies that melatonin may be involved in modulating synthesis of these metabolites to improve cold resistance (Krasensky and Jonak, 2012). Moreover, other metabolites including various carbohydrates, organic acids, and amino acids also increased in melatonin-treated plants. These results indicated that melatonin had comprehensive effects on multiple metabolic pathways, and these metabolic changes might be involved in cold resistance of Bermudagrass. Five sugars (arabinose, mannose, glucopyranose, maltose, and turanose) and one organic acid (propanoic acid) were significantly increased. However, valine and threonine acid contents were found to be decreased in melatonin-treated plants (Table 2), and hence roles of these metabolites in Bermudagrass response to cold need to be further investigated.

In summary, our findings reveal that melatonin plays a positive role in Bermudagrass to protect against cold stress in cold and non-cold acclimation conditions. This provides evidence that melatonin participates in cold stress through modulating photosynthesis and metabolism related pathways.

## AUTHOR CONTRIBUTIONS

JF and LC designed research; ZH and JF performed the experiments, analyzed the data and wrote the manuscript; YX analyzed the data of metabolism; EA, LC, ZC, and JF revised the manuscript. All authors declare no competing financial interests.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00925>

**Supplemental Figure S1 | Principal Component analysis (PCA) of the metabolite profiles in bermudagrass under control conditions and cold stress.** NT was normal temperature of 30°C. CA was cold acclimation, which bermudagrass were treated with 4°C for 7 d and then transferred to -5°C for 8 h. NA was cold stress without acclimation, which plants were treated with -5°C for 8 h without pre-treatment with 4°C. MLT, melatonin.

**Supplemental Figure S2 | Principal Component analysis (PCA) of the metabolite profiles in bermudagrass under cold stress.** NT was normal temperature of 30°C. CA was cold acclimation, in which Bermudagrass was

treated with 4°C for 7 d and then transferred to -5°C for 8 h. NA was cold stress without acclimation, in which plants were treated with -5°C for 8 h without pre-treatment with 4°C. MLT, melatonin.

**Supplemental Figure S3 | The representative leaves of the plants under different treatments.** (A) The representative leaves of the plants under control condition; (B) The representative leaves of the plants under cold acclimation condition; (C) The representative leaves of the plants under non-cold acclimation condition. CK was control (treated without melatonin). MLT, melatonin; CA, cold acclimation; NA, non-cold acclimation.

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# Effects of Melatonin on Anti-oxidative Systems and Photosystem II in Cold-Stressed Rice Seedlings

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Melatonin (*N*-acetyl-5-methoxytryptamine) plays important role in multiple plant developmental processes and stress responses. We investigated the possible mediatory role of melatonin in growth, photosynthesis, and the response to cold stress in rice by using three different experiments: soaking seed; immersing roots, and spraying to leaves with 0, 20, or 100  $\mu$ M melatonin. After 6 days of cold stress, the growth of rice seedlings was significantly inhibited, but this inhibition was alleviated by exogenous melatonin. Furthermore, exogenous melatonin pretreatment alleviated the accumulation of reactive oxygen species, malondialdehyde and cell death induced by cold stress. Melatonin pretreatment also relieved the stress-induced inhibitions to photosynthesis and photosystem II activities. Further investigations showed that, antioxidant enzyme activities and non-enzymatic antioxidant levels were increased by melatonin pretreatments. The treatment methods of seed soaking and root immersion were more effective in improving cold stress resistance than the spraying method. The results also indicated the dose-dependent response of melatonin on rice physiological, biochemical, and photosynthetic parameters.

**Keywords:** melatonin, cold stress, reactive oxygen species, photosynthetic parameters, chlorophyll fluorescence

## INTRODUCTION

Low temperature is one of the major abiotic stresses that limits crop growth, productivity, survival and geographical distribution of plants (Mishra et al., 2011; Hu et al., 2016), especially in temperate zones and high-elevation environments (Andaya and Mackill, 2003). Exposure of plants to cold stress results in changes in multiple physiological, biochemical, molecular and metabolic processes including alternations of membrane fluidity, enzyme activities and metabolism homeostasis (Bajwa et al., 2014; Hu et al., 2016). Plants growth at low temperature also lead to oxidative stress through increasing reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion, and hydroxyl radicals (Taşgin et al., 2006; Hu et al., 2016). The accumulation of ROS causes peroxidation of lipids and oxidation of proteins within cells, resulting in inhibition to plant growth (Cao et al., 2015; Nahar et al., 2015). Therefore, to prevent the oxidative injury induced by ROS, plants have evolved an complex antioxidant system including enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and non-enzymatic antioxidants such as glutathione and proline (Erdal, 2012; Erdal et al., 2015; Ghaderian et al., 2015; Chen et al., 2016).

Melatonin (*N*-acetyl-5-methoxytryptamine), a low molecular weight organic compound, is produced by the vertebrate pineal secretory (Pape and Lüning, 2006; Tan et al., 2012). It was first isolated from bovine pineal glands (Lerner et al., 1958), act as a animals hormone, which is involved in many physiological processes including circadian rhythm (Hardeland et al., 2012), immunological enhancement (Calvo et al., 2013), antioxidative processes (Galano et al., 2011; Reiter et al., 2013), sleep physiology (Jan et al., 2009), seasonal reproductive physiology (Barrett and Bolborea, 2012), sexual behavior and temperature homeostasis (Arnao and Hernández-Ruiz, 2015). Melatonin has also been found in almost all forms of organisms, like protists, invertebrates, algae, fungi, and bacteria (Pöggeler et al., 1991; Balzer and Hardeland, 1992; Tilden et al., 1997; Sprenger et al., 1999; Hardeland and Pöggeler, 2003). Dubbels et al. (1995) and Hattori et al. (1995) first identified melatonin in plants. Thereafter, further studies showed that melatonin was exist in various plant species (Manchester et al., 2000; Ramakrishna et al., 2012; Arnao and Hernández-Ruiz, 2013; Vitalini et al., 2013). Melatonin in plants may be involved in multiple developmental processes, including root architecture (Zhang et al., 2014), circadian rhythms regulation, regulation of flower development, photosynthesis promotion, regulation of fruit ripening, chlorophyll preservation (Arnao and Hernández-Ruiz, 2009; Tan et al., 2012), leaf senescence (Byeon et al., 2012; Wang et al., 2013a) and alleviation of oxidative damages through effectively scavenging ROS and reactive nitrogen species (RNS) (Arnao and Hernández-Ruiz, 2015). In addition, melatonin has been reported to protect plants against multiple abiotic stresses, such as salt, cold, drought and pathogen infections (Yin et al., 2013; Bajwa et al., 2014; Wang et al., 2014; Shi et al., 2015a,b,c). Recently, Shi et al. (2015a) found that cold stress activated the synthesis of melatonin in bermudagrass, and exogenous melatonin improved its cold stress tolerance by scavenging ROS directly and improving the antioxidative enzymes activities. However, the underlying physiological and molecular mechanism of melatonin in the induction of tolerance to cold stresses remains unclear in plants, and the effects of different melatonin-application method have not been compared. In this study, exogenous melatonin treatment was applied to investigate the potential roles of melatonin in rice response to cold stress. In addition, the effects of three different modes of melatonin application on physiological processes have been investigated. The first method was soaking seed with different concentrations of melatonin (Liu et al., 2015). The second method was immersing roots in Hoagland's nutrient solution containing different concentrations of melatonin (Shi et al., 2015a). The third method was spraying leaves with different concentrations of melatonin solution (Hu et al., 2016). To our knowledge, these treatment methods have not been compared before. We investigated plant growth, ROS production, membrane lipid peroxide, chlorophyll fluorescence, photosynthetic parameters, antioxidant enzyme activities, and photosystem (II) PSII proteins after the melatonin pretreatment and the subsequent cold stress. The results could be helpful in understanding the physiological functions of melatonin in plants under low temperature stress.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

The seeds (*Oryza sativa* L. cv. DM You 6188) were sterilized with 3% (m/v) sodium hypochlorite for 10 min, washed with distilled water for five times and soaked in distilled water or melatonin (20 or 100  $\mu$ M) for 36 h, then placed in Petri dishes with wetted filter paper, and germinated in the dark at 28°C in a growth chamber. After 4 days, the germinated seeds were transferred into soil with half strength Hoagland's solution. The seedlings were maintained at a 14-h photoperiod, a photosynthetic photon flux density of 150  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, day/night temperatures of 28/22°C and a relative humidity of 70%.

### Cold-Stress Treatment

Twelve-day-old seedlings were washed with tap water, and dried briefly with absorbent paper to remove surface water. There were three different ways for application of melatonin. The first method (I) was soaking seeds with 20 or 100  $\mu$ M melatonin and watered with the Hoagland's nutrient solution, and soaking seeds with water as a control. The second method (II) was that the rice seedlings were watered with the Hoagland's solution containing 20 or 100  $\mu$ M melatonin for 2 days. The seedlings were grown in the Hoagland's solution as a control. The third method (III) was that the rice seedling leaves were sprayed every 4 h with 20 or 100  $\mu$ M melatonin for 2 days. The control seedlings were sprayed with a similar solution without melatonin. After melatonin pretreatment, the 14-day-old seedlings were transferred to 12°C, and the control seedlings remain in day/night temperatures of 28/22°C. After 6-day treatment, the second leaves were used for the measurements, and all experiments were repeated at least three times.

### Extraction and Quantification of Melatonin by HPLC-MS/MS

Extraction of melatonin from rice was performed as described by Stürz et al. (2011). The endogenous melatonin quantification was performed using liquid chromatography (HPLC) system (1290 LC, Agilent, USA) couple to a mass spectrum (MS) system (6470 LC-MS/MS, Agilent, USA) according to Stürz et al. (2011) with some modifications. MS-MS 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; capillary current, 59 nA; capillary, 3368 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. Separations were carried out on a 150 × 2.1 mm, 1.8  $\mu$ m, Eldath RS-C18 column. Two different solvents were used in the mobile phase: solvent A (methanol) and solvent B (methanol with 0.1% formic acid, v/v), at a flow rate of 0.3 mL/min, injection volume of 1  $\mu$ L and methanol was from 20 to 80%.

### Determination of Shoot and Root Dry Weight

After 6 days cold stress, shoots and roots were separated, then washed with tap water and rinsed twice with distilled water, gently

wiped dry with a paper towel, and then were oven dried at 80°C to constant weight for DW determination, respectively.

## Determination of Leaf Relative Water Content (RWC) and Root Activity

The RWC was measured according to Cao et al. (2015). The second leaves were excised and their fresh mass (FM) was determined immediately, after floated on deionized water for 5 h, the saturated mass (SM) was recorded. Then the leaves were dried at 80°C to constant weight to measure their dry mass (DM). The RWC was calculated from the following equation:

$$\text{RWC [\%]} = [(FM - DM)/(SM - DM)] \times 100.$$

Root activity was assessed by TTC (triphenyl tetrazolium chloride) method (Chen, 2003), and was expressed as the deoxidization ability ( $\mu\text{g g}^{-1} \text{ h}^{-1}$ ).

## Determination of Chlorophyll and Carotenoids Contents

Chlorophyll (Chl) content was measured as previously described Lichtenthaler and Wellburn (1983). Fresh leaves (0.1 g) were cut and homogenized with 20 mL of 80% (v/v) acetone in a pre-chilled mortar and pestle, then centrifuged at 8000 g for 10 min. The supernatant was separated and the absorbance was measured with a spectrophotometer (UV-1750, Shimadzu, Japan) at 663, 646 and 470 nm. The content of chlorophyll and carotenoids were calculated using the Lichtenthaler and Wellburn formula (Lichtenthaler and Wellburn, 1983) and expressed in  $\text{mg g}^{-1}$  FW.

## Determination of Electrolyte Leakage (EL) and Malondialdehyde (MDA)

Electrolyte leakage was determined using a electrical conductivity meter (DDS-309+, Chengdu, China) following Ning et al. (2015). The second leaves were taken and cut into 5 mm segments, weighted and placed in 50 mL capped polypropylene centrifuge tubes containing 25 mL distilled water. The initial electrical conductivity (EC1) of the bathing solutions was measured after the samples were incubated at 25°C for 24 h. Then, the samples and bathing solutions boiled in water for 30 min and measured electrical conductivity (EC2) again after cooling the solution to room temperature. EL was calculated using the formula:  $\text{EL} = \text{EC1}/\text{EC2} \times 100$ .

The degree of membrane lipid peroxidation in leaves was estimated by MDA content. MDA was evaluated using thiobarbituric acid assay (Nahar et al., 2015). Fresh leaves (0.5 g) was homogenized in 5 mL of trichloroacetic acid (TCA) (5%, m/v) and centrifuged at 8000 g and 4°C for 10 min. 2 mL of the supernatant was mixed with the equal volume 0.5% thiobarbituric acid. The mixture was bathed at 95°C for 30 min and then instantly cooled on ice and centrifuged at 5000 g and 4°C for 10 min. The absorbance of the supernatant was recorded at 450, 532, and 600 nm, respectively. MDA content was calculated according to Nahar et al. (2015) and expressed as  $\mu\text{mol g}^{-1}$  FW.

## Determination of $\text{H}_2\text{O}_2$ and $\text{O}_2^-$

$\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  were visually detected in leaves with nitro blue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described previously method (Yang et al., 2004). The leaves were excised at the base and immersed into NBT (1 mg/mL) solution for 2 h or into DAB (0.5 mg/mL) solution for 12 h in the dark, then boiling in ethanol (90%, v/v) for 0.5–2 h to decolorize the leaves. At least five leaves were used for each staining.

The content of  $\text{H}_2\text{O}_2$  was determined as described by Velikova et al. (2000). 0.5 g fresh leaves were homogenized with 5 mL 0.1% (m/v) TCA in an ice bath and centrifuged for 15 min at 12 000 × g and 4°C. 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The absorbance of the mixture was recorded at 390 nm. Finally, a standard curve of  $\text{H}_2\text{O}_2$  reagent was used to calculated the  $\text{H}_2\text{O}_2$  content. The content of  $\text{H}_2\text{O}_2$  was expressed as  $\mu\text{mol g}^{-1}$  FW.

Production of  $\text{O}_2^-$  was estimated following Nahar et al. (2015) with some modification. 0.2 g fresh leaves was homogenized with 2 mL 65 mM phosphate buffer (pH 7.8), and centrifuged at 10000 g and 4°C for 10 min. Then, 0.5 mL supernatant was mixed with 0.5 mL 65 mM phosphate buffer (pH 7.8) and 0.1 mL 10 mM hydroxylamine hydrochloride. After 20 min incubated at 25°C, the solution was added to 1 mL 17 mM sulfanilamide and 1 mL 7 mM naphthylamine and incubated at 25°C for 20 min. Absorbance was recorded at 530 nm. The formation  $\text{O}_2^-$  was calculated using a standard curve of a  $\text{NaNO}_2$  reagent.

## Trypan-Blue Staining

The dead cells were visually detected using a trypan-blue staining method as described by Liang et al. (2015) with some modifications. 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.

## Enzyme Extraction and Assay

Fresh leaves (0.2 g) were homogenized with 2 mL of ice-cold 50 mM phosphate buffer (pH 7.8) containing 0.2 mM  $\text{Na}_2\text{EDTA}$ , 2 mM ascorbate and 2% (m/v) polyvinylpyrrolidone using a chilled mortar and pestle. The homogenate was centrifuged at 12000 g for 15 min at 4°C. The supernatant was used for assays of specific enzymatic activities. The activity of SOD was assessed according to Giannopolitis and Ries (1977) by assay 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 analysis of POD activity was based on the oxidation of guaiacol using  $\text{H}_2\text{O}_2$  according to the method as described by Zhang and Kirham (1994). The CAT activity was measured as decline in absorbance at 240 nm due to the consumption of  $\text{H}_2\text{O}_2$  according to the method of Hamurcu et al. (2013).

## Determination of Proline, Reduced and Oxidized Glutathione (GSH and GSSG) and Soluble Sugars Contents

Proline was extracted and determined by the method described by Mahdavian et al. (2016) with a minor modification. 0.5 g of fresh leaves were homogenized 5 mL 3% (m/v) sulphosalicylic acid and the homogenate was centrifuged at 5000 g for 10 min. 2 mL supernatant was added to 2 mL glacial acetic acid and 2 mL acid ninhydrin. The mixture was incubated in a boiling water bath for 1 h and terminated in an ice bath. The reaction mixture was then extracted with 4 mL of toluene with vigorous stirring. The upper toluene phase was separated and an absorbance was recorded at 520 nm. The proline content was calculated using a standard curve and expressed as  $\mu\text{mol g}^{-1}$  FW.

Reduced glutathione (GSH) content was determined using the method of Kraj (2016) with some modifications. 0.5 g samples were extracted in an ice bath with 5 mL 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM EDTA, and then centrifuged at 12000 g for 20 min. Two milliliter 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 was measured at 412 nm. For GSSG assay, the GSH in supernatant was cleared first, and GSSG content was quantified as described by Knörzer et al. (1996). The GSH and GSSG content was calculated according to their standard curves and expressed as  $\mu\text{mol g}^{-1}$  (FW).

Soluble sugar content was measured based on the anthrone method (Azarmi et al., 2016). Fresh leaf samples (0.5 g) were homogenized with 5 mL of 80% ethanol, and the homogenate was centrifuged at 5000 g for 15 min. Then, 0.1 mL of the supernatant was mixed with 3 mL of anthrone. The reaction mixture was placed in a boiling water bath for 30 min and then cooled on ice. Absorbance was recorded at 625 nm. The concentration of soluble sugars was calculated using a glucose standard curve and exhibited as  $\text{mg g}^{-1}$  FW.

## Measurement of Photosynthetic Parameters and Chlorophyll Fluorescence

The photosynthetic rate ( $P_n$ ), stomatal conductance ( $G_s$ ), intercellular  $\text{CO}_2$  ( $C_i$ ) and water use efficiency (WUE) of leaves were measured using a potable photosynthesis system (GSF-3000, Heinz-Walz Instruments, Effeltrich, Germany). Intact leaves were measured at a temperature of 25°C, a photosynthetically active radiation (PAR) of 750  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , a  $\text{CO}_2$  concentration of 400  $\mu\text{mol mol}^{-1}$  and relative humidity between 40 and 60%.

A modulated imaging chlorophyll fluorometer (IMAGING-PAM M-Series, Heinz-Walz Instruments, Effeltrich, Germany) was used to measure chlorophyll fluorescence parameters according to the instructions provided by the manufacturer. After dark adaptation for 30 min, the fluorescence ( $F_0$ ), maximum fluorescence ( $F_m$ ) and the non-photochemical quenching (NPQ) were determined (Zhou et al., 2010). The maximal quantum yield

of PSII photochemistry in the dark-adapted state was calculated as  $F_v/F_m$ .

## Protein Analyses

Thylakoid membrane proteins were isolated as described by Fristedt et al. (2011). Denaturing 12% (w/v) polyacrylamide containing SDS was prepared by the method of Aro et al. (1993). Western blotting analysis was performed according to Chen et al. (2009). Antibodies used (all raised in rabbits) were anti-Arabidopsis D1, D2, CP43, LHCb1, LHCb2, LHCb3 and ACTIN (against 100 amino acids of recombinant actin conserved peptide). These antibodies were purchased from Agrisera Comp. (Umeå, Sweden).

## Statistical Analysis

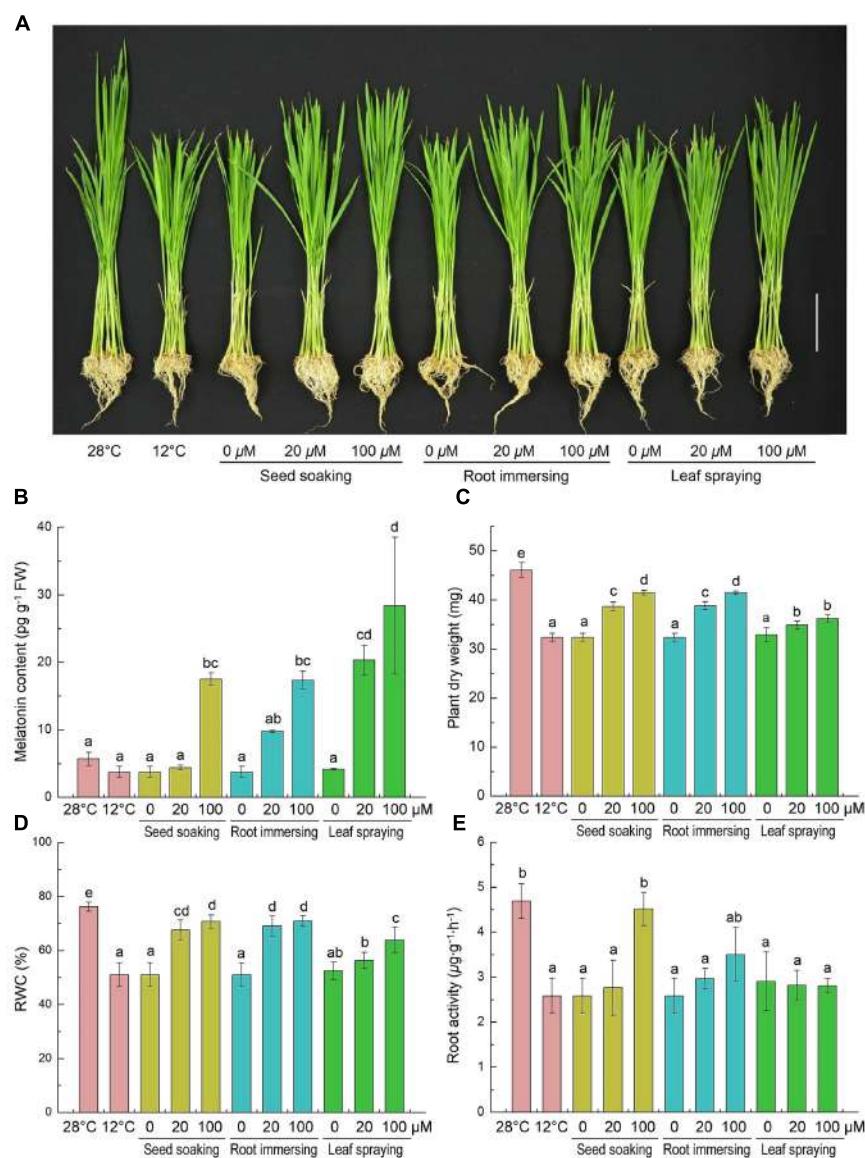
All experiments were repeated at least three times, and mean values were presented with standard deviations (SDs;  $n = 3$ ). Duncan's multiplication range test was used for comparison among different treatments. The difference was considered to be statistically significant when  $P < 0.05$ .

## RESULTS

Effects of cold stress and exogenous melatonin on growth of rice seedlings are shown in **Figure 1**. The low temperature stress inhibited the growth of rice seedlings, while melatonin pretreatment alleviated this inhibition to a certain extent (**Figure 1A**). And 100  $\mu\text{M}$  melatonin pretreated plants with higher endogenous melatonin levels grew better than that of 20  $\mu\text{M}$  melatonin pretreated plants (**Figures 1A,B**). Plant DM, RWC and root activity were significantly decreased by cold treatment compared with the control (**Figures 1C–E**). Consistently, melatonin pretreated rice seedlings exhibited significantly greater dry weight and higher RWC than control (**Figures 1C,D**). Seed soaking and root immersing methods showed better protection from cold stress than leaf spraying methods (**Figures 1C,D**). However, only Seed soaking methods in the 100  $\mu\text{M}$  melatonin showed a significant increase in root activity (**Figure 1E**). These results indicate that exogenous melatonin application improved cold stress resistance in rice seedlings.

Chlorophyll and carotenoid contents significantly decreased after 6-day cold stress (**Figures 2A,B**), and the decline of pigments caused by the cold stress was mitigated by melatonin application. The chlorophyll and carotenoid contents in melatonin-pretreated seedlings were higher than plants without melatonin pretreatment. The chlorophyll content of 100  $\mu\text{M}$  melatonin pretreated plants was higher than the plants that treated with 20  $\mu\text{M}$  melatonin with root immersing methods (**Figure 2A**). For carotenoid contents, there was no significant difference among the three methods.

The accumulations of MDA and the EL of leaf tissues are markers of lipid peroxidation and damage of cellular membranes. The content of MDA and EL increased significantly in response to cold treatment (**Figures 2C,D**). While the melatonin pretreatment significantly lowered MDA concentration and EL



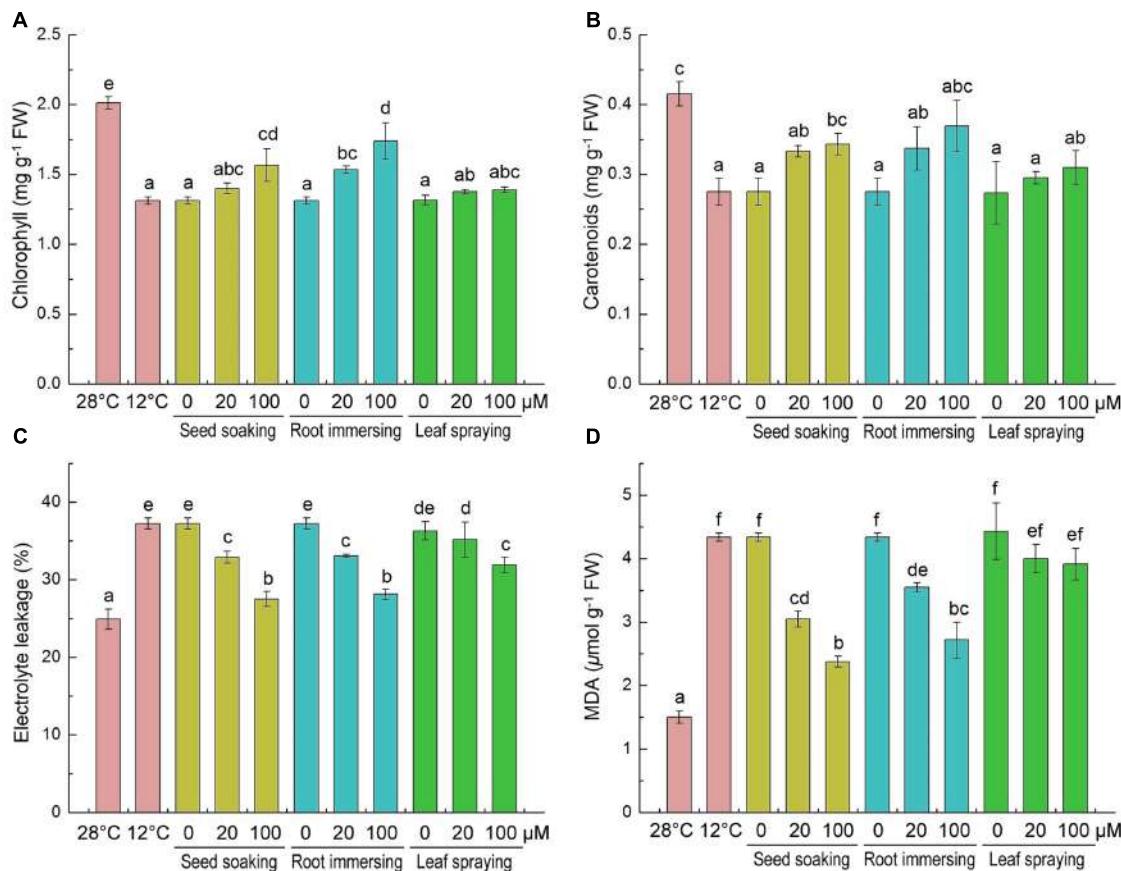
**FIGURE 1 | Effects of exogenous melatonin on growth (A), melatonin content (B), plant dry weight (C), leaf relative water content (D) and root activity (E) after 6-day cold stress (12°C). (A) Bar = 5 cm. (B–E) Data represent means  $\pm$  SDs of three replicate samples. Bars with different letters indicate significant differences according to Duncan's multiple range test at  $P < 0.05$ . I – seed soaking, II – root immersion, III – leaf spraying.**

And the 100  $\mu$ M melatonin pretreatment resulted in lower MDA content and EL than the 20  $\mu$ M melatonin pretreatment. These results indicate that melatonin effectively protected the membrane system, and seed soaking and root immersing methods provided better protective effect on membrane system against cold stress (Figures 2C,D).

$H_2O_2$  and  $O_2^-$  are two major ROS induced by environmental stresses (Chen et al., 2016). Slight  $H_2O_2$  and  $O_2^-$  staining was observed in the control leaves and intensive staining in the cold-treated plants was observed (Figures 3A,B). Histochemical staining showed that the  $H_2O_2$  and  $O_2^-$  accumulations were alleviated by melatonin application during cold stress. To validate the results of histochemical staining, we measured

$H_2O_2$  and  $O_2^-$  contents at the same time.  $H_2O_2$  and  $O_2^-$  were extensively accumulated under cold treatment, but they were lower in melatonin-pretreated plants than the control seedlings (Figures 3C,D). By seed soaking and root immersing methods of melatonin application, the 100  $\mu$ M melatonin pretreatment resulted in lower  $H_2O_2$  content than 20  $\mu$ M melatonin pretreatment. These results also suggested that the methods of seed soaking and root immersion could prevent the accumulation of ROS in leaf tissue more effectively than the method of leaf spraying (Figure 3).

We also detected cell death under cold stress with or without melatonin application by trypan-blue staining (Figure 4). After cold stress for 6 days, obvious cell death occurred in rice seedlings



**FIGURE 2 | Effects of exogenous melatonin on chlorophyll content (A), carotenoids content (B), electrolyte leakage (C) and malondialdehyde (MDA) (D) after 6-day cold stress (12°C). Others are the same as in Figure 1.**

in comparison with non-stressed plants. While, the cell dead was significantly alleviated in melatonin pretreated seedlings. The protective effect of 100  $\mu\text{M}$  melatonin pretreatment was more significant than 20  $\mu\text{M}$  melatonin pretreatment.

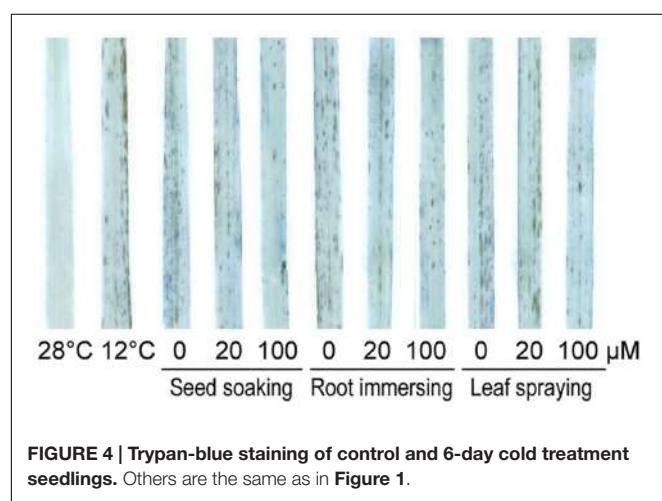
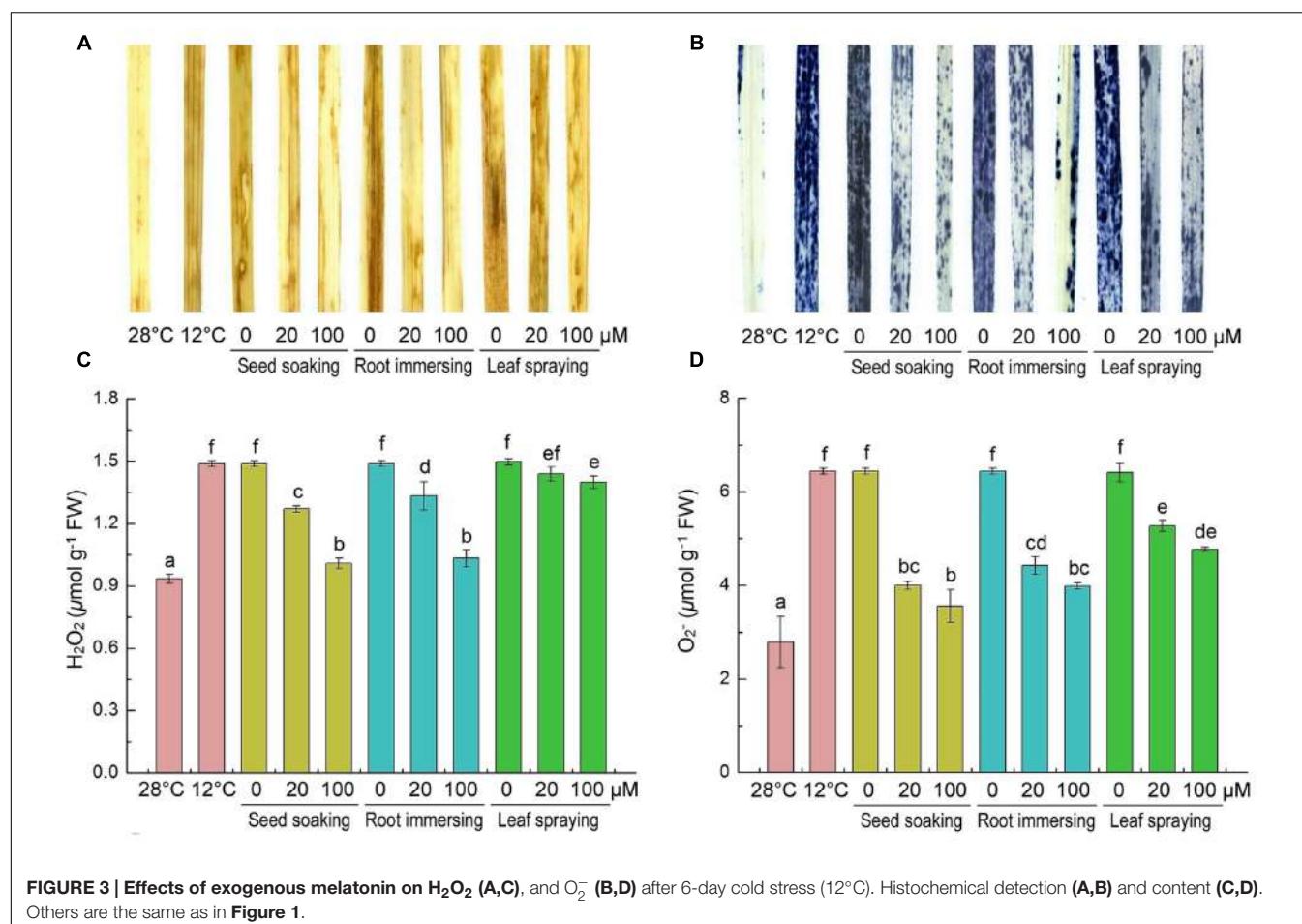
The effects of cold stress and melatonin applications on specific activities of antioxidant enzymes and on non-enzymatic antioxidants in rice seedlings are showed in Figure 5. The activities of antioxidant enzyme (SOD, CAT, and POD) were greatly induced by the cold stress (Figures 5A–C). Additionally, melatonin pretreated plants showed more higher activities of antioxidant enzymes (SOD, CAT, and POD), especially when the concentrations of melatonin were 100  $\mu\text{M}$  (Figures 5A–C). The three different methods of melatonin application did not show significant differences in SOD and POD activities (Figures 5A,B), while the seed soaking and root immersion caused more increasement of CAT activity (Figures 5C).

We also measured the contents of proline, GSH/GSSG and soluble sugar, because they are important non-enzymatic antioxidants. After cold stress for 6 days, compared with the control, the contents of proline, GSH, GSSG and soluble sugar were significantly increased (Figures 5D–F). Moreover, the melatonin application further increased their accumulation

under cold stress conditions. Meanwhile, compared to the cold treated seedlings alone, the 100  $\mu\text{M}$  melatonin pretreatment increased the proline, GSH and soluble sugar more than the 20  $\mu\text{M}$  melatonin pretreatment. Besides, the effects of three melatonin-application methods showed no significant difference (Figures 5D–F).

The effects of cold stress and melatonin application on photosynthesis were evaluated by monitoring gas exchange parameters [net photosynthetic rate ( $P_n$ ), stomatal conductance ( $G_s$ ), intercellular CO<sub>2</sub> ( $C_i$ ) and WUE]. Cold stress significantly decreased all of these parameters, as shown in Figures 6. These parameters were higher in melatonin pretreated rice seedlings than the control plants. Melatonin caused significant increases in net photosynthetic rate, stomatal conductance and intercellular CO<sub>2</sub> (Figures 6A–C), especially in the group of 100  $\mu\text{M}$  melatonin pretreatment by root immersing method. While, only the root immersing method caused a significant increase in water use efficiency (Figure 6D).

After cold treatment, the maximum photochemical efficiency of PSII (Fv/Fm) was significantly decreased, but it was higher in melatonin pretreated plants (Figures 7A,C). And there were no significant differences among the three melatonin-application methods. In contrast to the change of Fv/Fm, the

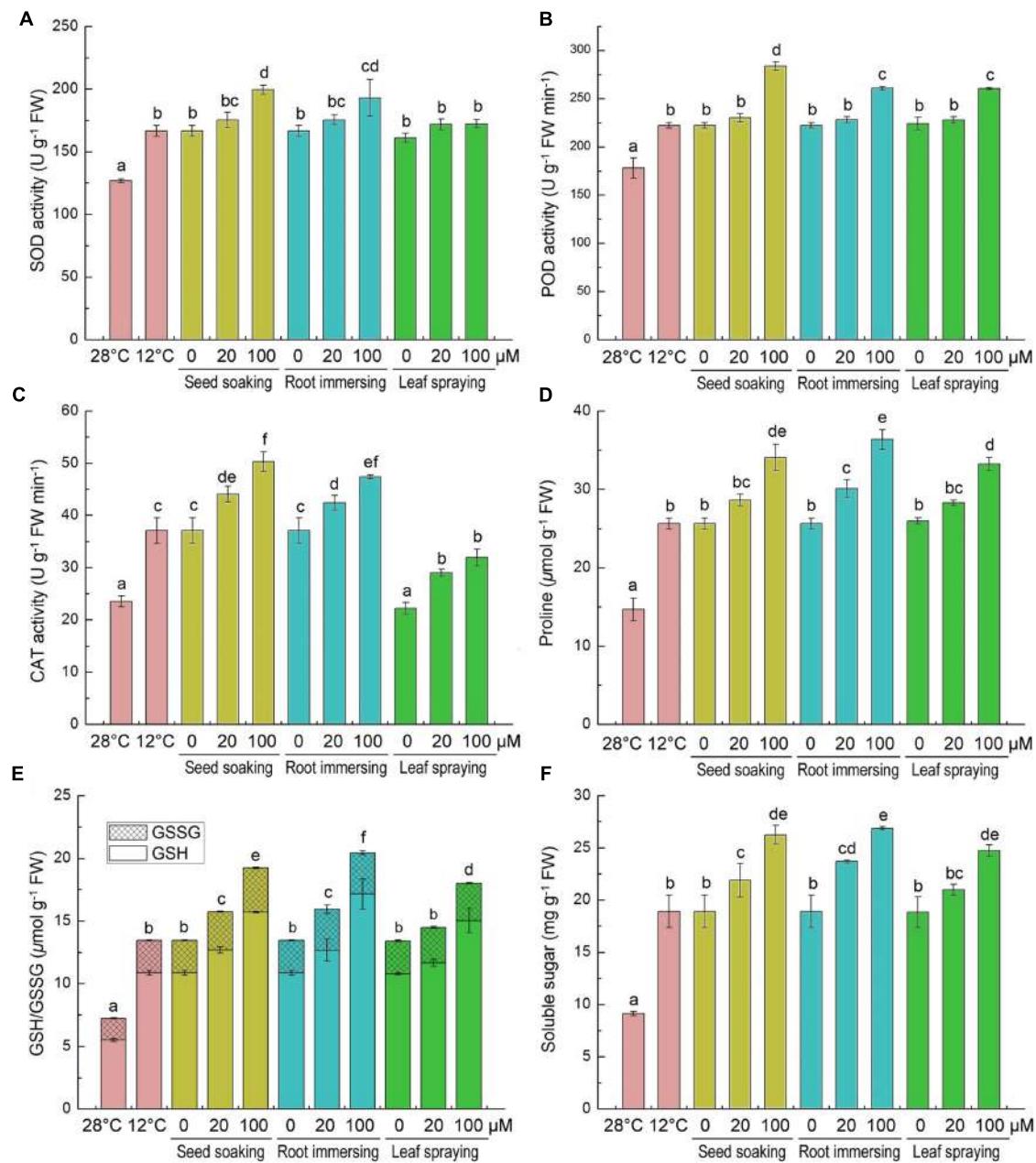


non-photochemical quenching (NPQ) was significantly increased after 6-day cold stress (Figures 7B,D). This increase was partly reversed by melatonin application (Figures 7B,D). The chlorophyll fluorescence data suggested that the seed soaking and root immersing applications were more effective than the leaf spraying (Figure 7).

PSII proteins were further detected by Western blotting. The results showed that the contents of D1, CP43, Lhcbl, and Lhcbl2 were significantly reduced after exposure to cold conditions, while the contents of D2 and Lhcbl3 proteins showed no significant change (Figure 8). However, higher D1, CP43, Lhcbl, and Lhcbl2 contents were found for the melatonin pretreated plants, compared with the seedlings without melatonin treatments. For these PSII proteins, seedlings of 100 μM melatonin pretreatment showed better protective effects than those of the 20 μM melatonin pretreatment. While the three methods did not show any significant difference.

## DISCUSSION

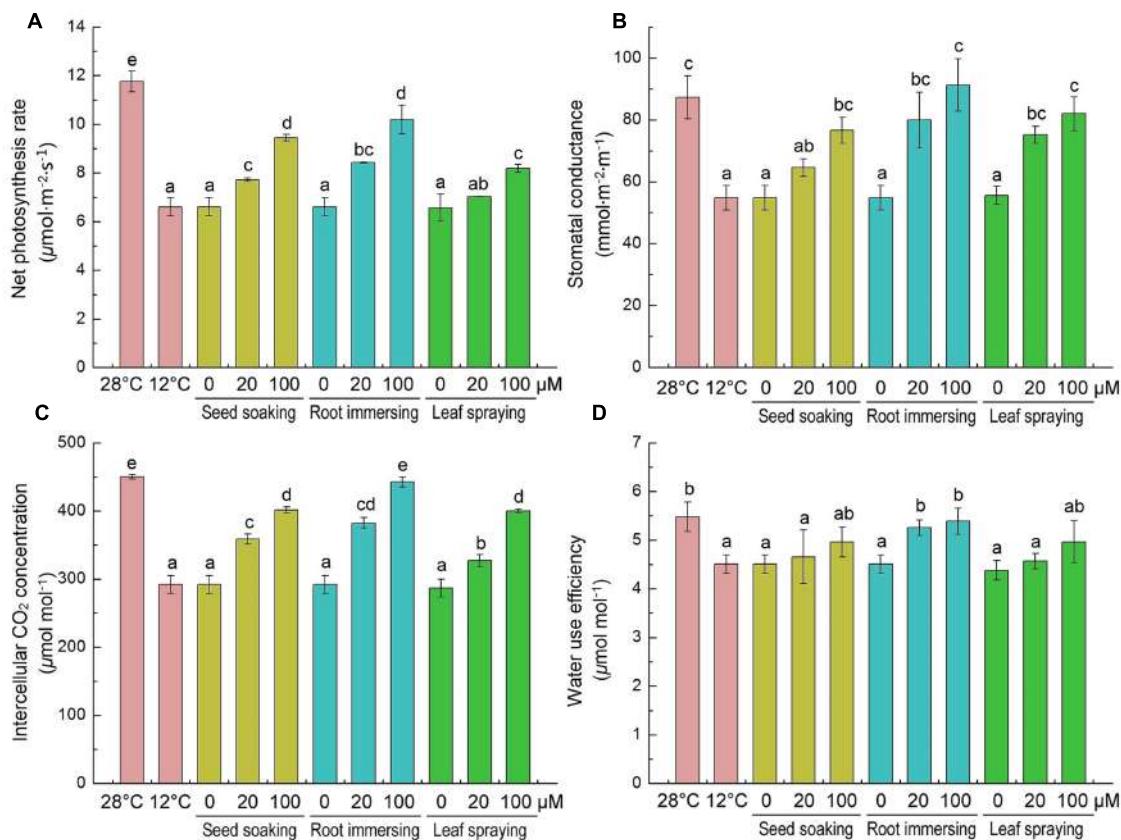
Low temperature stress critically inhibits plant growth. In the present study, we found that the growth of rice seedlings was significantly inhibited under low temperature conditions. However, the application of exogenous melatonin significantly alleviated the cold-stress-induced growth inhibition. These results are in agreement with the previous reports that melatonin application can enhance cold tolerance of *Arabidopsis* (Bajwa et al., 2014), wheat (Hulya et al., 2014), and bermudagrass



**FIGURE 5 | Effects of exogenous melatonin on SOD activity (A), POD activity (B), CAT activity (C), proline content (D), GSH content (E), and soluble sugar content (F) after 6-day cold stress (12°C). Others are the same as in Figure 1.**

(Hu et al., 2016). In the present study, we also investigated the effects of three different methods of melatonin application and our results showed the seed soaking and root immersion was more effective than leaf spraying. However, quantitative analysis of melatonin showed that the melatonin content of the leaf-spraying method was higher than the other two methods, especially in the plants treated with 100 µM melatonin. We presume that large amounts of melatonin may stay on the leaf surface and only a few was absorbed into the leaf tissue when melatonin was sprayed to the leaves.

Photosynthetic pigments are susceptible to environmental stresses (Ashraf and Harris, 2013). Chlorophylls, the most abundant and essential pigments in higher plants, responsible for capturing light energy and driving electrons to the reaction center during photosynthesis. Under biotic or abiotic stresses, seedlings have decreased chlorophyll contents due to impaired chlorophyll biosynthesis or accelerated chlorophyll degradation (Perveen et al., 2010). In the present study, we showed that chlorophylls and carotenoids were significantly decreased when the seedlings were exposed to cold stresses. However, this



**FIGURE 6 | Effects of exogenous melatonin on net photosynthetic rate ( $P_n$ ) (A), stomatal conductance ( $G_s$ ) (B), intercellular  $\text{CO}_2$  ( $C_i$ ) (C) and water use efficiency (WUE) (D) after 6-day cold stress (12°C). Others are the same as in Figure 1.**

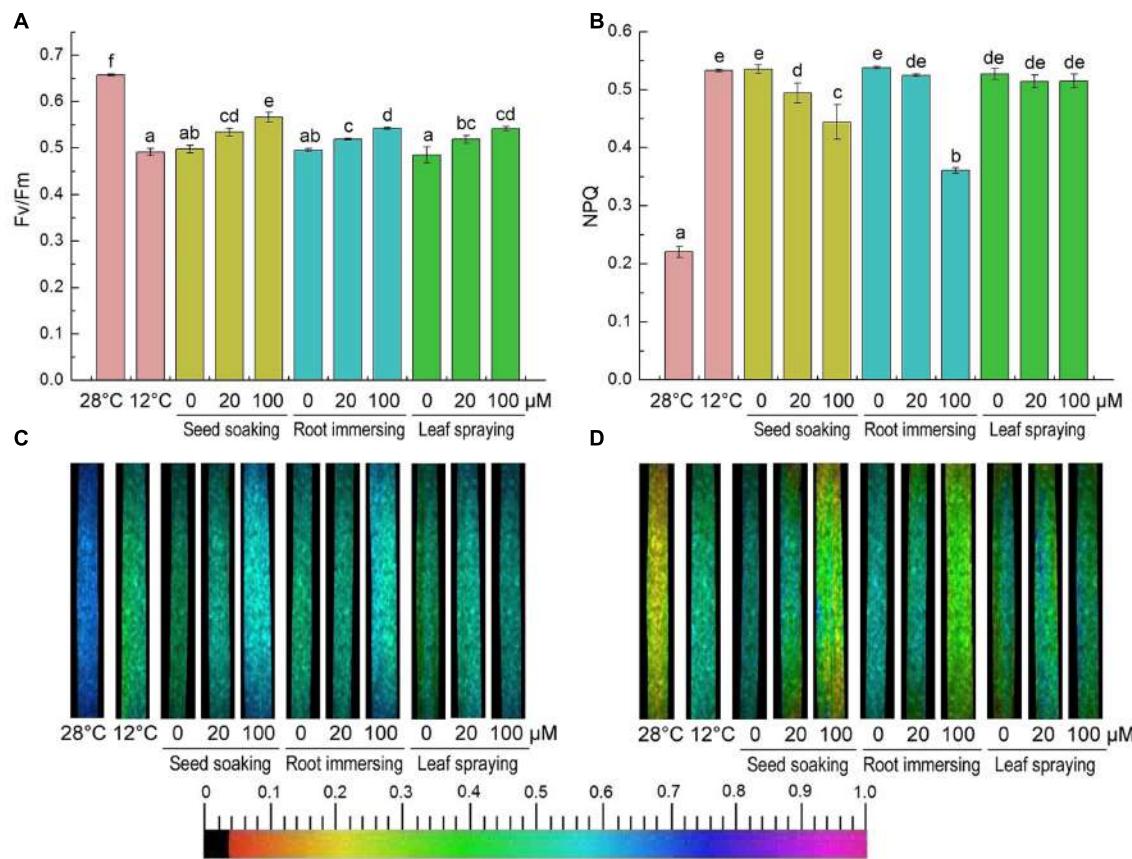
inhibitory effect of cold stress on pigments was alleviated by melatonin pretreatments, and they showed some dose-dependent response. A high concentration of 100 µM melatonin provided better protective effects than the 20 µM treatment. These results indicate that exogenous melatonin pre-treatment improved cold stress tolerance in rice seedlings.

Lipid peroxidation and the subsequent increase in MDA content are reported in previous studies during cold stress (Hulya et al., 2014; Hu et al., 2016). EL is a good indicator of the permeability of the plasma membrane and increased under stress conditions (Liu et al., 2015; Zeng et al., 2015). Compare with the control seedlings, the cold-stressed seedlings showed a 180% increase in MDA content, and a 49% increase in EL (Figures 2C,D). These findings are consistent with prior researchers that abiotic stress markedly enhanced MDA content and EL in plants (Hulya et al., 2014; Liu et al., 2015).

Reactive oxygen species are the byproducts of photosynthesis, respiration and other normal metabolic processes, and they play an important role in stress tolerance (Chen et al., 2016). ROS are also involved in programmed cell death, abiotic stress response and virus defense (Shi et al., 2015a,b,c). However, excessive accumulation of ROS can stimulate membrane lipid peroxidation, and then lead to the damage of cell membrane, loss of cellular integrity, and cell death (Chen, 2003; Zeng et al.,

2015). In the present study,  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  increased significantly under cold stress conditions. However, pretreatments with melatonin significantly decreased ROS levels in leaf tissues, and the alleviative effects were more obvious for the seed soaking and root immersion methods than the leaf spraying method.

Plants have evolved enzymatic antioxidant system or the non-enzymatic antioxidant system to maintain the redox balance. Among enzymatic antioxidant defense systems, SOD is a key enzyme that dismutates  $\text{O}_2^-$  to  $\text{O}_2$  and  $\text{H}_2\text{O}_2$ , while CAT and POD can break down  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  through different pathway in plant cells (Mittler, 2002; Hu et al., 2016). While the non-enzymatic antioxidant system plays an important role in maintaining normal cell metabolism and increasing plant tolerance to stresses (Liu et al., 2015). Melatonin is a broad spectrum antioxidant and a crucial radical scavenger and could stimulate the activities of antioxidant enzymes (Hulya et al., 2014; Hu et al., 2016), and exogenous melatonin application might activate enzymatic antioxidant system or the non-enzymatic antioxidant system to maintain the redox balance. Shi et al. (2015a) found that exogenous melatonin alleviated ROS accumulation and cold-induced oxidative damages by directly scavenging ROS and enhancing antioxidative enzymes in bermudagrass. In present study, melatonin pretreatments led to increasing in SOD, POD and CAT activities of rice seedlings. GSH



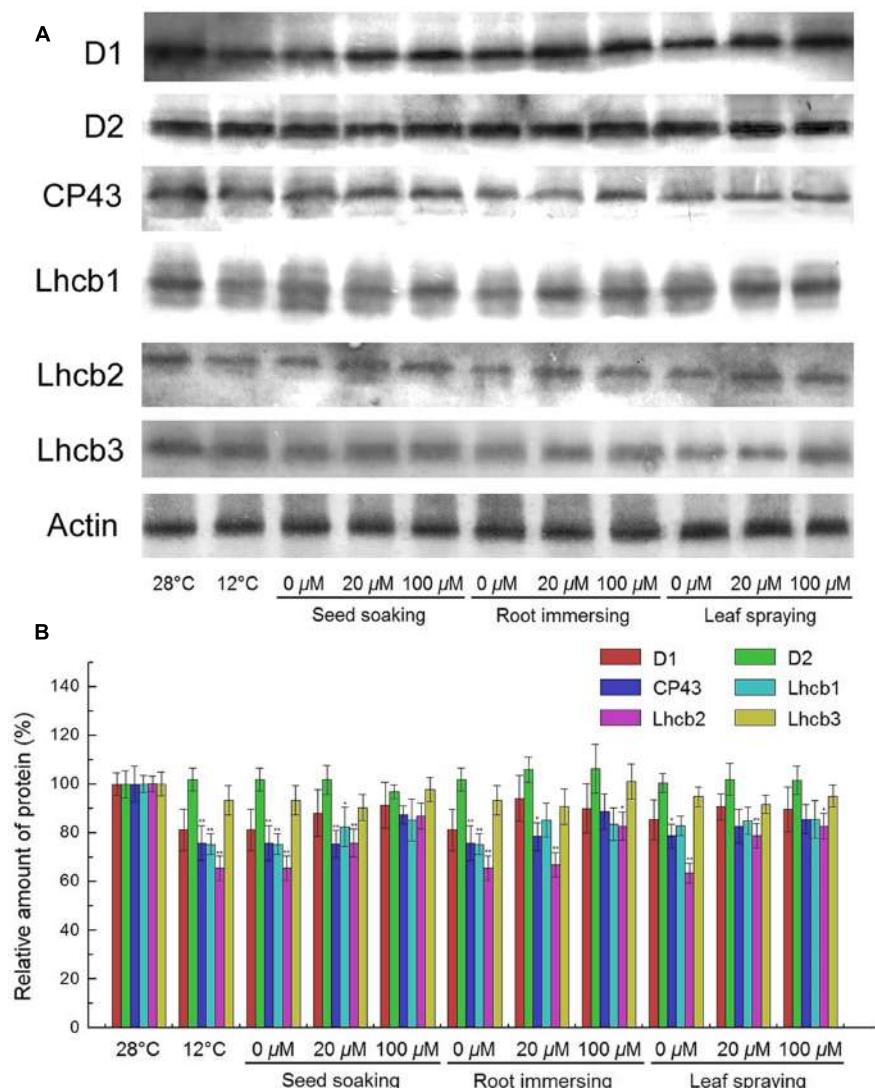
**FIGURE 7 | Effects of exogenous melatonin on maximum PSII quantum yield (Fv/Fm) (A,C), and non-photochemical quenching (NPQ) (B,D) after 6-day cold stress (12°C). Quantitative values (A,B) and fluorescence image (C,D). Others are the same as in Figure 1.**

is the main component of non-enzymatic antioxidant system in plants which is involved in direct and indirect regulations to ROS levels (Ning et al., 2015). GSH regulates the redox homeostasis of cell together with its oxidized form (GSSG) and based on the reduction potential of the GSH/GSSG couple (Szalai et al., 2009). Both of GSH and GSSG contents increased under the cold stress in this study, which could be further promoted by melatonin applications (Figures 5E). These results indicated that melatonin applications might promote GSH accumulation in plants suffered from cold stress.

Photosynthesis is an important physiological process occurring in green plants, which is the basis of the biological world, and is also an important medium for the earth's carbon and oxygen cycle. It is well known that photosynthesis is very sensitive to low temperatures (Dalal and Tripathy, 2012), which could induce the decrease of photosynthetic pigments, destruction of chloroplasts structure, and stomatal closure, and so forth (Cai et al., 2016). In the present study, rice seedlings experienced a pronounced depression in  $P_n$  under low temperature (Figure 6A), and this phenomenon has been also reported in other stress conditions (Wang et al., 2013a; Cai et al., 2016; Li et al., 2017). We observed that the decreased  $P_n$  was accompanied by significant declines in  $G_s$  and  $C_i$  (Figures 6B,C).

Therefore, we presume that decrease of  $P_n$  under low temperature conditions may be attributed to stomatal limitation. In addition, both stomatal and non-stomatal inhibitions could reduce  $P_n$ . The decrease in stomatal conductance could result in a declined  $P_n$  and reduced assimilation products, thus causing an inhibited growth and a lower yield (Easwar Rao and Chaitanya, 2016). Melatonin applications can alleviate cold-stress-induced inhibitions to photosynthetic activities, and the seed soaking and root immersion applications were more effective than leaf spraying.

Chlorophyll fluorescence analysis was one of the most powerful and widely used means for obtaining the functional status of PSII (Wang et al., 2013b), and the Fv/Fm ratio represents the maximal photochemical efficiency of PSII (Zhou et al., 2010). Fv/Fm decreased because of photoinhibition or PSII damages. While the thermal dissipation, represented by NPQ, increased (Wang et al., 2013a; Zhang et al., 2013). Under the cold stress, the value of Fv/Fm decreased significantly, but it was still higher in melatonin pretreated plants, and NPQ was also kept at a high value. Melatonin treated rice seedlings maintained lower NPQ, also suggesting that melatonin could protect photosynthetic system from cold-stress damages. Similar changes were also observed in other abiotic stresses (Wang et al., 2013a; Zhang



**FIGURE 8 | Effects of exogenous melatonin on thylakoid membrane proteins. (A)** Immunoblotting was performed with antibodies against D1, D2, CP43, Lhcb1, Lhcb2, Lhcb3 and Actin conserved peptide. Loading was according to an equal amount of chlorophyll. Results by SDS-PAGE after blue staining (CBS) are shown in the bottom panel. **(B)** The quantification of immunoblot data. Results are relative to the amount in the 28°C (100%). \*, \*\* indicate statistically significant differences at  $P < 0.05$  and  $P < 0.01$ , respectively, determined using Duncan's multiple range test. Others are the same as in Figure 1.

et al., 2013). For chlorophyll fluorescence, we did not find any significant difference among the three methods of melatonin application, while the decrease of NPQ was more obvious in the seed soaking method than the other two methods.

PSII is a multi-subunit pigment-protein complex that catalyzes the light-driven electron transfer from water to plastoquinone (PQ) in the thylakoid membrane of high plants, algae, and cyanobacteria (Baena-González et al., 2013). PSII plays an especially important role in the responses of photosynthesis in the higher plants to environment stresses (Yuan et al., 2005). Previous studies have shown that D1 protein, one of the core proteins of PSII, is the most vulnerable component in PSII reaction center under stress conditions (Luo et al., 2009; Li et al., 2017). It has been shown that the D1 protein can be

significantly damaged by environmental stresses (Luo et al., 2009). In the present study, the lighter decreases in PSII proteins indicated the protective role of melatonin to PSII under cold stress.

In summary, the applications of exogenous melatonin alleviated the cold-stress-induced inhibition to plant growth by enhancing the activities of antioxidative enzymes and the levels of non-enzyme antioxidants. Melatonin could protect photosynthetic system from oxidative damages. The melatonin application by the seed soaking and root immersion was more effective than the leaf spraying method. All the effects of melatonin depended on its concentrations and the application methods. Our results imply that melatonin could be considered as an utilizable substance to improve plant cold tolerance.

## AUTHOR CONTRIBUTIONS

SY and MY designed the experiments; Q-HH, BH, C-BD, Z-WZ, Y-EC, CH, L-JZ, YH, and J-QL performed the experiments and data analysis; and Q-HH, SY, and MY wrote the manuscript.

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# Comparative Physiological and Transcriptomic Analyses Reveal the Actions of Melatonin in the Delay of Postharvest Physiological Deterioration of Cassava

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Melatonin plays important roles in various aspects of biological processes. However, it is less known on the effects and mechanism of melatonin on the postharvest physiological deterioration (PPD) process of cassava, which largely restricts the potential of cassava as a food and industrial crop. In this study, we found that exogenous application of melatonin significantly delayed PPD of cassava tuberous roots by reducing H<sub>2</sub>O<sub>2</sub> content and improving activities of catalase and peroxidase. Moreover, 3425 differentially expressed genes by melatonin during the PPD process were identified by transcriptomic analysis. Several pathways were markedly affected by melatonin treatments, including metabolic-, ion homeostasis-, and enzyme activity-related processes. Further detailed analysis revealed that melatonin acted through activation of ROS-scavenging and ROS signal transduction pathways, including antioxidant enzymes, calcium signaling, MAPK cascades, and transcription factors at early stages. Notably, the starch degradation pathway was also activated at early stages, whereas it was repressed by melatonin at middle and late stages, thereby indicating its regulatory role in starch metabolism during PPD. Taken together, this study yields new insights into the effect and underlying mechanism of melatonin on the delay of PPD and provides a good strategy for extending shelf life and improvement of cassava tuberous roots.

**Keywords:** cassava, melatonin, postharvest physiological deterioration, reactive oxygen species, starch metabolism

## INTRODUCTION

In the late 1950s, melatonin was first identified from the bovine pineal gland (Lerner et al., 1958, 1959). In animals, melatonin exerts various biological roles, including effects on sleep physiology, circadian rhythms, sexual behavior, mood, immune system, body temperature regulation, and seasonal reproduction (Cardinali et al., 2012; Calvo et al., 2013; Reiter et al., 2014). In addition, melatonin was reported to be a direct scavenger of reactive oxygen species (ROS) and of other free radicals, but also through activating antioxidant enzymes and augmenting the efficiency of other

antioxidants (Tan et al., 1993, 2003; Barlow-Walden et al., 1995; Pablos et al., 1998; Rodriguez et al., 2004; Hardeland, 2005; Kolář and Macháčkova, 2005; Reiter et al., 2009; Galano et al., 2013; Zhang and Zhang, 2014). Dubbels et al. (1995), and Hattori et al. (1995) melatonin was discovered in plants. Further, endogenous concentrations of melatonin were found to be different in plants as function of growth state, growth location, plant tissue, and harvest time (Hattori et al., 1995; Burkhardt et al., 2001; Pape and Lüning, 2006; Hernández-Ruiz and Arnao, 2008; Arnao and Hernández-Ruiz, 2009, 2013; Murch et al., 2009; Okazaki et al., 2010; Ramakrishna et al., 2012; Wang L. et al., 2014). Subsequently, increasing evidences demonstrated that melatonin is involved in multiple biological processes in plants, such as seed germination, photo-protection, vegetative growth, flower development, leaf senescence, root architecture, fruit ripening, and response to biotic and abiotic stresses (Manchester et al., 2000; Arnao and Hernández-Ruiz, 2006; Paredes et al., 2009; Tan et al., 2012; Wang et al., 2012, 2013a,b; Zhao et al., 2013; Shi and Chan, 2014; Wang P. et al., 2014; Zhang et al., 2014b; Shi et al., 2015a,b). Because melatonin can act as an important antioxidant in animals, many studies emphasized the importance of melatonin in directly or indirectly scavenging ROS in plants (Arnao et al., 2001; Cano et al., 2006).

Cassava is the third most important crop besides rice and maize in Africa, Asia, and Latin America (Oliveira et al., 2014; Hu et al., 2015a,b). Due to its high starch content and limited input needs, cassava can provide a source of dietary carbohydrate for over 600 million people worldwide, and is also considered as a producer of industrial starch and bioethanol (Zidenga et al., 2012). The potential of cassava as a food and industrial crop is largely restricted by the rapid postharvest physiological deterioration (PPD) of the tuberous root that starts within 72 h after harvest (Zidenga et al., 2012; Vanderschuren et al., 2014). PPD is induced by mechanical injury, handling operations, and storage conditions (Vanderschuren et al., 2014). To this end, more attention has been paid to the physiology and biochemistry of PPD (Reilly et al., 2001, 2004; Iyer et al., 2010; Zidenga et al., 2012; Xu et al., 2013; Vanderschuren et al., 2014). These studies have revealed that ROS production is one of the earliest events in the PPD process, and reduction of ROS accumulation by overexpression of scavenging enzymes could lead to the delay of PPD. However, less is known on the effect and mechanism of melatonin on the PPD process of cassava tuberous roots.

In this study, to gain insight into the roles and regulatory mechanism of melatonin in PPD of cassava, we investigated the effect of melatonin on the phenotype, physiology, and gene transcription rate during the postharvest period of cassava tuberous roots, and found that melatonin plays a role in the delay of PPD by reducing ROS and improving ROS signaling network. We also found that exogenous application of melatonin alters the expression of genes involved in the starch metabolism pathway during the PPD process. This study provides insights into the roles and molecular mechanisms of the actions of melatonin in the delay of PPD in cassava.

## MATERIALS AND METHODS

### Plant Material and Treatment

Cassava tuberous roots were harvested from 10-month-old cassava varieties SC124 (*Manihot esculenta* cv SC124) grown in a greenhouse (60% humidity, 16 h light, 35/20°C). The tuberous roots were separated into two sample groups, water-treated PPD and melatonin-treated PPD. For water-treated PPD, whole tuberous roots were soaked in water for 2 h; for melatonin-treated PPD, whole tuberous roots were soaked in 100 µM melatonin for 2 h. Tuberous roots were cut into slices approximately 5 mm thick, then were moved into petri dishes containing a wet filter paper, and then were incubated at 28°C and 60% relative humidity in the dark (Vanderschuren et al., 2014). After incubation for 0, 6, 12, 24, 48, and 72 h, the slices were sampled and frozen in liquid nitrogen until extraction of total RNA and transcriptomic analysis. Each sample contains four replicates from independent experiments.

### Determination of H<sub>2</sub>O<sub>2</sub> Content and Antioxidant Enzyme Activities

The activities of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) and H<sub>2</sub>O<sub>2</sub> content were measured spectrophotometrically. Approximately 0.5 g of roots slices was ground and homogenized in 5 mL of extraction buffer with 0.05 M phosphate buffer (pH7.8). The homogenate was centrifuged at 10000 g for 10 min at 4°C and the resulting supernatant was collected for analysis of H<sub>2</sub>O<sub>2</sub> content and enzyme activities. Total POD activity was examined according to the changes in absorbance at 470 nm (Polle et al., 1994). H<sub>2</sub>O<sub>2</sub> content and activities of CAT and SOD were detected using H<sub>2</sub>O<sub>2</sub>, CAT and SOD Detection Kits (A064, A007 and A001, Nanjing Jiancheng, Nanjing city, China) according to manufacturer's instructions. Each sample contains four replicates consisting of three root slices each.

### Transcriptomic Analysis

Total RNA was extracted from tuberous roots of SC124 variety after 6, 12, and 48 h of incubation with or without melatonin according to the manufacturer's instructions for its plant RNA extraction kit (DP432, TIANGEN, Beijing city, China). Each sample contains three replicates from independent experiments. Three micrograms of total RNA from each sample were converted into cDNA with an AMV Reverse Transcriptase (Promega, Madison, WI, USA). Eighteen cDNA libraries were generated and sequenced by the use of Illumina GAI following the Illumina RNA-seq protocol. A total of 1667.1 million 51-bp raw reads was produced from the 18 samples. Using FASTX-toolkit, adapter sequences in the raw sequence reads were removed. After examining the sequence quality and removing low quality sequences by FastQC, 1589.5 million clean reads were generated. Using Tophat v.2.0.10, 84.2% clean reads were mapped to the cassava reference genome (version 4.1) (Trapnell et al., 2009). The transcriptome assemblies were performed by Cufflinks with alignment files (Trapnell et al., 2012). Gene expression levels were calculated as Fragments Per Kilobase

of transcript per Million mapped reads (FPKM). Differentially expressed genes (DEGs) were identified by DEGseq according to the read count for each gene (Wang et al., 2010).

## Statistical Analysis

Statistical analyses were carried out with SPSS (SPSS Inc., Chicago, IL, USA). Physiological data was analyzed by Duncan's multiple range test. Means denoted by the same letter do not significantly differ at  $P < 0.05$  (lower case letters) or  $P < 0.01$  (upper case letters) ( $n = 4$ ). Transcriptomic data was analyzed using Student's *t*-tests at a significance level of \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  ( $n = 3$ ).

## RESULTS

### Exogenous Application of Melatonin Delayed PPD of Cassava Tuberous Roots

To investigate the effect of melatonin on PPD of cassava tuberous roots, SC124 variety widely planted in China were treated with water (control) or melatonin for 2 h, then the PPD symptoms were observed at 0, 6, 12, 24, 48, and 72 h after treatment (Figure 1). After 6 h of water treatment, PPD symptoms could be observed in the tuberous roots with slight 'vascular streaking.' Further, 'vascular discoloration' symptom with brown color becoming spread on the whole surface of cassava tuber slices became visible after 12 h of water treatment. In comparison to water treatment, melatonin-treated cassava tuberous roots showed 'vascular streaking' after 12 h of treatment and 'vascular discoloration' after 48 h of treatment. These results indicate that exogenous application of melatonin delays PPD occurrence in cassava tuberous roots.

### Exogenous Application of Melatonin Decreased H<sub>2</sub>O<sub>2</sub> Content and Improved Activities of CAT and POD during the PPD Process

To test whether melatonin-induced delay in PPD is related to ROS scavenging in tuberous roots during the postharvest period, H<sub>2</sub>O<sub>2</sub> content was examined at different time points.

Generally, H<sub>2</sub>O<sub>2</sub> content increased in the tuberous roots of SC124 during 0–72 h postharvest with or without melatonin treatment, indicating the increasing of oxidative damage during postharvest. Compared with water-treated samples, melatonin-treated cassava tuberous roots showed lower H<sub>2</sub>O<sub>2</sub> accumulation during the 6 to 72 h postharvest period (Figure 2).

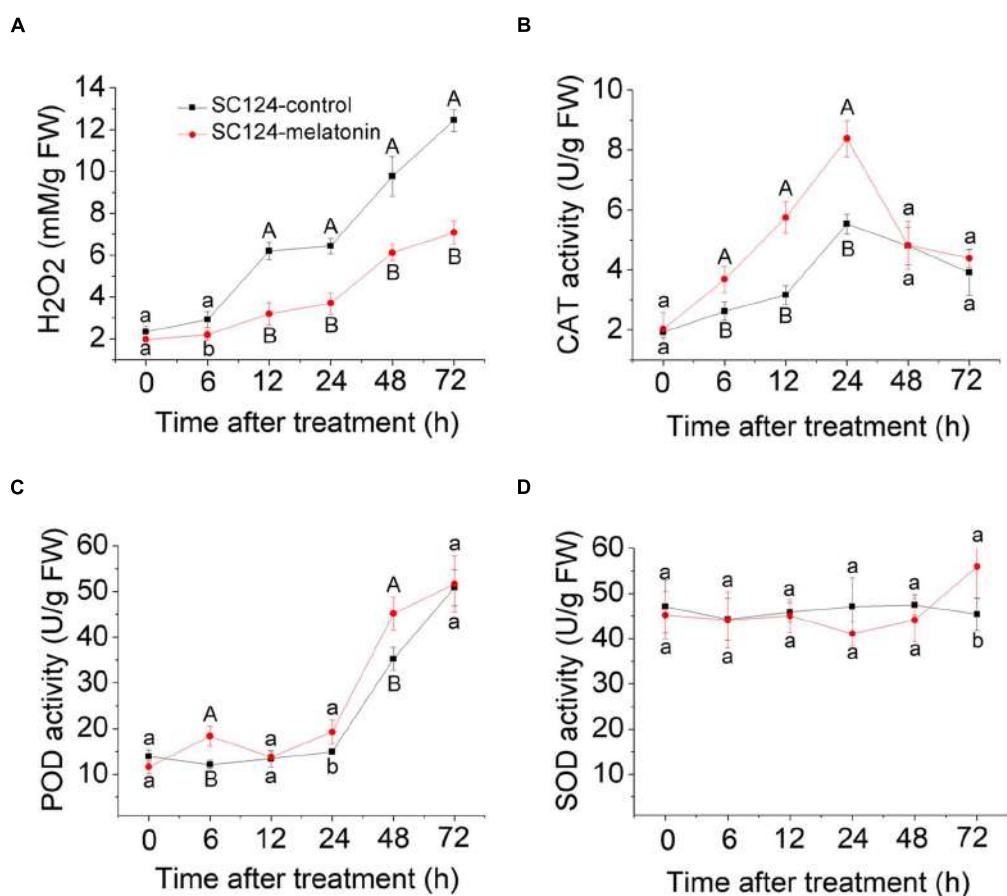
To detect whether melatonin-induced ROS scavenging is related to the activities of antioxidative enzymes during the postharvest period, CAT, POD, and SOD activities were tested at different time points (Figure 2). During the PPD process, CAT activity was significantly higher in melatonin-treated cassava tuberous roots than that in control samples at stages of 6, 12, and 24 h after treatment. Additionally, POD activity significantly increased in melatonin-treated samples compared with controls at stages of 6, 24, and 48 h after treatment. There were no significant differences for SOD activity between controls and melatonin-treated samples. These results indicate that increased activities of CAT and POD may be involved in melatonin-induced ROS scavenging during the PPD process.

### Differential Expression Profiling between Water-Treated and Melatonin-Treated Cassava Tuberous Roots

To study the transcriptionally regulatory mechanism underlying the melatonin-mediated delay of PPD in cassava, we performed comparative transcriptomic analysis between water-treated and melatonin-treated SC124 cassava tuberous roots during PPD. A total of 3425 differentially expressed genes (DEGs) (fold change  $\geq 2$ ;  $P$ -value  $\leq 0.05$ ) were identified by exogenous melatonin treatment, including 1439 (1079 up- and 360 down-regulated) genes from M6/CK6, 1211 genes from M12/CK12 (531 up- and 680 down-regulated), and 1844 genes from M48/CK48 (682 up- and 1162 down-regulated) (Figure 3A; Supplementary Tables S1–S4). Gene ontology (GO) enrichment analysis showed that many metabolic-, ion homeostasis-, and enzyme activity-related pathways were extensively changed after melatonin treatment during PPD (Supplementary Figure S1; Supplementary Table S5). Notably, some genes associated with response to oxidative stress, catalytic activity, antioxidant activity, and oxidation-reduction were enriched, indicating that melatonin-induced antioxidant response was activated during PPD process.



**FIGURE 1 | Effect of melatonin on PPD of tuberous roots in SC124 variety.** After soaking water (control) or 100  $\mu$ M melatonin (melatonin treatment) for 2 h, cassava tuberous roots were sliced into 5-mm-thick slices, following incubation in constant temperature of 28°C in the dark. Photos were taken at 0, 6, 12, 24, 48, and 72 h after treatment.



**FIGURE 2 | Effect of melatonin on H<sub>2</sub>O<sub>2</sub> accumulation (A) and activities of CAT (B), POD (C), and SOD (D) during the PPD process in SC124 variety.**  
Data are means  $\pm$  SE calculated from four biological replicates. Means denoted by the same letter do not significantly differ at  $P < 0.05$  (lower case letters) or  $P < 0.01$  (upper case letters) as determined by Duncan's multiple range test.

Additionally, Venn diagram analysis showed the amounts of 168 DEGs (fold change  $\geq 2$ ;  $P$ -value  $\leq 0.05$ ) present in all three stages, indicating that these genes were involved in the melatonin-mediated delay of PPD during 6–48 h (Figure 3B). Among these, some genes related to ROS scavenging were found, suggesting their crucial roles in the melatonin-mediated delay of PPD in cassava tuberous roots (Supplementary Table S6). GO enrichment analysis indicated that several pathways were significantly affected by melatonin treatments, including carbohydrate metabolic process, polysaccharide catabolic/metabolic process, biological process, glucosidase activity, hydrolase activity, and catabolic activity (Conesa et al., 2005) (Supplementary Figure S2; Supplementary Table S7).

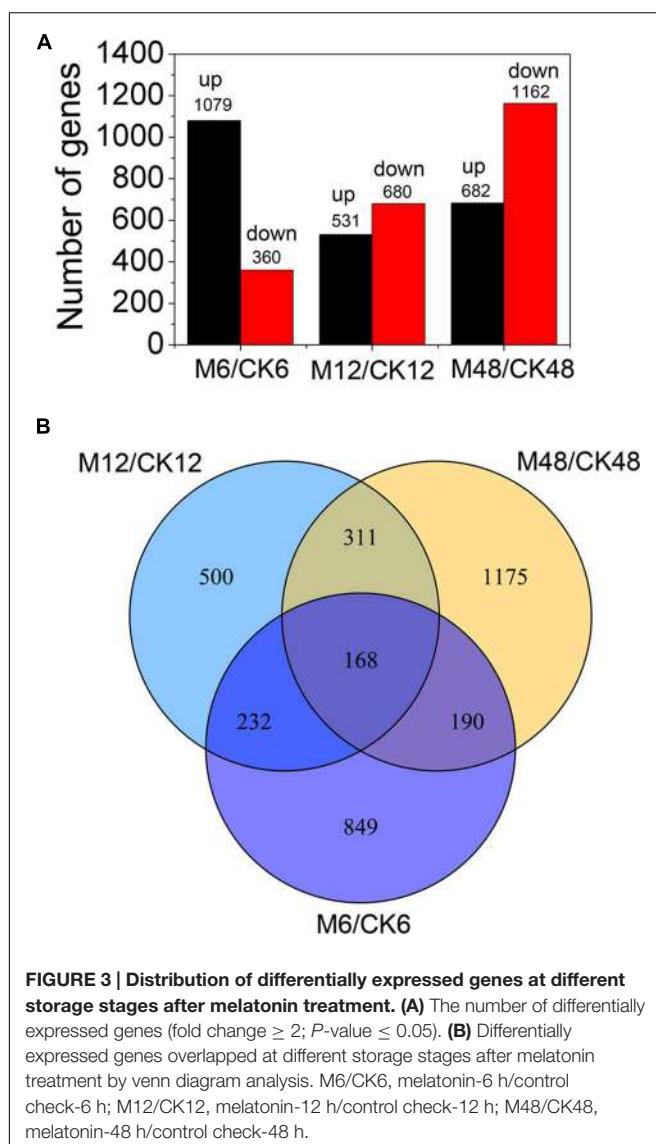
### Melatonin-Mediated ROS Scavenging Activity by Antioxidant Enzymes during PPD Process

To better understand the action of melatonin on ROS scavenging during the PPD process, 34 genes encoding antioxidant enzymes were identified from the DEGs (fold change  $\geq 2$ ;  $P$ -value  $\leq 0.05$

at least in one stage of PPD). Heat map analysis indicated that 30, 10, and 8 genes were up-regulated after 6, 12, and 48 h melatonin treatment, respectively; whereas 4, 24, and 25 genes were down-regulated after 6, 12, and 48 h of melatonin treatment, respectively (Figure 4; Supplementary Table S8). From these data, it can be concluded that many genes were transcriptionally induced at an early storage stage (6 h), whereas they were repressed following middle (12 h) and late stages (48 h), indicating the rapid activation of the expression of genes encoding antioxidant enzymes by melatonin.

### Melatonin-Mediated ROS Signaling Network during the PPD Process

To gain insight into the melatonin-mediated ROS signaling network during the PPD process, 161 genes related to ROS response were identified from the DEGs (fold change  $\geq 2$ ;  $P$ -value  $\leq 0.05$  at least in one stage of PPD), including calcium signaling-, phospholipase signaling-, MAPK cascades-, NADPH oxidase-, and transcription factor-related genes (Figure 5; Supplementary Table S9). Expression profiles showed that 114, 98, and 83 genes showed up-regulation after 6, 12, and 48 h melatonin



treatment, respectively; whereas 47, 61, and 76 genes showed down-regulation after 6, 12, and 48 h melatonin treatment, respectively. This indicates that more ROS-responsive genes were activated at early stages relative to middle and late stages by melatonin.

## Melatonin-Mediated Starch Metabolism during the PPD Process

To get some clues on the roles of melatonin in regulating the starch synthesis and degradation processes, 17 starch metabolic associated genes were identified from the DEGs (fold change  $\geq 2$ ;  $P$ -value  $\leq 0.05$  at least in one stage of PPD). Transcriptomic data indicated that 9, 3, and 3 genes were up-regulated after 6, 12, and 48 h melatonin treatment, respectively; whereas 8, 14, and 14 genes were down-regulated after 6, 12, and 48 h melatonin treatment, respectively (Figure 6; Supplementary Table S10). Genes related to starch synthesis, including sucrose synthase,

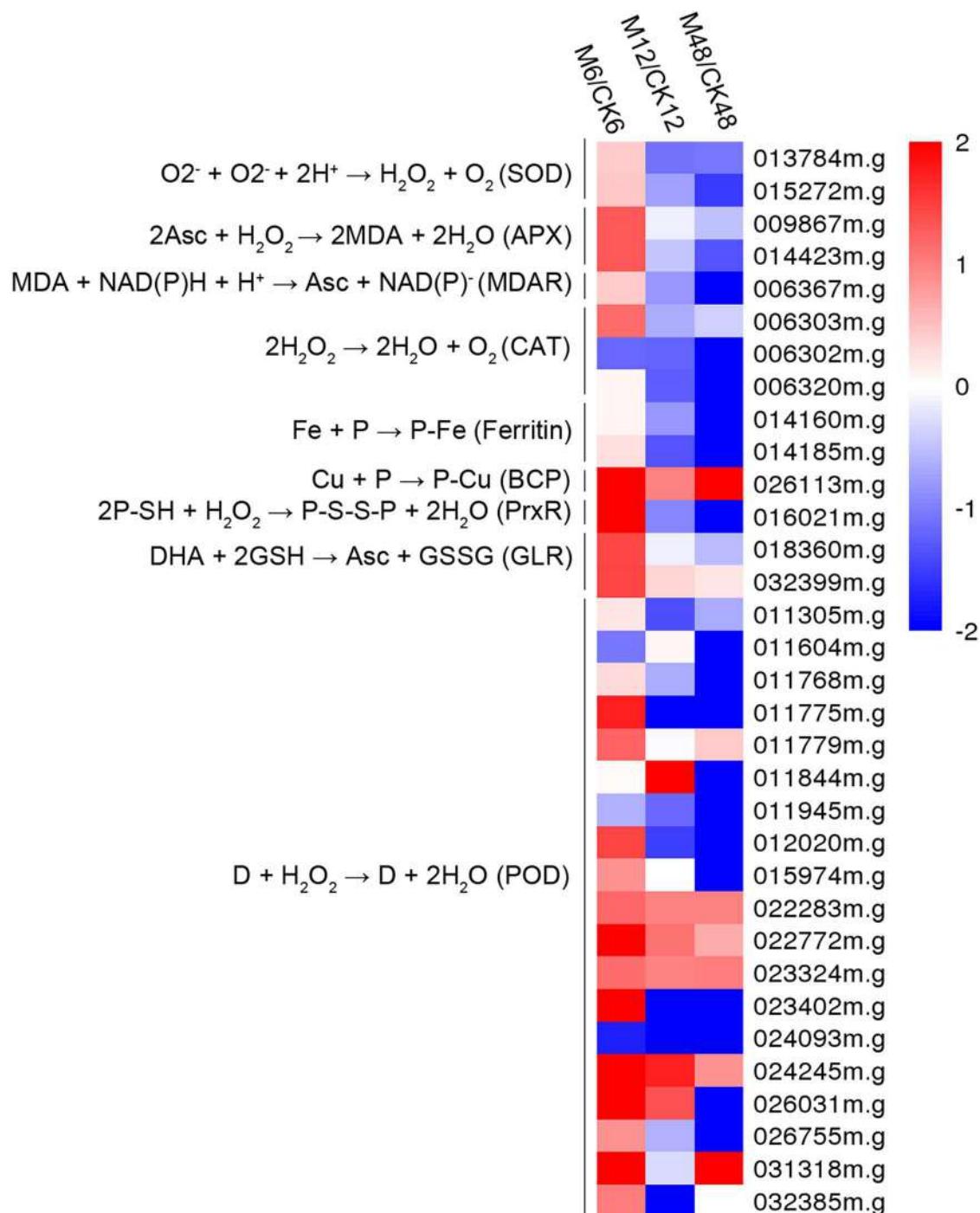
glucose phosphomutase, and ADP-glucose pyrophosphorylase were mainly repressed, indicating the repression of starch synthesis process by melatonin during PPD. Most of genes involved in starch degradation, such as starch phosphorylase, alpha-amylase, and beta-amylase were induced at the early stage (6 h); however, repressed at the middle and late stages (12 and 48 h). These results suggest that melatonin may be a crucial regulator of starch metabolism during PPD process of cassava tuberous roots.

## DISCUSSION

Cassava is one of the most important crop in Africa, Asia, and Latin America. Nevertheless, the rapid PPD of its tuberous roots, a unique phenomenon compared with other root crops, renders the roots unpalatable and unmarketable, thereby adversely impacting farmers, processors, and consumers alike (Xu et al., 2013). Previous studies demonstrated that ROS production is one of the earliest events in the deterioration process and reduction of ROS accumulation could lead to a delayed onset of PPD (Reilly et al., 2001, 2004; Iyer et al., 2010; Zidenga et al., 2012; Xu et al., 2013; Vanderschuren et al., 2014). Melatonin was reported to function in the reduction of ROS through scavenging ROS directly and enhancing the levels of antioxidants and the activities of antioxidative enzymes (Tan et al., 1993, 2003; Barlow-Walden et al., 1995; Pablos et al., 1998; Rodriguez et al., 2004; Hardeland, 2005; Kolář and Macháčkova, 2005; Reiter et al., 2009; Galano et al., 2013; Zhang and Zhang, 2014; Zhang et al., 2014a; Shi et al., 2015d). Therefore, we hypothesized that exogenous application of melatonin might delay PPD of cassava tuberous roots by scavenging ROS. To confirm this, cassava tuberous roots were treated with melatonin. Compared with controls, melatonin-treated cassava tuberous roots showed delayed PPD both for 'vascular streaking' and 'vascular discoloration' symptoms, indicating that melatonin has a clear effect on PPD (Figure 1). Physiological analyses also support that melatonin-mediated delay of PPD is related to reduced  $H_2O_2$  and increased activities of CAT and POD (Figure 2).

To provide the possible molecular evidence, transcriptomic analysis was carried out between water-treated and melatonin-treated cassava tuberous roots during the PPD process. Among the DEGs, 30 out of 34 genes encoding enzymes associated with ROS scavenging, including *SOD*, *POD*, *CAT*, *APX*, *PrxR*, were transcriptionally induced by melatonin at early storage stage (6 h) during PPD (Figure 4; Supplementary Table S8). These genes provide cells with highly efficient machinery for detoxifying  $O_2^-$  and  $H_2O_2$  in plants (Mittler et al., 2004). Accordingly, the decreased  $H_2O_2$  content, increased activities of POD and CAT, and lightened PPD symptoms after melatonin treatment were also observed at early storage stage (6 h) during PPD (Figures 1 and 2). These results further support that the melatonin mediated ROS-scavenging system may contribute to reducing oxidative injury of cassava tuberous roots.

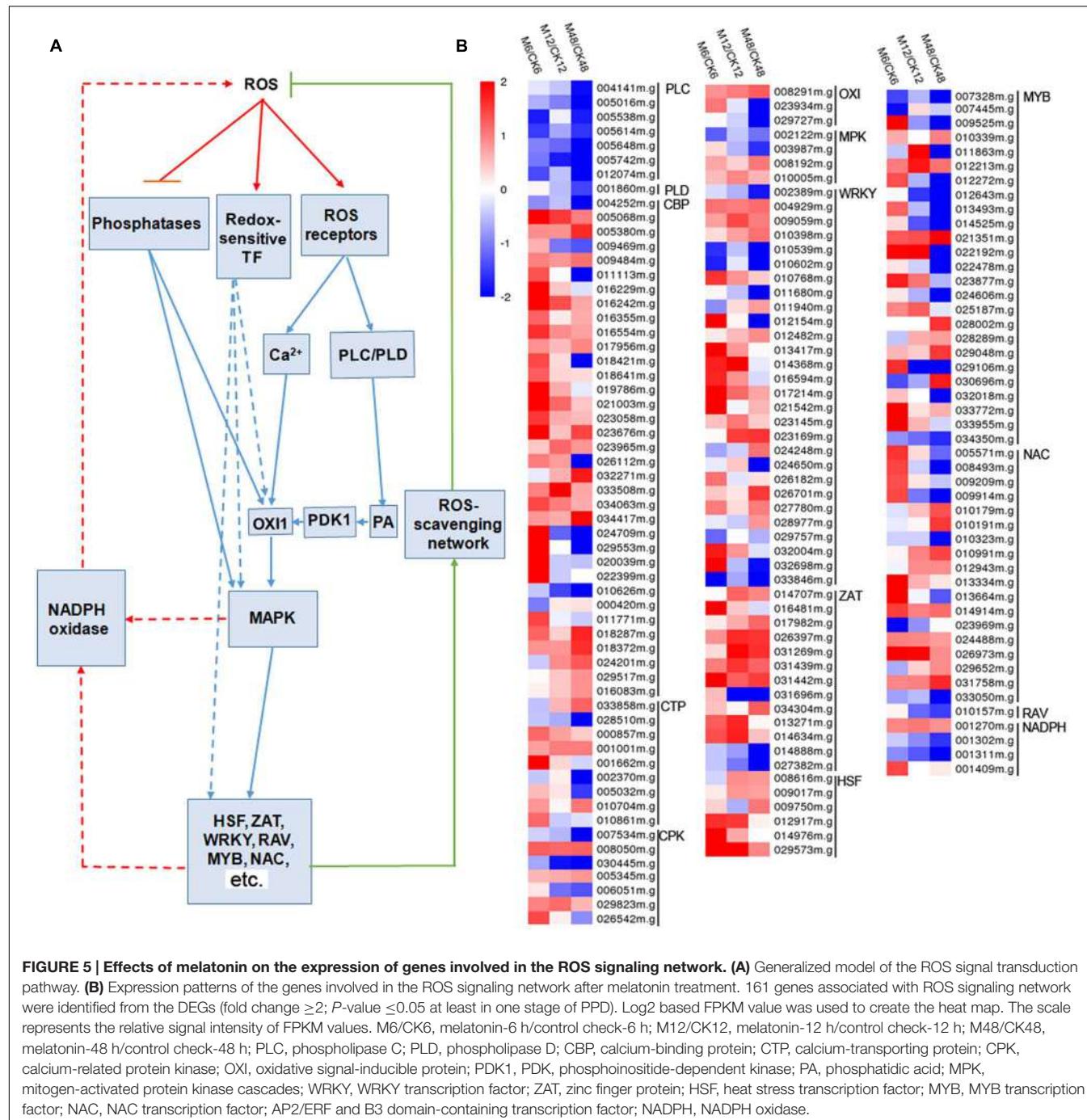
Previous studies demonstrated that an oxidative burst occurred at the early stage of PPD (Reilly et al., 2001, 2004; Iyer



**FIGURE 4 | Effects of melatonin on the expression of genes encoding antioxidant enzymes.** Thirty-three genes encoding antioxidant enzymes were identified from the DEGs (fold change  $\geq 2$ ;  $P$ -value  $\leq 0.05$  at least in one stage of PPD). Log2 based Fragments Per Kilobase of transcript per Million mapped reads (FPKM) value was used to create the heat map. The scale represents the relative signal intensity of FPKM values. M6/CK6, melatonin-6 h/control check-6 h; M12/CK12, melatonin-12 h/control check-12 h; M48/CK48, melatonin-48 h/control check-48 h; SOD, superoxide dismutase; APX, ascorbate peroxidase; MDAR, monodehydroascorbate reductase; CAT, catalase; BCP, blue copper protein; PrxR, peroxiredoxin; GLR, glutaredoxin; POD, peroxidase.

et al., 2010; Zidenga et al., 2012; Xu et al., 2013; Vanderschuren et al., 2014). Coincidentally, melatonin acted through scavenging ROS by antioxidant enzymes, thus assisting in reduction of

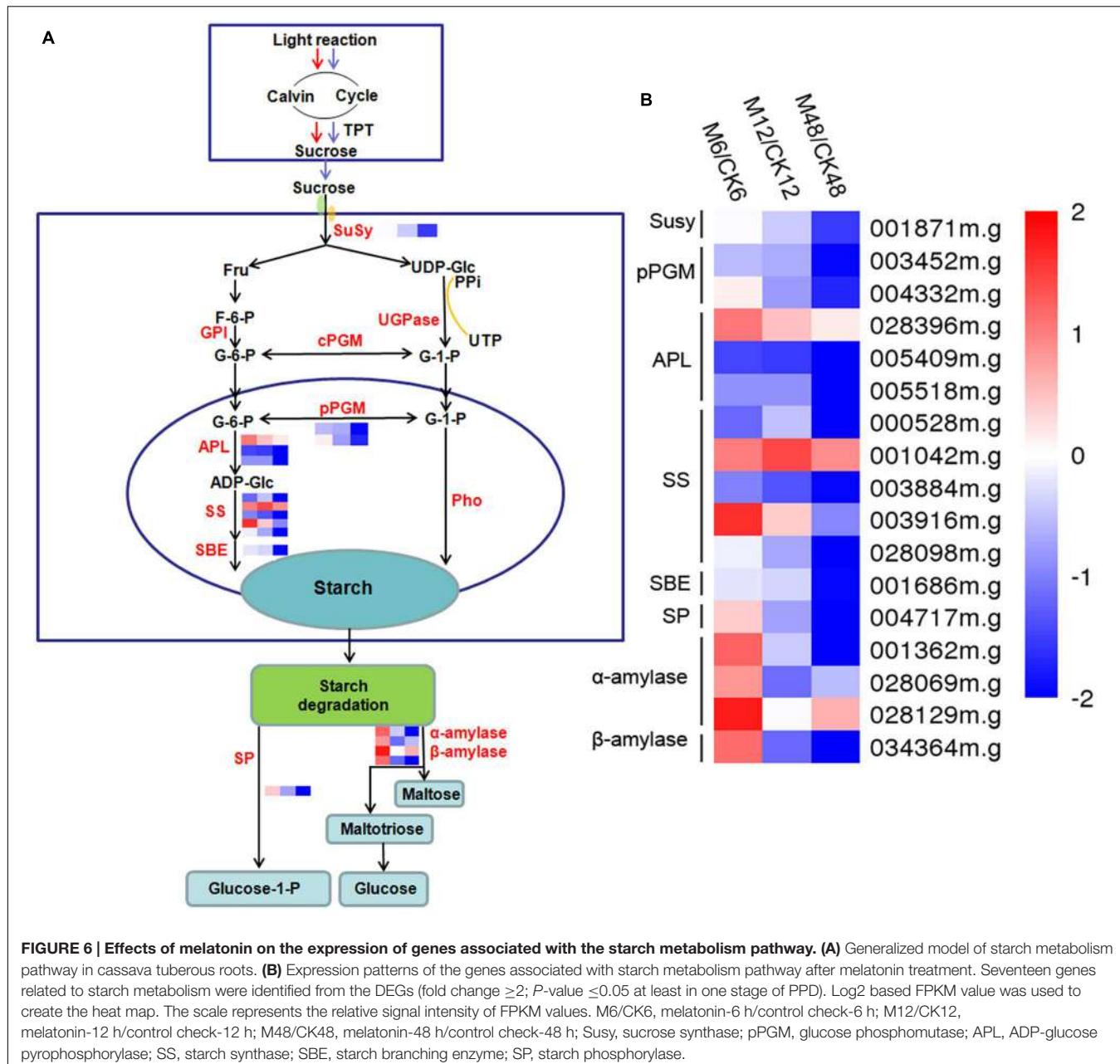
cellular ROS levels at early stage. After oxidative burst, cells still need to maintain a homeostasis of ROS. Thus, the downregulation of genes encoding ROS-scavenging enzymes at



middle (12 h) and late stages (48 h) by melatonin implied that melatonin may be a flexible regulator of ROS during the PPD process (Figure 4; Supplementary Table S8). In addition, although melatonin repressed the expression of genes encoding ROS-scavenging enzymes at middle and late stages, melatonin-treated samples still maintained lower H<sub>2</sub>O<sub>2</sub> levels and higher activities of CAT and POD relative to control samples (Figures 1 and 2). This implies that melatonin may extend the effects of early transcriptional activation to enzyme activity levels at middle and

late stages through a series of post-transcriptionally regulatory mechanisms.

Plant cells sense ROS through at least three different mechanisms, including unidentified receptor proteins, redox-sensitive transcription factors, and direct inhibition of phosphatases. Downstream signaling events include calcium and phospholipid signaling pathways, and hence activate oxidative signal-inducible protein (OXI1), MAPK cascades, NADPH oxidase, and transcription factors (Mittler et al.,



2004; Kim et al., 2011). To address the question how does melatonin regulate cells to perceive and transduct ROS signaling, those genes related to the ROS network were identified in the melatonin-treated cassava tuberous roots. Notably, many calcium signaling pathway connected genes, including calcium-sensing, -transport, -transduction genes, and downstream transcription factors, were significantly induced, whereas almost all of the phospholipase C (*PLC*) and phospholipase D (*PLD*) genes in phospholipid signaling pathway were repressed by melatonin during the PPD process (Figure 5; Supplementary Table S9). Accordingly, phosphoinositide-dependent kinase (PDK1), a downstream component of phospholipid signaling pathway, did not show significant difference at transcriptional levels with

melatonin treatment. This suggests that melatonin-induced ROS signaling is positively transduced through calcium signaling components. In response to melatonin, 14 genes related to calcium-dependent signaling were induced in *Arabidopsis* by RNA-seq analysis (Weeda et al., 2014). Calcium-related protein kinase (CRK), calcium-dependent protein kinase (CDPK), and calcineurin B-like (CBL)-interacting protein kinase (CIPK) were reported to be commonly regulated by melatonin treatment in Bermuda grass (Shi et al., 2015a). Many transcription factors, including WRKY, NAC, ZAT, and HSF were up-regulated after melatonin treatment and some of them were confirmed to play a melatonin-mediated protective role in abiotic stress response (Shi and Chan, 2014;

Zhang et al., 2014b; Shi et al., 2015a,c). Also, the crosstalk between melatonin and calcium signaling has been demonstrated to modulate various calcium-dependent cellular functions in animal cells (Posmyk and Janas, 2009). Additionally, calmodulin proteins were also reported to be up-regulated during early PPD of cassava (Owiti et al., 2011). Therefore, the extensive activation of calcium signaling and related transcription factors may be an important event in melatonin-mediated ROS pathway during PPD.

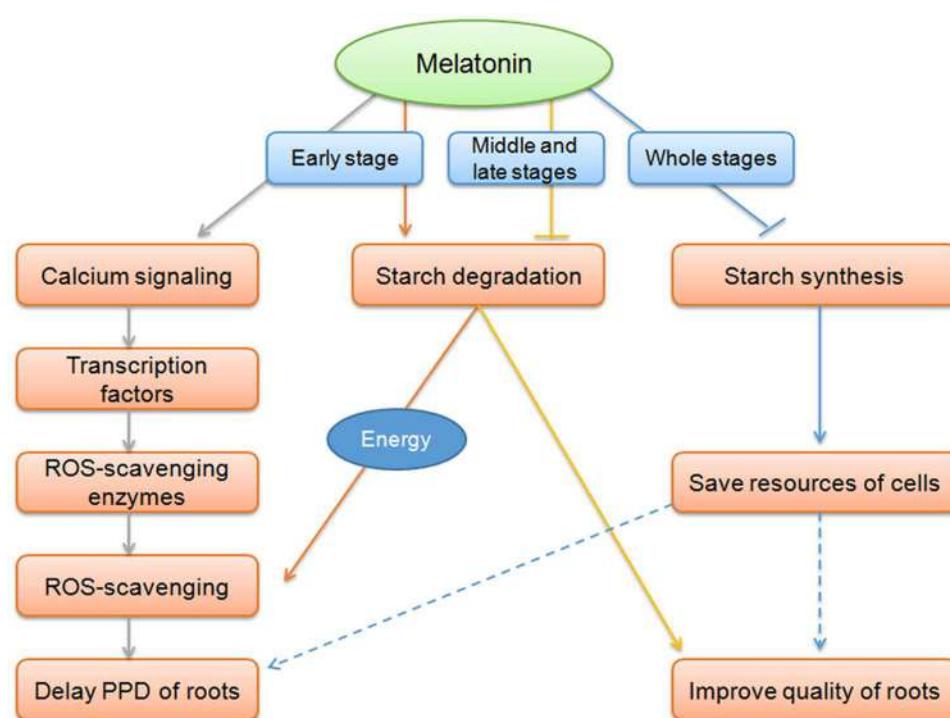
Besides, we also noted that the number of genes related to ROS network induced by melatonin was greater at early stages than that at middle and late stages (Figure 5; Supplementary Table S9). This is consistent with the expression patterns of genes encoding ROS-scavenging enzymes after melatonin treatment in general (Figure 4; Supplementary Table S8). Based on this consistent trend, it is concluded that coincidentally with an oxidative burst melatonin acted by inducing cells to sense and transduct ROS signaling through calcium signaling and related transcription factors, thus resulting in the induction of ROS-scavenging genes to relieve ROS injury.

Interestingly, we observed that numerous starch metabolism-related genes showed transcriptional changes after melatonin treatment according to GO analyses (Supplementary Figures S1 and S2). Most of genes related to starch synthesis were

repressed during the PPD process (Figure 6; Supplementary Table S10). After harvest, the primary carbon source supplied by photosynthesis was cut off, which limited the function of starch synthesis-related genes. The repression of starch synthesis pathway by melatonin may represent an action of melatonin-mediated saving of cell resources in tuberous roots after harvest.

Additionally, exogenous application of melatonin resulted in upregulation of starch degradation related genes at early stage of PPD (6 h) (Figure 6; Supplementary Table S10), which is consistent with the expression profiles of genes in ROS-scavenging and ROS signal transduction pathways. Starch degradation produces glucose that supplies energy for various biological process. It is possible that melatonin-mediated starch degradation may provide energy for the active ROS-scavenging process, such as ascorbate-glutathione cycle, at early stage of PPD.

As reported by Uarrota et al. (2016), degradation of starch occurred during the PPD process, which resulted in reducing starch content, thus decreasing the commercial value of cassava. Exogenous application of melatonin decreased the transcripts of starch degradation associated genes at the middle and late stages (12 and 48 h) of postharvest (Figure 6; Supplementary Table S10). This indicates that melatonin cannot only alleviate PPD symptoms, but also can synchronously inhibit starch



**FIGURE 7 | Overview of melatonin action on the delay of PPD of cassava tuberous roots.** At early stage of the PPD process, coincidently with oxidative burst, melatonin acted on inducing cells to sense and transduct ROS signaling through calcium signaling and related transcription factors, thus resulting in the induction of ROS-scavenging genes to relieve ROS injury and delay of PPD. Melatonin-mediated starch degradation may provide energy for the active ROS-scavenging process at early stage of PPD. At middle and late stages, melatonin function on repressing starch degradation, benefit for improving food characteristics and quality of cassava. During all three stages, the repression of starch synthesis pathway by melatonin may represent an action of melatonin-mediated saving resources of cells in tuberous roots.

degradation, which is of great importance for improving food characteristics and quality of cassava. ROS have been implicated in the oxidative-reductive depolymerization of carbohydrates (Uarrota et al., 2016). The early activation of ROS-scavenging events by melatonin may contribute to the subsequent repression of starch degradation during PPD process.

Recent studies suggested that low doses of melatonin accelerated starch catabolism at night, whereas high doses of melatonin significantly decreased this process and led to starch accumulation in photosynthetic tissues of maize (Zhao et al., 2015). Additionally, melatonin could alter the metabolic status and increase the levels of starch in leaf tissue of *Malus hupehensis* (Wang et al., 2013a). These studies indicate the physiological effect of melatonin on starch metabolism in photosynthetic tissues. The evidences presented here expand the role of and transcriptionally regulatory mechanism of melatonin on starch metabolism in non-photosynthetic tissue.

## CONCLUSION

This study demonstrated the effect of melatonin on the delay of PPD in cassava tuberous roots is related to ROS-scavenging pathway, ROS-signaling transduction, and starch metabolism (Figure 7). These findings would contribute to extend shelf life and improve quality of cassava tuberous roots.

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## AUTHOR CONTRIBUTIONS

HS, HK, MP, and AG conceived the study. WH, YG, YZ, and YY performed the experiments. ZD, QH, and WT carried out the analysis. WH and HS designed the experiments and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00736>

**FIGURE S1 | Differentially expressed genes at 6, 12, and 48 h storage after melatonin treatment by GO enrichment analysis.** Asterisks indicate a significant difference (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

**FIGURE S2 | Common genes differentially expressed at 6, 12, and 48 h storage after melatonin treatment by GO enrichment analysis.** Asterisks indicate a significant difference (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

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# Melatonin-Producing Endophytic Bacteria from Grapevine Roots Promote the Abiotic Stress-Induced Production of Endogenous Melatonin in Their Hosts

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Endophytes form symbiotic relationships with plants and constitute an important source of phytohormones and bioactive secondary metabolites for their hosts. To date, most studies of endophytes have focused on the influence of these microorganisms on plant growth and physiology and their role in plant defenses against biotic and abiotic stressors; however, to the best of our knowledge, the ability of endophytes to produce melatonin has not been reported. In the present study, we isolated and identified root-dwelling bacteria from three grapevine varieties and found that, when cultured under laboratory conditions, some of the bacteria strains secreted melatonin and tryptophan-ethyl ester. The endophytic bacterium *Bacillus amyloliquefaciens* SB-9 exhibited the highest level of *in vitro* melatonin secretion and also produced three intermediates of the melatonin biosynthesis pathway: 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin. After *B. amyloliquefaciens* SB-9 colonization, the plantlets exhibited increased plant growth. Additionally, we found that, in grapevine plantlets exposed to salt or drought stress, colonization by *B. amyloliquefaciens* SB-9 increased the upregulation of melatonin synthesis, as well as that of its intermediates, but reduced the upregulation of grapevine tryptophan decarboxylase genes (*VvTDCs*) and a serotonin *N*-acetyltransferase gene (*VvSNAT*) transcription, when compared to the un-inoculated control. Colonization by *B. amyloliquefaciens* SB-9 was also able to counteract the adverse effects of salt- and drought-induced stress by reducing the production of malondialdehyde and reactive oxygen species ( $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ ) in roots. Therefore, our findings demonstrate the occurrence of melatonin biosynthesis in endophytic bacteria and provide evidence for a novel form of communication between beneficial endophytes and host plants via melatonin.

**Keywords:** melatonin, endophytic bacteria, UPLC-MS/MS, grapevine, abiotic stress

**Abbreviations:** IAA, indole-3-acetic acid; MDA, malondialdehyde; ROS, reactive oxygen species; TEE, tryptophan-ethyl ester.

## INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) was first isolated from the bovine pineal gland (Lerner et al., 1958) and is now recognized as ubiquitous among living organisms, including humans, animals, plants, bacteria, fungi, and macroalgae (Tilden et al., 1997; Rodriguez-Naranjo et al., 2012; Tan et al., 2012). In vertebrates, the molecule functions as a biological modulator of mood, sleep, sexual behavior, seasonal reproductive physiology, and circadian rhythms (Reiter, 1993; Hardeland, 2008; Reiter et al., 2010); as a potent antioxidant, with free radical-scavenging activities; and as a stimulator of antioxidant enzyme activities (Reiter et al., 2005). Meanwhile, the occurrence of melatonin in higher plants wasn't documented until almost 50 years later, when it was reported by both Dubbels et al. (1995) and Hattori et al. (1995). Since then, melatonin has been recognized to function as an abiotic antistressor, by protecting plants against ROS that are produced as a result of harmful abiotic stresses (Posmyk et al., 2008, 2009; Arnao and Hernández-Ruiz, 2009a; Nawaz et al., 2016). In addition, melatonin also functions as a plant regulator, with growth-promoting effects similar to those of IAA, which is a plant hormone in the auxin class (Hernández-Ruiz and Arnao, 2008).

Since the discovery of melatonin in higher plants, the factors that influence the endogenous melatonin levels in plants have remained relatively unexplored. Recently, reports have demonstrated that different abiotic stressors are able to elevate the endogenous melatonin levels of plants (Arnao and Hernández-Ruiz, 2013a,b; Shi et al., 2015; Hernández-Ruiz and Arnao, 2016) and that stress-induced ROS bursts may be the common factor that triggers the accumulation of melatonin (Arnao and Hernández-Ruiz, 2015). Under natural conditions, the internal organs of plants are frequently colonized by a vast number of diverse microbes that are able to interact with their hosts and, thereby, modulate plant growth and development (Sturz et al., 2000). Soil microbes, for example, have been shown to enter and proliferate within plant roots (Hardoim et al., 2008), and in grapevines, naturally occurring endophytes have been isolated from roots, stems, leaves, and various reproductive tissues (e.g., inflorescences, seeds, and fruits; Compant et al., 2011). These symbiotic organisms are important in defending their hosts against phytopathogens (Lindow and Brandl, 2003; West et al., 2010) and may also promote the growth of their host plants *via* nitrogen fixation (Elbeltagy et al., 2001), phosphorus solubilization (Richardson et al., 2009), and the enhancement of plant hormones levels (Ali et al., 2009). Therefore, endophytes are generally recognized as important and beneficial components of plant micro-ecosystems. In fact, since endophytes often supply their hosts with plant hormones, we speculate that endophytes are capable of producing melatonin and that they provide melatonin to their plant hosts. This conjecture is based on observations that (i) melatonin has been identified in microorganisms, such as aerobic photosynthetic bacteria (Tilden et al., 1997), recombinant *E. coli* (Byeon and Back, 2016) and some fungi (Manchester et al., 1995; Hardeland and Poeggeler, 2003); (ii) melatonin biosynthesis is likely to be evolutionarily conserved (Tan et al., 2014); (iii) the cellular machinery for melatonin synthesis in

eukaryotes may have been inherited from bacteria, as a result of endosymbiosis (Tan et al., 2013); and (iv) bioinformatic analyses has revealed that enzymes involving in melatonin synthesis occur in bacterial genomes (Pavlicek et al., 2010; Falcón et al., 2014). However, to the best of our knowledge, the ability of endophytic bacteria to produce melatonin has not been reported, and the synthetic pathway of melatonin in heterotrophic bacteria remains to be elucidated.

Therefore, in the present study, we used a culture-dependent method to isolate endophytic bacteria from the roots of three grapevine varieties and screened the resulting cultures for their *in vitro* capacity to produce melatonin. We subsequently used a promising endophytic strain that produced high levels of melatonin to investigate the intermediates of the melatonin biosynthesis pathway, and in addition, we also performed root colonization experiments, in order to evaluate the effect of the strain on the endogenous melatonin production of host plants under abiotic stress. Finally, to examine whether stress-induced changes in melatonin levels were associated with the induction of melatonin synthesis, we performed qRT-PCR analysis of *VvSNAT* and several *VvTDCs* genes, both of which play a pivotal role in regulating melatonin biosynthesis in plants (Byeon et al., 2013; Zhao et al., 2013).

## MATERIALS AND METHODS

### Isolation of Endophytic Bacteria from Grapevine Roots

We selected three grapevine varieties for our experiments, including the Chinese wild grapevine *Vitis amurensis* 'Changbai 9', *V. vinifera* 'Cabernet Sauvignon', and *V. labruscana* 'Summer Black', and collected root samples from plants that were cultivated at the National Grape Germplasm Repository (113°70' E; 34°72' N), Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Zhengzhou, Henan, China. The root samples were collected from one vine of each variety, when the vines were in flower, and a total of 5 g roots were collected from five randomly chosen root sections (~20 cm below ground) each plant and pooled in sterile 15-mL tubes. The pooled root samples were then kept in refrigerated boxes and transported to the laboratory within 1 h of collection for subsequent processing.

The roots were surface-sterilized in 70% ethanol for 3 min, followed by soaking in sodium hypochlorite (3% available chlorine) for 2 min, and were then rinsed three times with sterile water. Next, each sample was ground and homogenized in 1.5-mL of PBS using sterile quartz sand in individual mortars. The resulting homogenate was serially diluted ( $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ ) and plated on nutrient agar (3 g/L beef extract, 10 g/L tryptone, 5 g/L NaCl, and 20 g/L agar, pH 7.4) with 100 mg/L actidione to inhibit the growth of fungi, and each dilution was prepared in triplicate. In parallel, we also checked the efficiency of our surface sterilization procedure by plating 100  $\mu$ L of the last washing solution (i.e., sterile water used for third rinse) onto nutrient agar. Then, after incubation at 28°C for 2–3 days, the number of colony-forming units was counted, and four to six

representative isolates of each morphology were collected and purified by streaking onto fresh nutrient agar plates. Each purified isolate was maintained at  $-80^{\circ}\text{C}$  in 1 mL sterile nutrient broth that contained 20% glycerol.

## Genomic DNA Extraction and Species Assessment

DNA extraction was performed using the TIANamp bacteria DNA Kit (Tiangen, Beijing, China), according to the manufacturer's instructions, and in order to identify each of the isolates, we amplified the 16S rRNA sequence, using the primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1063R (5'-ACGGG CGGTGTGTRC-3') (Wang and Qian, 2009), as well as the *gyrB* sequence, which encodes the B subunit of the type II topoisomerase DNA gyrase, using the degenerate primers UP-1 (5'-GAAGTCATCATGACCGTTCTGCAYGCNGNGNAARTTY GA-3') and UP-2r (5'-AGCAGGATAACGGATGTGCGAGCCRTC NACRTCNGCRTCNCTCAT-3') reported by Yamamoto and Harayama (1995). The resulting PCR products were purified and bi-directionally sequenced, using the same primers that were used for PCR amplification. The sequences were then compared with reference sequences in GenBank, using the online Blastn software<sup>1</sup>, and identification was considered valid when the identity of a contiguous sequence was  $\geq 99\%$ .

## Screening for Melatonin-Producing Endophytic Bacteria

After determining the identities of the individual isolates to the lowest possible taxonomic level (i.e., species or genus), we randomly selected individual strains to represent each taxon. The selected strains were inoculated into 10 mL lysogeny broth medium, and incubated for 24–36 h, until they reached an  $\text{OD}_{600}$  of  $\sim 1.0\text{--}1.5$ . Once this concentration was achieved, the liquid medium was removed by centrifugation at  $8000 \times g$  and  $4^{\circ}\text{C}$  for 10 min, and the resulting pellets were washed once with 10 mL sterile PBS and re-suspended in 10 mL minimal medium (Voigt et al., 2007). The cultures were then incubated at  $28^{\circ}\text{C}$  for 8 h to allow for the depletion of amino acids, after which the cell concentration of the cultures was determined using a Petroff–Hausser counting chamber and the cultures were standardized to  $10^8$  cells/mL. Next, 1 mL of each of the standardized bacterial cultures was inoculated into individual brown bottles (250 mL) that contained 100 mL of nutrient broth with 200 mg/L tryptophan, and the cultures were incubated in a rotary shaker at  $28^{\circ}\text{C}$  with a rotational speed of 150 rpm in the dark. Cell viability was quantified using the plate counting method with appropriate dilutions. After 36 h, the bacterial cultures were centrifuged and the supernatants were diluted 1:1 with methanol, and the resulting mixtures were passed through a 0.22- $\mu\text{m}$  filter and used for preliminary screening of melatonin and TEE production.

To investigate the potential conversion of L-tryptophan to melatonin in the bacterial strain that produced the highest

concentration of melatonin, we measured the concentrations of several intermediate molecules of melatonin biosynthesis in its culture medium. The strain was cultivated using the procedure described above. In addition, every 6 h, we sampled the bacterial cultures, centrifuged the samples at  $8000 \times g$  for 10 min, and diluted the supernatant 1:1 with methanol. Then, after being passed through a 0.22- $\mu\text{m}$  filter, the resulting mixtures were analyzed for tryptamine, 5-hydroxytryptophan, serotonin, N-acetylserotonin, and melatonin contents using UPLC-MS/MS.

## Colonization Assay

To determine whether the melatonin-producing endophytic bacteria could influence the endogenous melatonin level in roots, grapevine plantlets were inoculated with a bacterial isolate that produced the highest amount of melatonin. The bacterial inoculum was prepared by inoculating the strain into 100 mL nutrient broth, incubating the culture for 24–36 h at  $28^{\circ}\text{C}$ , centrifuging the culture at  $6000 \times g$  for 10 min, and re-suspending and standardizing the inoculum to  $10^7\text{--}10^8$  cells/mL with 0.9% sterilized saline solution. The standardized inoculum was then used to inoculate 6-week-old tissue-cultured *V. labruscana* 'Summer Black' plantlets. The roots of the experimental plantlets were immersed in the bacterial inoculum for 1 min, whereas the roots of the control plantlets were treated with 0.9% sterile saline solution, and afterward, all the plantlets were transferred to 500 mL culture bottles that contained 150 g sterile nutrient soil (Pindstrup, Ryomgaard, Denmark) and 40 mL nutrient-rich water that was prepared with the MS (Murashige and Skoog) basic nutrient medium (Cat# M519; Phytotechnology<sup>2</sup>). The plants were randomly distributed in a greenhouse with a 16-h light/8-h dark cycle at  $26^{\circ}\text{C}$  and irrigated with distilled water (5 mL) every 2 days.

After 20 days of endophyte colonization, we randomly selected 12 plantlets from each of the inoculated and control plantlets, in order to compare their growth, which we assessed by measuring root length, root fresh weight (FW), plant height, and chlorophyll content. The chlorophyll content of fully expanded leaves was analyzed using a chlorophyll ELISA Kit, according to the manufacturer's instructions (Lvyuan, Beijing, China). In addition, the roots were also sampled to determine counts of viable bacteria in the roots of inoculated and control plants, as described in the "Isolation of endophytic bacteria from grapevine roots" section above, and the plate counts of viable bacteria were considered an indicator of bacterial invasion capacity.

Meanwhile, the rest of plantlets were assigned to one of four experimental treatment groups: (i) inoculated plantlets subject to salt stress; (ii) control plantlets subject to salt stress; (iii) inoculated plantlets subject to drought stress; and (iv) control plantlets subject to drought stress. Briefly, 20 mL NaCl solutions (60 or 120 mM) were applied to a series of inoculated and control plantlets in order to simulate salt stress, and 20 mL 10% PEG-6000 solution was added to another series of inoculated and control plantlets in order to simulate drought stress, with 12 plantlets per treatment. After 4 days, the roots were sampled

<sup>1</sup><http://www.ncbi.nlm.nih.gov/BLAST>

<sup>2</sup><http://www.phytotechlab.com/>

from each plantlet at 9–10:00 AM and ground into powder using liquid nitrogen in individual mortars, and 0.5 g of each root powder was extracted with 2 mL of methanol, as described previously (Boccalandro et al., 2011). The resulting extracts were mixed with 2 mL ultrapure water, centrifuged, passed through a 0.22- $\mu\text{m}$  filter, and stored in amber vials for analysis of 5-hydroxytryptophan, tryptamine, serotonin, *N*-acetylserotonin, and melatonin via UPLC-MS/MS.

The  $\text{H}_2\text{O}_2$  levels of the roots were measured according to Patterson et al. (1984), and superoxide production was estimated using the method of Elstner and Heupel (1976). In addition, we assessed the extent of lipid peroxidation in the roots by measuring the MDA content as described by Zhao et al. (2013) with little modification. Briefly, 0.1 g of each root powder was extracted with 1 mL of 10% (w/v) trichloroacetic acid (TCA), vortexed, and centrifuged at  $8000 \times g$  for 10 min. Then, 0.2 ml of each supernatant was mixed with 0.2 ml 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA. The mixtures were heated at 100°C for 20 min, cooled, and centrifuged at  $8000 \times g$  for 10 min. Absorbances were read at 440, 532, and 600 nm, and the MDA concentration (nmol/g FW) was calculated according to the formula:  $[6.45 \times (\text{A}532 - \text{A}600) - 0.56 \times \text{A}450] \times V/W$ , where V (mL) is the volume of the tissue extract, and W (g) is the FW.

## RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated from the root tissue of stress-induced plantlets, following the CTAB method (Reid et al., 2006), and the resulting RNA was treated with DNase I and converted to cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China).

Three TDC homologs, which shared conserved functional domains and >30% homology with the amino acid sequence of rice tryptophan decarboxylase (GenBank No. XP\_015648768), were identified by searching the non-redundant protein and nucleotide sequence data of grapevine (*Vitis Vinifera* L.) at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD, USA), using the tBLASTn, BLASTp, and PSI-BLAST programs (Altschul et al., 1997); and sequence of the grapevine serotonin *N*-acetyltransferase gene (*VvSNAT*; GenBank No. XM\_002266325) was previously predicted by Byeon et al. (2014a). The expression levels of all four genes in the roots of stress-induced plantlets were determined using quantitative real-time PCR (qRT-PCR) analysis with a Roche 480 light cycler System and SYBR Fast qPCR Mix (TaKaRa, Dalian, China). All primers were designed using the NCBI Primer-BLAST service<sup>3</sup> (Table 1), and the qRT-PCR amplification was performed with the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 15 s; and melting curve analysis was performed using 95°C for 5 s, 60°C for 1 min, 97°C continuously, and then 40°C for 30 s. All the qRT-PCR reactions were performed in triplicate, and the expression levels of the target genes were normalized using the *EF1- $\alpha$*  gene as an internal reference.

<sup>3</sup><http://www.ncbi.nlm.nih.gov/tools/primer-blast/>

**TABLE 1 |** Primers used for quantitative real-time PCR.

Gene	Primers sequences (5'-3')
<i>EF-1<math>\alpha</math></i>	F: GAACTGGGTGCTTGATAGGC R: AACCAAAATATCCGGAGTAAAAGA
<i>VvSNAT</i>	F: GCGCGTGCATCATCAGATCA R: TTGATGCCCTCTGGGTCA
<i>VvTDC1</i>	F: CTGCCAGATTCCGCACCTAA R: TCGCCGAGGAGAAGTAATC
<i>VvTDC2</i>	F: CGGAGCTATGGTGTGTC R: TCCCCAACAAATGGCATGAG
<i>VvTDC3</i>	F: CCAGAGAAGAAGGGGAAAGCA R: GGCTCCTGCAGTACGAGTTG

## UPLC-MS/MS Analysis of Metabolites

Tryptophan-ethyl ester, tryptamine, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, and melatonin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced using a Millipore Milli-Q ultrapure water purification unit (Millipore, Bedford, MA, USA). Other solvents, including methanol and formic acid (HPLC grade), were purchased from Merck (Darmstadt, Germany). Stock solutions were prepared by dissolving 10 mg of each standard in 1 mL methanol under low light conditions; the solutions were then stored at –80°C to prevent degradation. Fresh working solutions were prepared in a methanol:water solution (50:50, v/v).

Quantitative detection was conducted using a UHPLC-ESI-QQQ-MS (Agilent 1290 and 6460 triple quadrupole mass spectrometry series; Agilent Corporation, Santa Clara, CA, USA). In the solvent system, Milli-Q water that contained 0.1% (v/v) formic acid was used as eluent A, and methanol was used as eluent B. The analytes were separated using an Agilent ZORBAX Eclipse XDB-C18, Rapid Resolution HT column (1.8  $\mu\text{m}$ , 3.0 mm × 50 mm) at 42°C with linear elution gradient protocols of 0–6 min, 5–55% B, 6–15 min, 55–100% B, at 0.2 mL/min flow rate. Next, 100% B was kept constant for 2 min and the column was re-equilibrated for 5 min using the initial solvent composition. The injection volume was 1  $\mu\text{L}$ . The metabolites were quantitatively detected using the multiple reactions monitoring mode under unit mass-resolution conditions (tryptamine  $m/z$  161→144, 5-hydroxytryptophan  $m/z$  221→204, serotonin  $m/z$  177→160, *N*-acetylserotonin  $m/z$  219→160, and melatonin and TEE  $m/z$  233→174) in positive ion mode. To quantify the analytes, we constructed eight-point calibration curves, using diluted working solutions of external standards. All points on the curves represented the average of three independent determinations. The linearity of the calibration graphs was determined using regression analysis. The limits of detection (LOD) were calculated based on the S/N ratio of 3:1. The limits of quantitation (LOQ) were defined as the lowest level that had an S/N ratio of 10:1. All the investigated analytes displayed excellent linearity, with correlation coefficients ( $R^2$ ) ranging from 0.9975 to 0.9988 (Table 2).

**TABLE 2 | UPLC-MS/MS quantitation data for six analytes.**

Analyte	Linearity range (ng/mL)	R <sup>2</sup>	LOD (ng/mL)	LOQ (ng/mL)
Tryptamine	0.24–24	0.9975	0.08	0.24
5-Hydroxytryptophan	0.45–45	0.9984	0.15	0.45
Serotonin	0.36–360	0.9988	0.12	0.36
N-acetylserotonin	0.18–18	0.9985	0.06	0.18
Melatonin	0.12–1.2	0.9984	0.04	0.12
Tryptophan-ethyl ester	0.1–10	0.9979	0.03	0.1

LOD, limit of detection; LOQ, limit of quantification.

## Statistical Analysis

For each experiment, the results were expressed as the mean  $\pm$  standard deviation of data from 3–12 replicates. Statistical evaluation was performed using one-way ANOVA, followed by Tukey's test for the data of preliminary screening of melatonin production and Student's *t*-test for the colonization assay. All the statistical analyses were performed using SPSS (version 19.0; IBM, Armonk, NY, USA), and a *P*-value of  $<0.05$  was considered statistically significant.

## RESULTS

### Melatonin and TEE Production by Endophytic Bacteria from Grapevine Roots

No colonies grew on the plates that were inoculated with water from the final washing step of the root sterilization procedure, suggesting that the surface sterilization procedure was effective. For the remaining plates, the highest bacterial count ( $5.75 \pm 0.26 \log_{10}$  CFU/g FW) was detected on those inoculated with the homogenized roots of *V. labruscana* 'Summer Black' followed by those inoculated using *V. vinifera* 'Cabernet Sauvignon' roots ( $5.23 \pm 0.18 \log_{10}$  CFU/g FW) and *V. amurensis* 'Changbai 9' roots ( $4.89 \pm 0.22 \log_{10}$  CFU/g FW). A total of 98 endophytic bacteria strains were isolated from the surface-sterilized roots, and 16S rRNA sequences were amplified from each strain, whereas *gyrB* sequences were only amplified from 64. Based on comparison with related sequences deposited in the GenBank DNA database, we identified seven different bacterial genera, which included *Agrobacterium*, *Bacillus*, *Variovorax*, *Pseudomonas*, *Streptomyces*, *Sphingomonas*, and *Ensifer*.

The *Streptomyces* strains were excluded from the screening of melatonin- and TEE-producing abilities, owing to their abnormal growth and low biomass in nutrient broth. We randomly screened eight endophytic bacterial strains that represented eight species (Table 3). With the exception of *B. cereus* CS-17 and *Sphingomonas* sp. VA-16, all the investigated strains secreted tryptophan derivatives into the medium and exhibited species-specific levels of production (Figure 1A). Five of the strains secreted melatonin *in vitro*, and the highest level ( $7.75 \log_{10}$  cells;  $0.87 \text{ ng/mL}$ ; cell count,  $11.15 \log_{10}$  CFU/mL) was produced by *B. amyloliquefaciens* SB-9, followed

by *B. thuringiensis* CS-9 ( $3.33 \log_{10}$  cells;  $0.53 \text{ ng/mL}$ ; cell count,  $11.20 \log_{10}$  CFU/mL) and *Agrobacterium tumefaciens* CS-30 ( $2.90 \log_{10}$  cells;  $0.22 \text{ ng/mL}$ ; cell count,  $10.88 \log_{10}$  CFU/mL). TEE has previously been considered as one of the melatonin isomers, and this compound produced the same fragmentation pattern of melatonin using the triple quadrupole mass spectrometry (Figure 1B). We found that six strains, including all the melatonin-producing isolates, were able to produce TEE, with amounts ranging from  $0.24$  to  $19.83 \log_{10}$  cells.

### Characterization of Melatonin Formation in *B. amyloliquefaciens* SB-9

The melatonin synthetic pathways of animals and plants have been reported previously (Figure 2A); however, the mechanism of melatonin synthesis in bacteria is currently unknown. We detected 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, and melatonin after 6 h of incubation; however, we did not find tryptamine during the incubation. The concentrations of 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin increased throughout the incubation period, and reached maximum values of  $8.82 \pm 1.08$ ,  $3.81 \pm 0.46$ , and  $8.41 \pm 0.82 \text{ ng/mL}$ , respectively, after 30 h (Figure 2), whereas the concentration of melatonin reached a maximum value of  $1.19 \pm 0.12 \text{ ng/mL}$  after 24 h of incubation and declined slightly thereafter. When the results were expressed as  $\log_{10}$  viable cells, the production capacity for all the investigated metabolites peaked at 6 h (cell number,  $9.87 \log_{10}$  CFU/mL) and, thereafter, declined with increasing cell density ( $11.76 \log_{10}$  CFU/mL at 30 h).

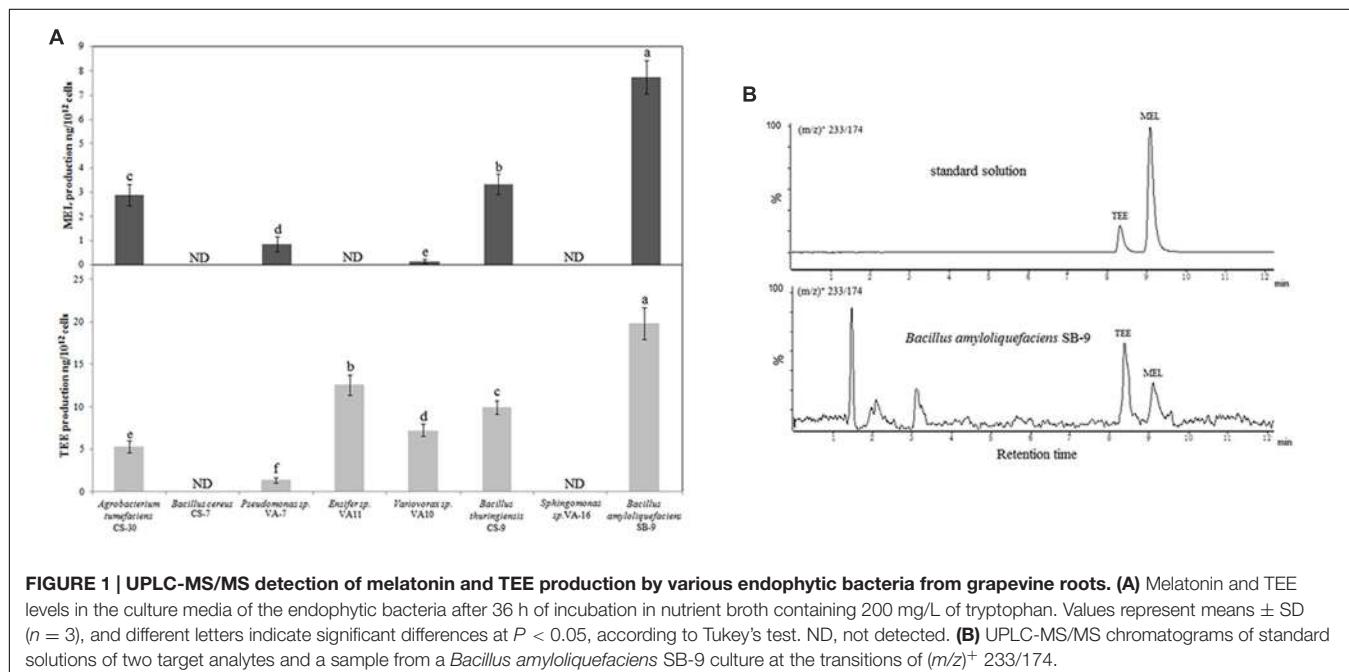
### Growth Responses of *V. labruscana* 'Summer Black' Plantlets Treated with *B. amyloliquefaciens* SB-9

Based on its high melatonin-producing capacity, we selected *B. amyloliquefaciens* SB-9 for the inoculation of grapevine plantlets. We observed no symptoms of pathogenicity in the inoculated *V. labruscana* 'Summer Black' plantlets. At 20 days after inoculation, *B. amyloliquefaciens* SB-9 was successfully re-isolated from inoculated roots, and its identity was verified via sequencing of the 16S rRNA region. In addition, the colonies recovered from the inoculated seedling roots exhibited a single morphotype (Figure 3A), and the population density was  $5.74 \pm 0.22 \log_{10}$  CFU/g FW (Figure 3B), which indicated a high level of colonization, whereas no colonies were isolated from the control plant roots. Furthermore, we also observed that inoculation with *B. amyloliquefaciens* SB-9 significantly promoted the growth of the grapevine plantlets. In fact, the root length, plant height, FW, and leaf chlorophyll content of the inoculated plantlets were  $48.58$ ,  $19.46$ ,  $41.82$ , and  $41.76\%$  greater, respectively, than those of the control plantlets (Figures 3C,D), which indicates that the strain can be regarded as a valuable plant growth-promoting rhizobacteria.

**TABLE 3 |** Endophytic bacterial strains screened for melatonin production ability.

Species	Code	Origin	GenBank accession no.	
			16S rRNA gene	gyrB
<i>Agrobacterium tumefaciens</i>	CS-30	Cabernet Sauvignon	KU522188	KX346711
<i>Bacillus thuringiensis</i>	CS-9		KU522196	KX346714
<i>B. cereus</i>	CS-17		KU522189	KX346713
<i>B. amyloliquefaciens</i>	SB-9	Summer Black	KX346710	KX346712
Variovorax sp.	VA10	Changbai 9	KX065462	—
Pseudomonas sp.	VA-7		KU522198	KX423685
Ensifer sp.	VA11		KX065463	—
Sphingomonas sp.	VA-16		KU522199	—

—, no PCR product.



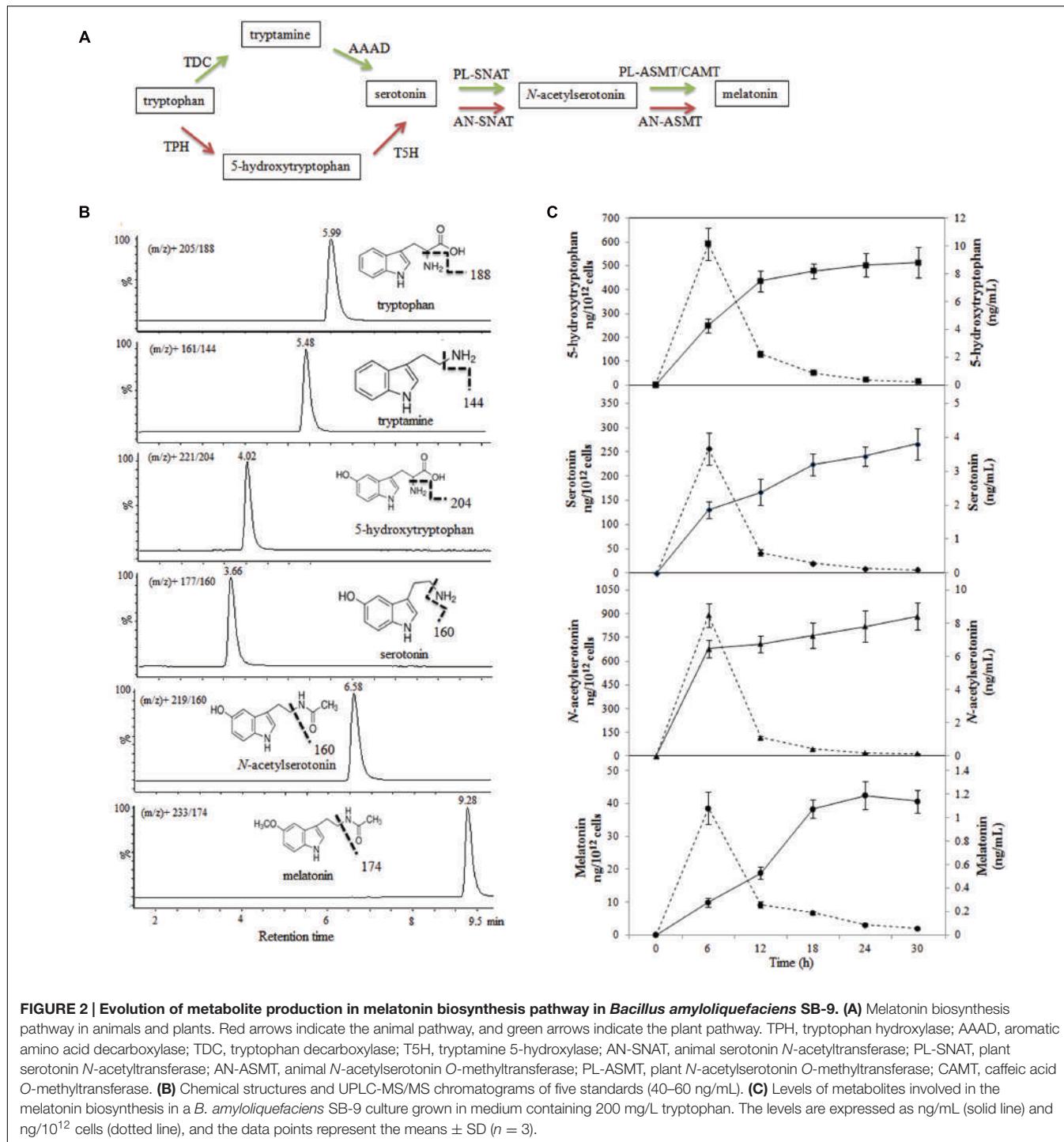
## Effect of *B. amyloliquefaciens* SB-9 on Levels of Endogenous Melatonin and Its Intermediates in Roots under Stress

When the roots of inoculated and control plantlets were subjected to salt or drought stress, they responded by synthesizing melatonin and its intermediates (tryptamine, 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin), albeit to different extents. We found that melatonin synthesis was similar in the inoculated and control plants under normal growth conditions; however, after exposure to salt or drought stress, the melatonin levels in the roots of inoculated plantlets were 52.61, 37.90, and 53.07% higher, respectively, than those in the roots of control plantlets (Figure 4E). Similarly, in the absence of abiotic stresses, 5-hydroxytryptophan levels in inoculated plants and control plants were similar, but after NaCl or 10% PEG 6000 treatment, the synthesis of 5-hydroxytryptophan in the roots of inoculated plantlets was significantly higher than that in the roots of control plants (Figure 4B). A similar trend was also observed

for *N*-acetylserotonin, and in salt-stress plantlets (120 mM only), its level was significantly upregulated in inoculated plantlets, when compared to control plants (Figure 4C); however, the tryptamine levels in the roots of inoculated plantlets were significantly lower than those in the roots of control plants after exposure to abiotic stresses (Figure 4A), and the serotonin levels in inoculated and control plantlets were statistically similar ( $P > 0.05$ ; Figure 4D). Therefore, it is likely that the synthesis of melatonin and its intermediates in the roots of plants exposed to abiotic stress is influenced by *B. amyloliquefaciens* SB-9 colonization.

## Effect of *B. amyloliquefaciens* SB-9 on Stress-Induced Oxidative Damage

The MDA level in plant tissue is an indicator of lipid peroxidation status, and it is accompanied by ROS production ( $H_2O_2$  and  $O_2^-$ ). In the absence of abiotic stresses, the MDA level was markedly lower in the roots of inoculated plantlets than



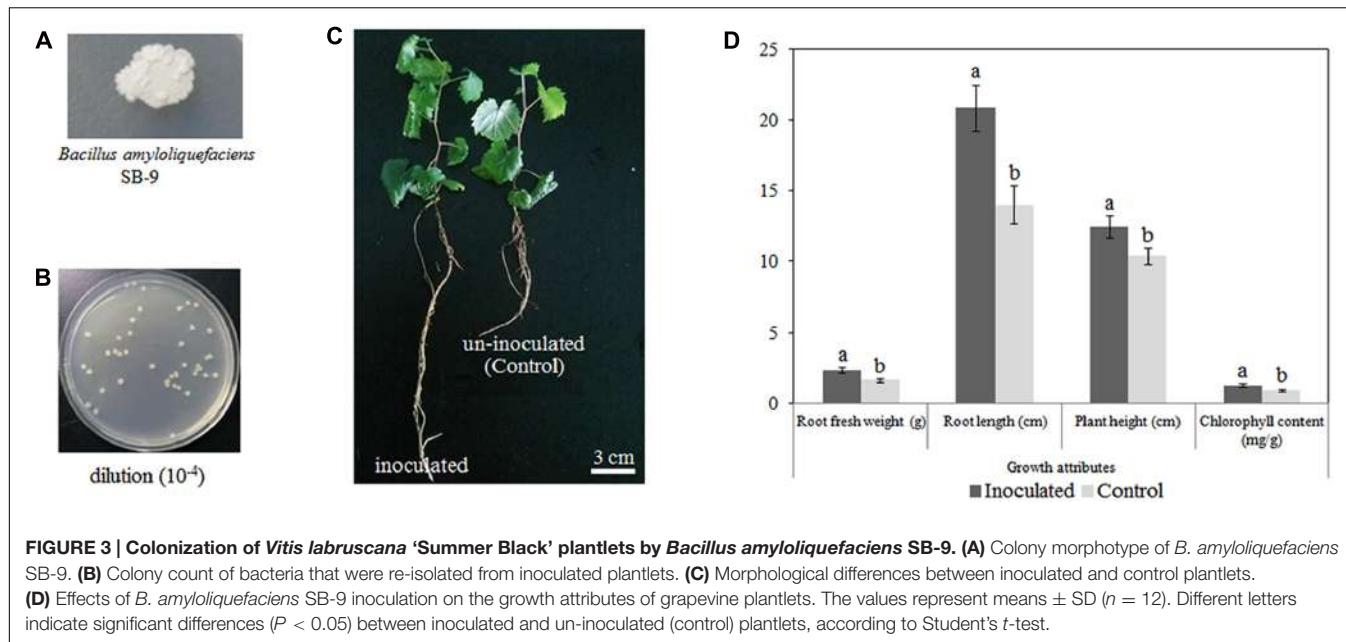
**FIGURE 2 | Evolution of metabolite production in melatonin biosynthesis pathway in *Bacillus amyloliquefaciens* SB-9. (A)** Melatonin biosynthesis pathway in animals and plants. Red arrows indicate the animal pathway, and green arrows indicate the plant pathway. TPH, tryptophan hydroxylase; AAAD, aromatic amino acid decarboxylase; TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxylase; AN-SNAT, animal serotonin N-acetyltransferase; PL-SNAT, plant serotonin N-acetyltransferase; AN-ASMT, animal N-acetylserotonin O-methyltransferase; PL-ASMT, plant N-acetylserotonin O-methyltransferase; CAMT, caffeic acid O-methyltransferase. **(B)** Chemical structures and UPLC-MS/MS chromatograms of five standards (40–60 ng/mL). **(C)** Levels of metabolites involved in the melatonin biosynthesis in a *B. amyloliquefaciens* SB-9 culture grown in medium containing 200 mg/L tryptophan. The levels are expressed as ng/mL (solid line) and ng/10<sup>12</sup> cells (dotted line), and the data points represent the means ± SD ( $n = 3$ ).

in the roots of un-inoculated plantlets (3.8 nmol/g FW vs. 5.2 nmol/g FW). Additionally, inoculated and un-inoculated plantlets exposed to salt and drought stress had increased MDA levels; however, the MDA levels were significantly lower in the roots of endophyte-associated plantlets than in the roots of un-inoculated plantlets (Figure 5A). We observed similar trends in ROS production between un-inoculated and inoculated plantlets (Figures 5B,C). Our findings indicate that colonization

with *Bacillus amyloliquefaciens* SB-9 counteracted the adverse effects of abiotic stress by reducing the production of MDA and ROS.

## Relative Expression of Melatonin Synthesis Genes

The *VvTDC1*, *VvTDC2*, and *VvTDC3* genes are located on grapevine chromosomes 7, 10, and 4, respectively, and their



predicted amino acid sequences possessed 48.50, 47.87, and 47.49 % homology with rice tryptophan decarboxylase (*TDC1*). Similar to the pattern observed for endogenous melatonin levels, we observed that the relative expression levels of *VvTDC1*, *VvTDC2*, *VvTDC3*, and *VvSNAT* in the roots of control plantlets were significantly upregulated by both salt and drought stress (Figure 6), again suggesting that melatonin synthesis is stress-inducible. Interestingly, the transcript levels of the *VvTDCs* and *VvSNAT* in the roots of SB-9-inoculated plants were also increased by both salt and drought stress; however, the extent of upregulation for these genes was significantly lower ( $P < 0.05$ ) than that of control plants when they were subject to identical stressors, with the exception of *VvTDC1* under 60 mM salt stress.

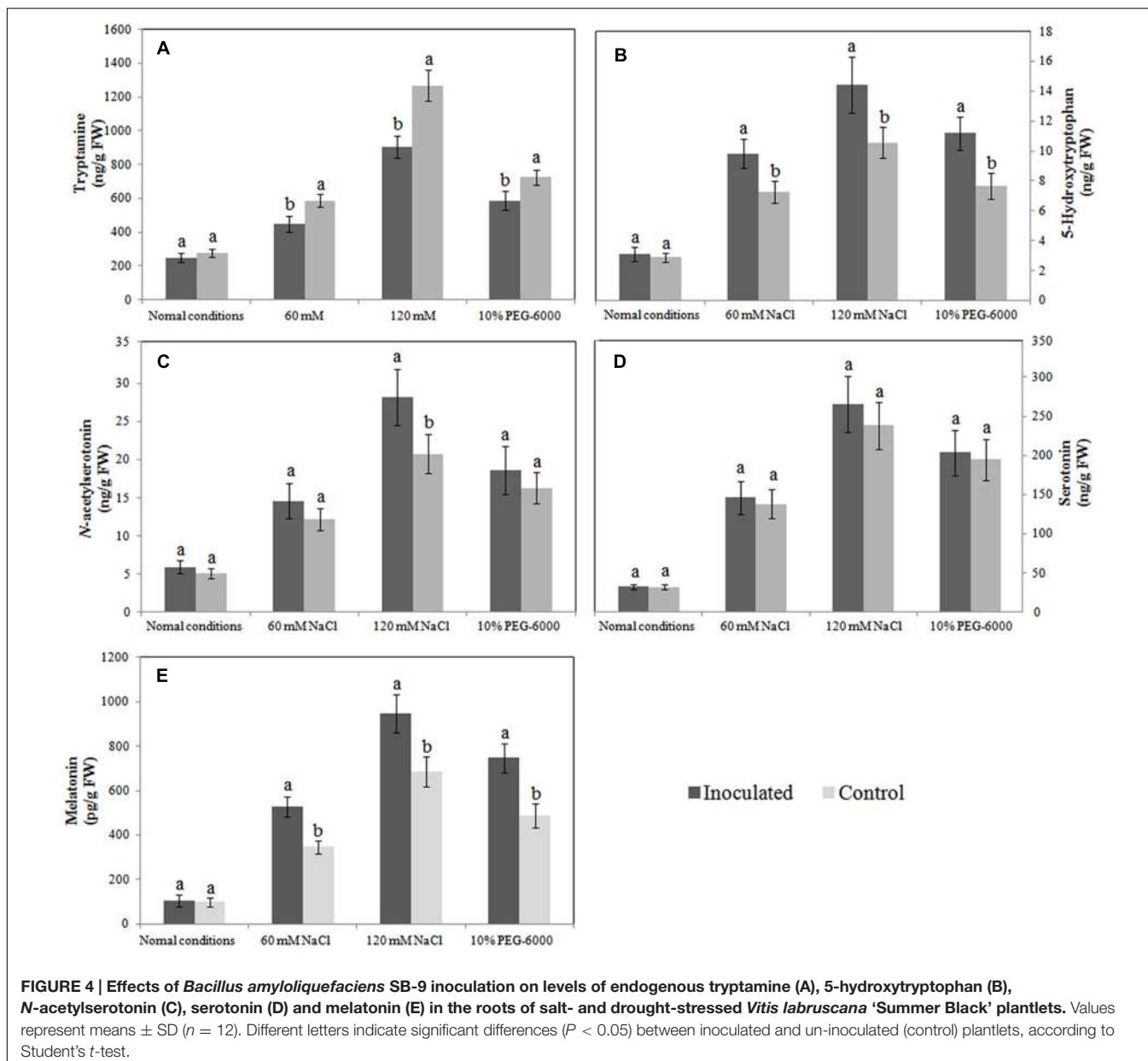
## DISCUSSION

Melatonin was previously identified in the primitive photosynthetic bacterium *Erythrobacter longus* (Tilden et al., 1997) and in the gram-negative bacterium *Escherichia coli* (Hardeeland and Poeggeler, 2003); however, few other bacteria are known to produce melatonin. In the present study, we demonstrated that endophytic bacteria, such as *A. tumefaciens* and *B. amyloliquefaciens*, are capable of secreting melatonin into extracellular media (Figure 1A). In addition, we also found that the level of melatonin in seedling roots was greater when the roots were colonized by *B. amyloliquefaciens* SB-9 and subjected to salt or drought stress (Figure 2A). These findings are in accordance with those of Arnao and Hernández-Ruiz (2015), who proposed an association between beneficial endophytes and the enhanced melatonin levels in their host plants. However, we cannot be sure that the enhanced levels of endogenous melatonin were derived from the endophytic bacteria. Alternatively,

root cells might utilize intermediate metabolites of melatonin that are produced by endophytic bacteria. In fact, in the present study, *B. amyloliquefaciens* SB-9 secreted serotonin and *N*-acetylserotonin.

The melatonin biosynthesis pathway of plants was recently described (Byeon et al., 2014b) and was shown to differ markedly from that of vertebrates (Tan et al., 2014). One difference is that plants initially decarboxylate tryptophan to form tryptamine and subsequently hydroxylate tryptamine to form serotonin, whereas vertebrates initially hydroxylate tryptophan to form 5-hydroxytryptophan. In the present study, we failed to detect tryptamine in the *B. amyloliquefaciens* SB-9 culture; however, the concentration of 5-hydroxytryptophan increased throughout the incubation period (Figure 2C). This suggests that the first step of melatonin biosynthesis in the endophytic bacterium *B. amyloliquefaciens* SB-9 may be similar to that in vertebrates. However, the entire melatonin biosynthesis pathway remains to be elucidated, and further studies of the genes involved in the melatonin biosynthesis pathway of *B. amyloliquefaciens* SB-9 are required.

Endophytic bacteria play an important role in promoting plant growth; however, their influence might be the result of cumulative effects from the various bioactivities of individual endophytes. Indeed, N<sub>2</sub>-fixing bacteria are also capable of producing IAA (Pedraza et al., 2004), solubilizing phosphate, and releasing amino acids (Liba et al., 2006). Furthermore, it has also been reported that IAA and gibberellins frequently occur simultaneously in culture broth (Piccoli et al., 2011), which indicates that individual endophytic bacterium may be capable of synthesizing multiple phytohormones. All of the above-mentioned factors are beneficial to plant growth. We found that endophytic bacteria produced an additional growth regulator, melatonin, which was previously reported to stimulate

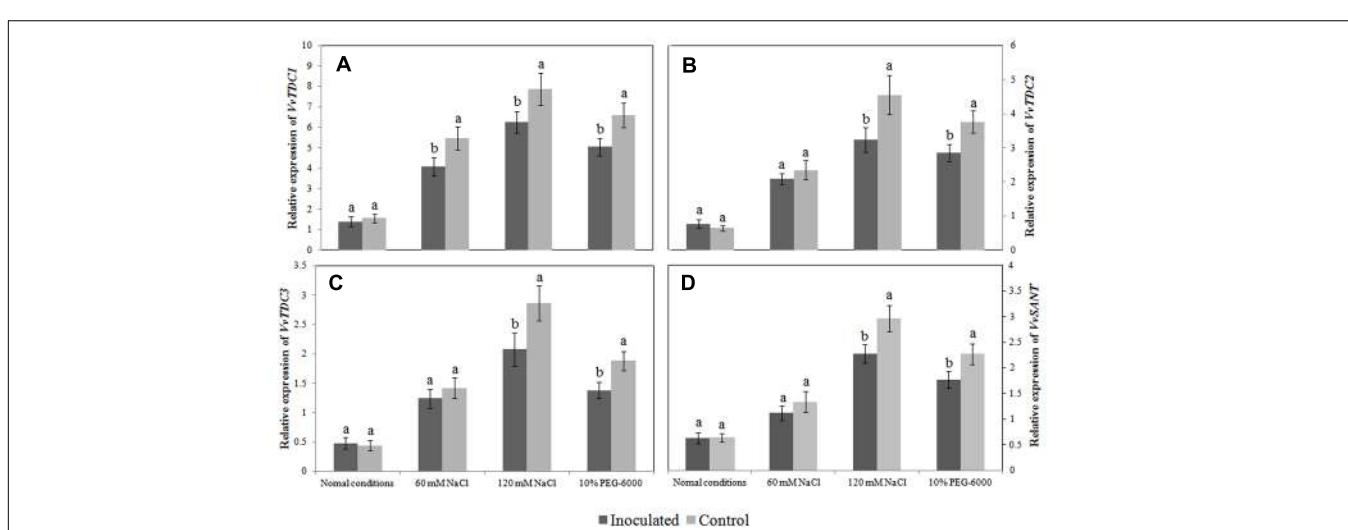
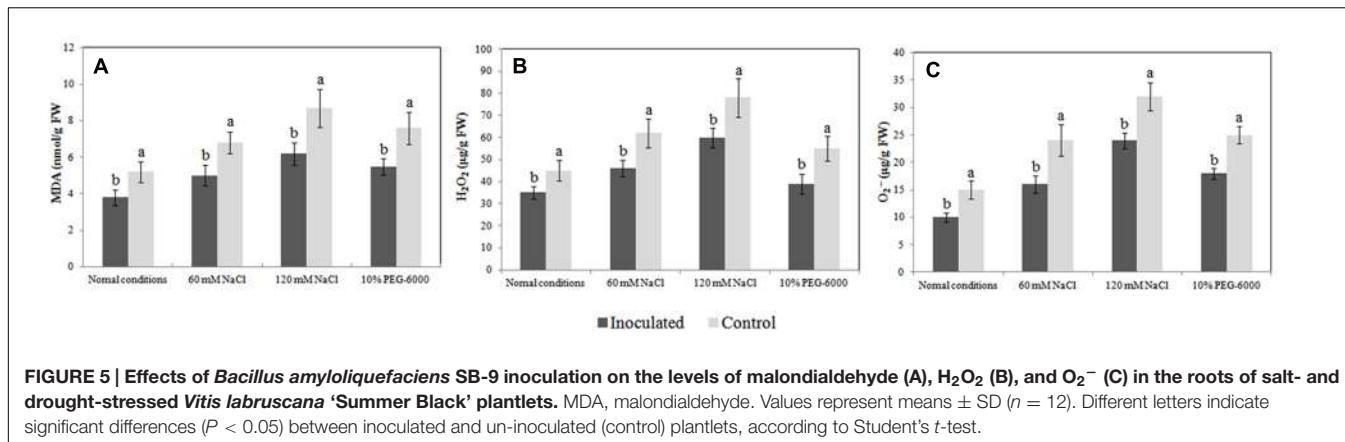


**FIGURE 4 |** Effects of *Bacillus amyloliquefaciens* SB-9 inoculation on levels of endogenous tryptamine (A), 5-hydroxytryptophan (B), *N*-acetylserotonin (C), serotonin (D) and melatonin (E) in the roots of salt- and drought-stressed *Vitis labruscana* 'Summer Black' plantlets. Values represent means  $\pm$  SD ( $n = 12$ ). Different letters indicate significant differences ( $P < 0.05$ ) between inoculated and un-inoculated (control) plantlets, according to Student's *t*-test.

lateral root and shoot growth in several plants, even at low concentrations (Chen et al., 2009; Park and Back, 2012; Bajwa et al., 2014; Wei et al., 2014; Hernández-Ruiz and Arnao, 2016). In the present study, plant height, FW, leaf chlorophyll content, root length, and number of lateral roots (data not shown) were all enhanced by SB-9 colonization. Although we are not sure whether the other *B. amyloliquefaciens* strains could enhance the endogenous melatonin level in roots, this growth-promoting activity has also been confirmed by other researchers (Idriss et al., 2002; Zhang et al., 2015). Until now, however, we have no direct evidence that the SB-9-derived melatonin enhancement played a role in the growth attributes we measured. This is especially true since the growth promotion, including main/lateral roots development, were likely derived from the

combined effects of plant growth-promoting rhizobacteria (PGPR), such as nitrogen fixation, phosphorus solubilization, the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase or other PGPR-induced physical and chemical (gibberellin, auxin, cytokinin, and unknown metabolites) changes in plants. Further studies, using a mutant that is unable to increase the endogenous melatonin level of roots, are needed to determine the correlation between the enhanced level of melatonin in endophytic bacteria and the promotion of growth in their host plants.

Recent studies have suggested that some plants accumulate melatonin as a defense against a variety of environmental abiotic stressors, such as salt (Mukherjee et al., 2014), chemical agents (Arnao and Hernández-Ruiz, 2009b; Byeon et al., 2015),



low temperature (Shi et al., 2015), and drought (Arnao and Hernández-Ruiz, 2013a,b). The results of the present study concur with those of previous reports, and we also found that stress-induced melatonin synthesis was accompanied by the upregulation of several *VvTDCs* and *VvSNAT*, as well as the increased production of melatonin intermediates, such as tryptamine, 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin (Figure 4).

Endophytes have mostly been reported to counteract the adverse effects of stress by reducing the production of MDA and ROS in plants (Jungwook et al., 2009; Khan et al., 2012). This behavior is likely derived from the combined effects of endophytes, such as the enhancement of plant antioxidant enzyme (POD, CAT, POD and APX) activities and the production of phenolic compounds or other antioxidant compounds (Torres et al., 2012). We found that, in grapevine plantlets exposed to salt or drought stress, the production of MDA and ROS, as well as the transcript levels of the grapevine *VvTDCs* and *VvSNAT* in

inoculated roots were relatively lower than those in un-inoculated controls. Therefore, it seems logical that the endogenous levels of melatonin and its intermediates in inoculated roots could be also lower than those in the un-inoculated controls. However, only tryptamine levels exhibited this trend, whereas levels of the other intermediates in the roots of inoculated plants were similar or higher than those in the un-inoculated controls. We speculate that the lower expression of *VvTDCs* and *VvSNAT* could be associated with lower oxidative damage in the roots of inoculated plantlets (Figures 5 and 6) because the transcript levels of melatonin synthesis genes were reported to show a positive correlation with ROS levels caused by abiotic stress (Li et al., 2014); however, the higher endogenous 5-hydroxytryptophan, *N*-acetylserotonin and melatonin levels, as well as the statistically similar serotonin level, in the roots of these plantlets was due to SB-9 colonization, which may promote the production of these compounds via supplementary bacterial melatonin biosynthesis, a possible exchange of metabolites between plants and the strain,

or additional promoting factors produced by the strain. In fact, in the present study, *B. amyloliquefaciens* SB-9 was able to secrete these compounds *in vivo*, except tryptamine. In primitive bacteria, melatonin is thought to function as an antioxidant and free radical scavenger that reduces the harmful effects of ROS (Tan et al., 2014). This is the primary role that was reported in both animals and plants (Tan et al., 2002, 2003; Reiter et al., 2005). Therefore, based on these observations, if abiotic stress induces a burst of ROS in the internal tissues of host plants, the living conditions for root-inhabiting microbes presumably become toxic, and as a result, endophytes upregulate their melatonin biosynthesis accordingly, in order to alleviate ROS-induced cell damage.

In summary, the findings of the present study demonstrate that the melatonin-producing ability of endophytic bacteria and the potential application of these bacteria in promoting endogenous melatonin level in plants. However, it remains unclear (i) whether the enhanced levels of endogenous melatonin in roots were derived from production by endophytic bacteria; (ii) whether endophytic fungi produce melatonin; (iii) whether fruit-colonizing endophytes enhance the melatonin levels of fruit tissue, especially in grapevines; (iv) whether any endophytic bacteria, even those with low melatonin-producing abilities, are able to enhance the endogenous melatonin levels in their host plants by providing intermediate metabolites; or (v) which specific internal and external elements influence melatonin production in endophytes and their host plants? Therefore, more comprehensive and detailed investigations are needed to characterize the role

of melatonin biosynthesis in naturally occurring symbiotic relationships.

## AUTHOR CONTRIBUTIONS

YL, CY, JJ, YS, and YQ conceived the study; JJ and YM performed the experiments, analyzed the data, and wrote the manuscript; SC analyzed the UPLC-MS/MS data; CL provided suggestions and revised the manuscript. All authors approved the final manuscript and agreed to be accountable for all aspects of the work, i.e., ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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# Melatonin Protects Cultured Tobacco Cells against Lead-Induced Cell Death via Inhibition of Cytochrome c Translocation

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Melatonin was discovered in plants more than two decades ago and, especially in the last decade, it has captured the interests of plant biologists. Beyond its possible participation in photoperiod processes and its role as a direct free radical scavenger as well as an indirect antioxidant, melatonin is also involved in plant defense strategies/reactions. However, the mechanisms that this indoleamine activates to improve plant stress tolerance still require identification and clarification. In the present report, the ability of exogenous melatonin to protect *Nicotiana tabacum* L. line Bright Yellow 2 (BY-2) suspension cells against the toxic exposure to lead was examined. Studies related to cell proliferation and viability, DNA fragmentation, possible translocation of cytochrome c from mitochondria to cytosol, cell morphology after fluorescence staining and also the *in situ* accumulation of superoxide radicals measured via the nitro blue tetrazolium reducing test, were conducted. This work establishes a novel finding by correcting the inhibition of release of mitochondrial cytochrome c in to the cytoplasm with the high accumulation of superoxide radicals. The results show that pretreatment with 200 nm of melatonin protected tobacco cells from DNA damage caused by lead. Melatonin, as an efficacious antioxidant, limited superoxide radical accumulation as well as cytochrome c release thereby, it likely prevents the activation of the cascade of processes leading to cell death. Fluorescence staining with acridine orange and ethidium bromide documented that lead-stressed cells additionally treated with melatonin displayed intact nuclei. The results revealed that melatonin at proper dosage could significantly increase BY-2 cell proliferation and protected them against death. It was proved that melatonin could function as an effective priming agent to promote survival of tobacco cells under harmful lead-induced stress conditions.

**Keywords:** BY-2 tobacco cells, cytochrome c, DNA fragmentation, melatonin, programmed cell death

## INTRODUCTION

In their natural environment, plants are exposed to many different biotic and abiotic stresses. Among various stressors, heavy metals, especially lead (Pb), are major environmental pollutants, particularly in areas with high anthropogenic pressure (Gill, 2014) and its accumulation has adverse effects on plant growth and crop productivity. Pb is phytotoxic and found in dust, fumes, mists,

vapors and in soil as minerals ( $\text{PbCO}$ ,  $\text{PbS}$ ,  $\text{PbSO}_4$ ) (Nicholls and Mal, 2003). Although the level of heavy metals in agricultural soil is normally very low, the repeated use of phosphate fertilizers over long periods may cause dangerously high concentrations of some of these toxins (Gill, 2014). Pb is taken up *via* roots along with water, or it can be absorbed from the air *via* shoots and foliage (Fahr et al., 2013). Unfortunately, plant roots are not selective and absorb Pb with other minerals where accumulates. In a number of species, high Pb levels cause abnormal plant morphology, reduced plant growth and finally it induces cell death (Pourrut et al., 2012). Toxic Pb concentrations inhibit the activity of key enzymes, e.g., acid phosphatase, esterases, peroxidases, malic dehydrogenase, by reacting with their sulfhydryl groups. Moreover, Pb contributes to water imbalance, alterations in cell membrane permeability and it limits mineral nutrition. Pb excess also induces oxidative stress in tissues by increased reactive oxygen species (ROS) generation. Simultaneously, Pb provokes DNA damage, gene mutations, protein oxidation, lipid peroxidation and finally it promotes signal transduction cascades that promote cell death (Wierzbicka, 1999; Gill, 2014).

Programmed cell death (PCD) is an indispensable process for animals and plant development. In plant systems, PCD falls within two broad categories, environmentally induced and developmentally regulated cell death. Environmentally induced PCD is usually a consequence of external factors including heat shock (Vacca et al., 2006; Lord and Gunawardena, 2012), cold (Lei et al., 2004), pathogen infection leading to a hypersensitivity response (HR) (Mur et al., 2008; Pietrowska et al., 2015) and death caused by heavy metals (Iakimova et al., 2007; Iwase et al., 2014). PCD is an event displayed by many different organisms throughout evolution; however, despite the enormous evolutionary distance across organisms there are some common features including: increased formation of vesicles, cytoplasmic condensation, nuclear condensation, DNA laddering and translocation of cytochrome c (Cyt c) from mitochondria to the cytosol (Isbat et al., 2009; Martínez-Fábregas et al., 2014). In plant cells, Cyt c release occurs during PCD and is a result of many stimuli such as menadione, D-mannose, heat or ROS (Sun et al., 1999; Stein and Hansen, 1999; Tiwari et al., 2002; Vacca et al., 2004).

Petrosillo et al. (2003) documented that mitochondrial-induced ROS production promotes Cyt c release from mitochondria by a two-step process, including dissociation of Cyt c from cardiolipin, followed by permeabilization of the outer membrane, probably by interaction with voltage dependent anion channels. However, the function of cytoplasmic Cyt c is still controversial since Vacca et al. (2006) found that Cyt c release depended on ROS production, but it may not trigger PCD. Furthermore, after Cyt c translocation, caspase-like proteases inactivate it, leading to Cyt c degradation *en route* to PCD (Vacca et al., 2006). However, data of Martínez-Fábregas et al. (2014) indicated that extra-mitochondrial Cyt c had a double role in causing living cells to die, by triggering the pro-apoptotic routes, e.g., cysteine protease response to dehydration 21 - RD21, hydroxyacylglyutathione

hydrolase 2 (GLY2) as well as by inhibiting the pro-survival factors including SET protein (which acts as an inhibitor of p53 acetylation and blocks both p53-mediated cell cycle arrest and apoptosis after stress) or luminal binding protein 1 and 2 (BiP1 and BiP2) whose overexpression increased cell tolerance to endoplasmic reticulum stress as shown in tobacco protoplast (Leborgne-Castel et al., 1999; Martínez-Fábregas et al., 2014).

To reduce the negative impact of various stresses, including Pb pollution, the best solution may be biostimulators, which improve plant tolerance and protect them against harmful factors. Among many different protective substances naturally occurring in plants, melatonin (*N*-acetyl-5-methoxytryptamine) seems to have great biostimulatory potential (Janas and Posmyk, 2013). Melatonin has been detected in numerous plant species (Reiter et al., 2015). This indoleamine is a broad-spectral antioxidant. It stimulates antioxidant enzymes and synthesis of glutathione, and activates other antioxidants (Bałabusta et al., 2016). It also increases the efficiency of mitochondrial electron transport chain thereby decreasing electron leakage thus limiting free radical generation (Kładna et al., 2003; Rodriguez et al., 2004; Leon et al., 2005; Tan et al., 2007; Reiter et al., 2015; Bałabusta et al., 2016). Moreover, the work of Galano et al. (2013), Tan et al. (2014) and Kołodziejczyk et al. (2015) indicated that the melatonin metabolites, e.g., cyclic-3-hydroxymelatonin, 2-hydroxylmelatonin and especially *N*1-acetyl-*N*2-formyl-5-methoxykynu-ramine (AFMK) also possessed antioxidant activity. These facts, together with melatonin small size makes it particularly capable of translocating easily between cell compartments and of protecting cell structures against excessive ROS.

Melatonin is also useful to protect plants against heavy metal-induced stresses (Tan et al., 2007). Presowing melatonin treated seeds eliminated the toxic effects of copper ions in *Brassica oleracea rubrum* during germination (Posmyk et al., 2008) and zinc sulfate in *Hordeum vulgare* L. roots (Arnao and Hernández-Ruiz, 2009). Relatively little is known about the specific mechanisms of melatonin action at the subcellular level in plants. Lei et al. (2004) showed that pretreatment with melatonin of carrot suspension cells attenuated cell damage caused by cold exposure.

Studying the molecular pathways of PCD in whole plants introduces many difficulties, because it often occurs in a small number of directly stress-affected cells (McCabe and Leaver, 2000). Thus, for analysis of cytotoxic effects, cell lines are of particular suitable. *Nicotiana tabacum* L. cv Bright Yellow 2 (BY-2) suspension cells are fast growing higher plant cells, which provide an excellent model for examining plant physiology, biochemistry and molecular biology (Nagata et al., 1992). They allow research both at the level of a single cell and in its compartments. The objective of the present study was to determine if pretreatment of a suspension *Nicotiana tabacum* BY-2 cells with melatonin inhibits Pb-induced PCD. The findings show that melatonin significantly limited the negative effects of this heavy metal and acted as a biostimulating, pro-survival factor.

## MATERIALS AND METHODS

### Plant Material

Sterile suspensions of *in vitro* cell cultures of *Nicotiana tabacum*, L. cv Bright Yellow 2 (BY 2) were used. The cells were cultivated in Linsmaier and Skoog (1965) basal medium (LS) supplemented with 30 g l<sup>-1</sup> sucrose, 0.2 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D; synthetic auxin), 1 mg l<sup>-1</sup> thiamine, 0.1 g l<sup>-1</sup> myo-inositol and 10<sup>-2</sup> M KH<sub>2</sub>PO<sub>4</sub>. The initial pH of the medium was established as 5.3.

### Cell Culture and Growth Conditions

BY-2 suspended cells were routinely propagated and cultured at 25°C. From the stationary growth phase (day 7th) of the base culture, 2 ml of cell suspension were passaged into the fresh LS medium as a control (C) and LS with 200 nM melatonin (MEL). The optimal dose of melatonin was chosen experimentally. In the middle of the logarithmic phase of growth (day 4th) Pb(NO<sub>3</sub>)<sub>2</sub> was added to LS (Pb) and LS with melatonin (MEL + Pb) media to the final Pb<sup>2+</sup> concentration 15 µM. Thus, the experiments were performed in four variants: (i) C: BY-2 cells cultured under optimal conditions on LS medium, (ii) MEL: BY-2 cells cultured on LS medium supplemented with melatonin from the start of new culture; (iii) Pb: BY-2 cells cultured on LS medium with Pb<sup>2+</sup> added on the 4th day of culture and (iv) MEL + Pb: BY-2 cells cultured on LS medium with melatonin added from the start of culture and stressed with Pb<sup>2+</sup> added on the 4th day of culture. The cultures were maintained to the 7th day (stationary phase of the control cell growth). The applied concentration of lead was chosen after measurement of LC<sub>50</sub> on the 7th day.

### Determination of Cell Growth and Viability

The cell number was determined with the use of a Fuchs-Rosenthal haemocytometer under a light microscope Olimpus CX-31 equipped with MicroScan v.15. digital system of image analysis; additionally the number of dead cells was assessed after selective staining with methylene blue. Living cells do not take up the stain and retain their natural color whereas damaged cells are stained blue as they are unable to keep the methylene blue from penetrating their membranes. The number of cells and their viability were analyzed every experimental day.

### Melatonin Determination

Melatonin was extracted according to the modified methods of Guerrero et al. (2001) and Hernandez-Ruiz et al. (2004). Its concentration was measured during lag, log and the stationary phases of growth. After filtration and separation of the cells from the medium concentrations of melatonin in the extracts were determined using high-performance liquid chromatography (HPLC-MS/MS). For extraction, 5 g of fresh weight of the cells was homogenized with 5 mL of 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 5 µM butylated hydroxytoluene (BHT) as an antioxidant. The homogenate was

maintained for 15 h at room temperature in darkness with minimal shaking, in order to ensure complete extraction of melatonin.

The homogenate was centrifuged at 15000 g for 10 min at 5°C. Initial purification consisted in two steps by solvent-partitioning using ethyl acetate and 50 mM sodium phosphate buffer (first at pH 8.0 and second at pH 3.0). The two organic phases were evaporated together under vacuum. Dry residue was re-dissolved in 1 mL of mobile phase, filtered through Supelco ISO-Disc filters (PTEF-4 – 2.4 mm × 0.2 m; Supelco, Bellefonte, PA, United States), and frozen at -70°C until HPLC-MS analysis. The purified extract was subjected to HPLC-MS/MS analysis using an Agilent 1200 LC System coupled with AB Sciex 3200 QTRAP mass detector equipped with TurboSpray Ion Source (ESI). Each sample was injected onto Agilent SB-C18 column.

### Assay of Cell Death by Fluorescent Microscopy

Detection and verification of cell death in the suspension of cells were carried out according to Byczkowska et al. (2013) procedure: (1) 0.5 mL of the culture medium with 0.5 mL of the appropriate cell suspension was supplemented with 0.5 mL of 0.02 M phosphate buffer pH 7.4 (PHB). (2) The cells were stained with the AO/EB mixture containing 50 µg cm<sup>-3</sup> of acridine orange and 50 µg cm<sup>-3</sup> of ethidium bromide in PHB. (3) Drops of cell suspension were immediately put on glass slides and analyzed for 5 min using fluorescent microscopy with a blue light excitation filter of the Optiphot-2 epi-fluorescence microscope (Nikon) equipped with a camera and Act-1 software (Preoptic, Poland) for fluorescent microscopy and preparation of microphotographs according to Byczkowska et al. (2013). AO/EB staining included the use of acridine orange which penetrates whole cells and stains the nuclei green and with ethidium bromide which dyes nuclei red and it is only absorbed by damaged cells with impaired cellular and nuclear membrane integrity. From the above data, a curve of the fluorescence intensity of nuclear chromatin after AO/EB staining was prepared, as described by Byczkowska et al. (2013). This scale allows the recognition of living, dying and death cells. Living cells have intact nuclei stained green, while dying cells have green-yellow, yellow, yellow-orange, or bright orange nuclei with slightly condensed or fragmented chromatin at the early stage of death whereas with condensed and fragmented chromatin at the late stage. Necrotic cells have structurally normal orange nuclei.

When the color is changed from green to red, values of fluorescence intensity of acridine orange and ethidium bromide increase (Byczkowska et al., 2013).

### Cell Fractionation

Fractionation of cells was performed using the digitonin method according to Ganju and Eastman (2003) with modification of Kobylińska et al. (2006). In all experimental variants the cells were washed twice with PBS and next permeabilized for 30 min in a buffer containing: 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 75 mM NaCl, 250 mM sucrose, digitonin (0.05% of cells weight),

20 µl/g cells 1 mM phenylmethylsulfonyl fluoride (proteases inhibitor), and cocktail of enzymes for cell wall lysis (CellLytic Sigma). Cell homogenate was obtained by centrifugation at 3000 g for 1 min. at 4°C to remove cell debris. The cleaned homogenate after centrifugation at 12000 × g was divided into two fractions: the supernatant was removed as the cytosolic fraction and the pellet (mitochondrial fraction) was resuspended in the above buffer (without digitonin). To both fractions sufficient volumes of Laemmli sample buffer supplemented with 10% β-mercaptoethanol were added (Laemmli, 1970) and the mixtures were boiled for 5 min.

### Western Blot Analysis

Fractionated BY-2 cell lysates (50 µg of proteins) were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gel (Laemmli, 1970) and transferred to Immobilon P<sup>SQ</sup> at the voltage of 20 V overnight, at 4°C according to Towbin et al. (1979). After blocking in 3% non-fat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 60 min, the membranes were incubated with primary antibodies specific to Cyt c in TBST in a cold room overnight. Subsequently, the membranes were washed several times in TBST and incubated with appropriate secondary antibodies conjugated with alkaline phosphatase (Sigma Chemical Co.) in TBS for 2 h at room temperature. Next the membranes were washed several times with TBST, and the proteins were visualized by incubation with the substrate solution (0.33 mg/ml of nitro blue tetrazolium, 0.17 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM MgCl<sub>2</sub>), prepared according to Leary et al. (1983).

### DNA Isolation

DNA digestion was performed using the cetyl-trimethylammonium bromide (CTAB) method previously described by Murray and Thompson (1980). BY-2 cells from log phase of growth (4th day, 4 h after Pb<sup>2+</sup> addition) and from the beginning of the stationary phase (6th day, 48 h after Pb<sup>2+</sup> addition) were frozen in liquid nitrogen and ground in a mortar to a fine powder. Then, CTAB buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA and 2% CTAB) was added and extraction was performed for 30 min at 65°C. After immediate cooling on ice, DNA preparation was continued in the extraction mixture of chloroform/isoamyl alcohol (24:1) until a fine emulsion was created. The organic phase was separated from the aqueous phase by centrifugation at 12000 × g for 15 min at 4°C. DNA was precipitated with isopropanol at -20°C, 20 min. The DNA precipitates were spun at 12000 × g for 10 min at 4°C, washed two times in 70% ethanol and air dried. DNA pellets were dissolved in 100 µl TE buffer (10 mM Tris-HCl pH 7.5, and 1 mM EDTA) containing 10 µl 1% RNase A. RNA digestion was conducted 2 h at 37°C. Purity of the obtained DNA preparations was determined spectrophotometrically by analysis of the absorbance spectra in the range of 230–320 nm. The value of A<sub>260/280</sub> within the limits of 1.8 – 2.0 was the criterion of DNA purity. Then, 5 µl of a loading buffer was added to each tube, and the DNA preparations were electrophoresed in 2% agarose gels

and run at 5 V/cm. The gels were stained with ethidium bromide and visualized under ultraviolet (UV) light.

### Statistical Analysis

The data represent the means ± standard deviation (±SD). Each variant of culture was replicated three times and at least three independent samples were used for measurement. The data were analyzed using STATISTICA v.10.0\_MR1\_PL [StatSoft] software. One-way or two-way analysis of variance (ANOVA) and then the *post hoc* Duncan multiple range test was carried out to find the significant differences at *p* < 0.001 in each experiment.

## RESULTS

### Cell Growth and Viability

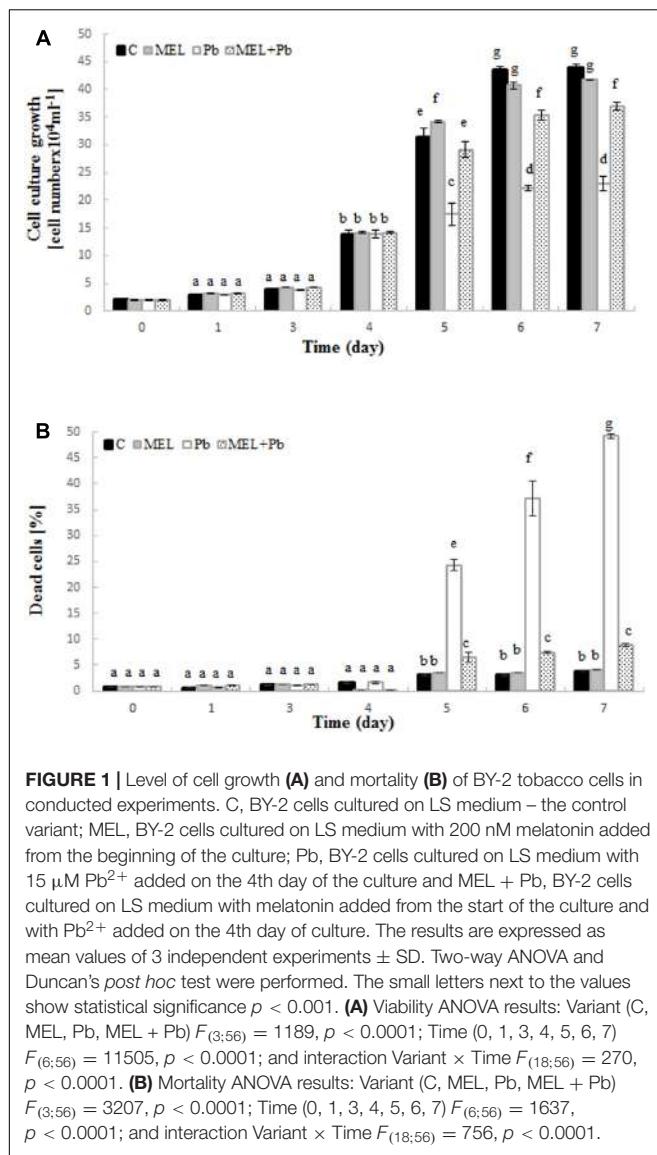
Preincubation with melatonin prior to Pb treatment protected tobacco suspension cells from death and improved cell proliferation. Cell growth intensity in C and MEL variants was similar during culture time. After Pb addition on the 4th day, a significant inhibition of tobacco cell proliferation was observed (**Figure 1A** – see the variants MEL + Pb and especially Pb). From the first day after heavy metal stress induction, proliferation of the MEL + Pb cells was about 40% higher in comparison to those treated with Pb but not primed with melatonin (Pb) (**Figure 1A**) this tendency was maintained throughout the duration of the Pb-stress.

The effects of melatonin pretreatment on viability of Pb-stressed tobacco suspension cells were verified in all experimental samples. Methylene blue staining documented the protective effect of melatonin against cell death induced by Pb. The mortality of cells exposed to Pb but preincubated with melatonin (MEL + Pb) was slightly higher than in C and MEL variants. Culture medium supplementation with melatonin did not result in cell death acceleration. The number of dead cells in the Pb exposed cells increased significantly and it was 24.2, 37.3, and 49.2% for the 1st, 2nd, and 3rd day after Pb application, respectively (**Figure 1B**). In contrast, mortality of MEL + Pb cells was about 80% lower than in the Pb cells (**Figure 1B**).

### Detection of Cell Death

Fluorescence analyses after successive addition of AO/EB fluorochromes showed that after Pb treatment BY-2 cells died via PCD. Yellow and green-yellow nuclei with slightly condensed chromatin dominated among dying cells (**Figure 2**), but some yellow-stained nuclei with condensed chromatin were also observed. This observation indicates that already 4 h after Pb treatment, BY-2 cells underwent the initial stages of cell death. At the end of cell culture (the 7th day; the 3rd day after Pb stress) nuclei with dark orange chromatin were not found, indicating that necrotic type of cell death after Pb exposure in BY-2 tobacco cells was not detected.

Unexpected effects were obtained for cells exposed to Pb but pre-incubated with melatonin (MEL + Pb). The fluorescence intensity of randomly selected nuclei for this treatment was estimated at 16% and it was similar to the Pb-untreated samples:



14 and 17% for control and melatonin treated cells respectively. In contrast fluorescence intensity in Pb variant increased to 53%, and was expressed as yellow/yellow-orange nuclei color (Figure 3).

## Profile of DNA Fragmentation

To gain insight into the mechanism of plant PCD induced by Pb, DNA fragmentation and release of Cyt c from mitochondria into cytosol was checked. We found an inhibitory effect of melatonin on DNA laddering, one of the hallmarks of PCD. Figure 4 shows that DNA isolated from control and melatonin treated cells remained intact, whereas DNA from Pb samples exhibits significant fragmentation; this was more intensive 4 h after Pb stress than 2 days later (the 6th culture day, the 2nd day after lead treatment). The analyses showed that melatonin completely blocked/reversed the cytotoxic Pb influence and

protected tobacco cells against DNA damage caused by the heavy metal.

## Cytochrome c Translocation

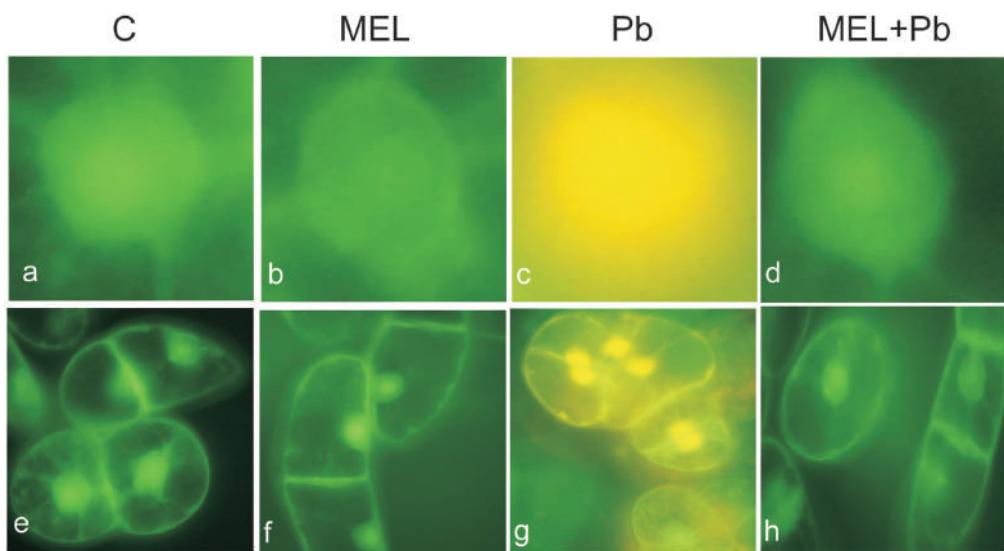
To further confirm the protective action of melatonin, immunodetection of Cyt c in mitochondrial and cytosolic fractions was examined. There is growing evidence that in plants, as in mammals, translocation of Cyt c from mitochondria to cytosol plays an important role in PCD mediated events. Detection of this protein with an antibody recognizing whole Cyt c molecule was performed in mitochondrial pellet and cytosolic fractions of BY-2 cells in all experimental groups. Unexpected effects were obtained in the 4th hour after Pb treatment, despite DNA fragmentation at that time, Cyt c was detected only in the mitochondrial pellet, suggesting that Cyt c release from mitochondria to cytosol is a later stage of PCD and takes place independent on DNA damage (Figure 5A). Relatively low, but different levels of Cyt c accumulated in mitochondrial pellet of BY-2 cells in control (C) and melatonin-treated samples (MEL and MEL + Pb) on the 2nd day after Pb treatment (Figure 5B). Release of Cyt c from mitochondria into cytosol was observed after Pb exposure where translocation of Cyt c was accompanied by almost complete disappearance of this protein from the mitochondria and its accumulation in the cytosolic fraction. MEL + Pb samples were deficient of Cyt c in cytosol, similar to Pb-untreated cells. This cytological and molecular evidence demonstrates that melatonin preincubation protects tobacco suspension cells from Pb-induced PCD.

## Content and Cellular Localization of $O_2^-$

To investigate whether the observed a Cyt c release is related to Pb-induced ROS production, we measured *in situ* accumulation of  $O_2^-$  via the nitro blue tetrazolium reducing (NBT) test. In the non-stressed cells (C, MEL), few formazan precipitants were apparent indicating the physiological origin of ROS. Cytological analyses of  $O_2^-$  production in tobacco suspension cells demonstrated, abundant formazan deposits after Pb exposure. They were especially visible in the boundary cytoplasm and in nuclei which appeared almost black (Figure 6). In contrast, in the MEL + Pb samples the amounts of formazan precipitants was similar to that in the control, confirming a reduction in  $O_2^-$  by melatonin.

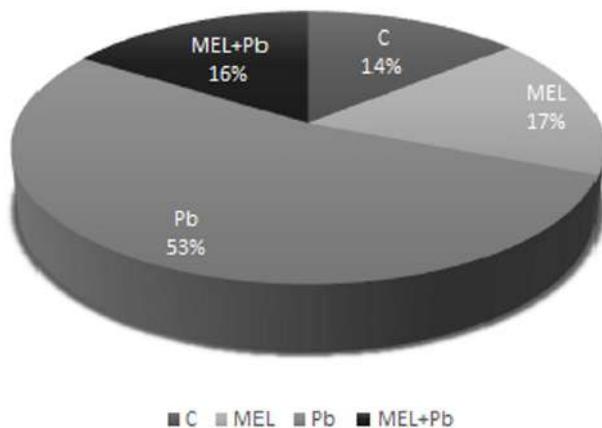
## Melatonin Content in BY-2 Cells

To test if the protective function of melatonin treatment is related to its uptake from the environment and accumulation in cells, the content of this indoleamine in cell lysates was determined at the main experimental points, i.e., in the lag, log and stationary phases of growth. Generally, BY-2 tobacco cells have low melatonin levels (Table 1). In the non-melatonin-primed cells (C and Pb), melatonin increased from zero (the 1st day of culture) to  $\sim 1$  ng/gFW (on the last day). Tobacco suspension cells synthesize endogenous melatonin, but at an extremely low level in comparison with the melatonin-treated cells (MEL and MEL + Pb). In addition, in cells under Pb stress the endogenous level of this indoleamine in comparison to the



**FIGURE 2 |** Micrographs of living and dying BY-2 nuclei (**a–d**) and whole cells (**e–h**) detected by AO/EB staining. Green nuclei of living cells (**a,b,d,e,f,h**), yellow nuclei (**c,g**) of PCD-dying cells. C, BY-2 cells cultured on LS medium – the control variant; MEL, BY-2 cells cultured on LS medium with 200 nM melatonin added from the beginning of culture; Pb, BY-2 cells cultured on LS medium with 15  $\mu$ M  $Pb^{2+}$  added on the 4th day of culture and MEL + Pb, BY-2 cells cultured on LS medium with melatonin added from the start of the culture and with  $Pb^{2+}$  added on the 4th day of culture. Micrographs were done 4 h after lead administration.

### Fluorescence intensity



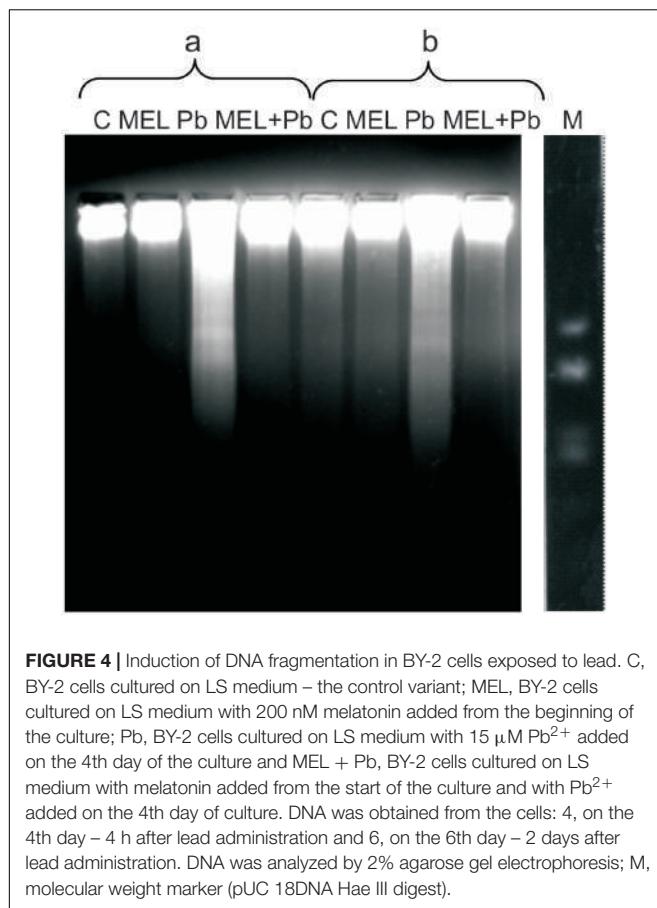
**FIGURE 3 |** The fluorescence intensity of nuclear chromatin stained with AO/EB 4 h after lead administration. C, BY-2 cells cultured on LS medium – the control variant; MEL, BY-2 cells cultured on LS medium with 200 nM melatonin added from the beginning of culture; Pb, BY-2 cells cultured on LS medium with 15  $\mu$ M  $Pb^{2+}$  added on the 4th day of culture and MEL + Pb, BY-2 cells cultured on LS medium with melatonin added from the start of the culture and with  $Pb^{2+}$  added on the 4th day of culture.

control (C) cells was about 30% lower at the end of the culture period (7th day). Concentrations of melatonin significantly increased in the cells during the period of melatonin treatment (Table 1). The data indicate that BY-2 cells absorbed melatonin from the medium. Interestingly, MEL + Pb cells absorbed 20% more than MEL-treated cells alone.

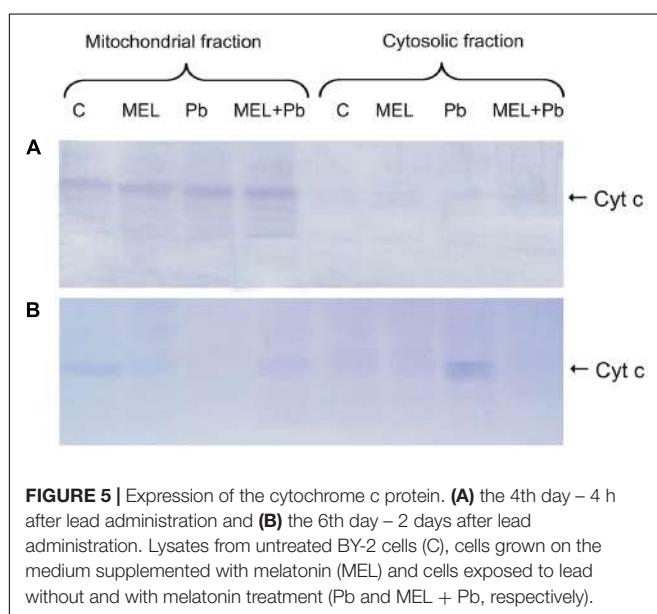
### DISCUSSION

Melatonin was discovered in the plant kingdom in Dubbels et al. (1995), Hattori et al. (1995) and significant progress has been made in defining its multiple roles in plants. Many researchers underline the fact that among its various roles, its antioxidant effectiveness and free radical scavenging ability, that protect plants against oxidative stress and alleviate or counteract cell damage, are crucial to plant physiology as in animals (Reiter et al., 1997; Tian et al., 2001). Melatonin is widely present in many higher plants (Arnao and Hernandez-Ruiz, 2015; Reiter et al., 2015). Elevated levels of melatonin protect plants against water and soil pollutants by acting as a direct free radical scavenger (Tan et al., 2007; Manchester et al., 2015) and/or as an indirect antioxidant stimulating antioxidant enzymes (Rodriguez et al., 2004; Bałabusta et al., 2016). Melatonin metabolites also possess antioxidant properties and they act in synergy with other antioxidants, such as ascorbic acid, glutathione, etc. (Gitto et al., 2001; Arnao and Hernández-Ruiz, 2009; Kołodziejczyk et al., 2015). Melatonin protects plant tissues and organs, particularly reproductive tissues, fruit and germ tissues of the seeds, from secondary oxidative stress caused by unfavorable environmental conditions, such as drought, salinity, cold, heat, ultraviolet light and ozone (Van Tassel et al., 2001; Dawood and El-Awadi, 2015; Reiter et al., 2015). The data indicate that exogenously applied melatonin also acts as plant biostimulator especially under suboptimal environmental conditions (Posmyk et al., 2008, 2009; Janas and Posmyk, 2013; Kołodziejczyk et al., 2016).

There is growing evidence that in plants melatonin action is associated with its antioxidant properties. Moreover melatonin as the antiapoptotic factor is well documented in various animal cells, but not in plants (Reiter et al., 1997, 2015). Thus, the aim



**FIGURE 4 |** Induction of DNA fragmentation in BY-2 cells exposed to lead. C, BY-2 cells cultured on LS medium – the control variant; MEL, BY-2 cells cultured on LS medium with 200 nM melatonin added from the beginning of the culture; Pb, BY-2 cells cultured on LS medium with 15  $\mu$ M  $Pb^{2+}$  added on the 4th day of the culture and MEL + Pb, BY-2 cells cultured on LS medium with melatonin added from the start of the culture and with  $Pb^{2+}$  added on the 4th day of culture. DNA was obtained from the cells: 4, on the 4th day – 4 h after lead administration and 6, on the 6th day – 2 days after lead administration. DNA was analyzed by 2% agarose gel electrophoresis; M, molecular weight marker (pUC 18DNA Hae III digest).



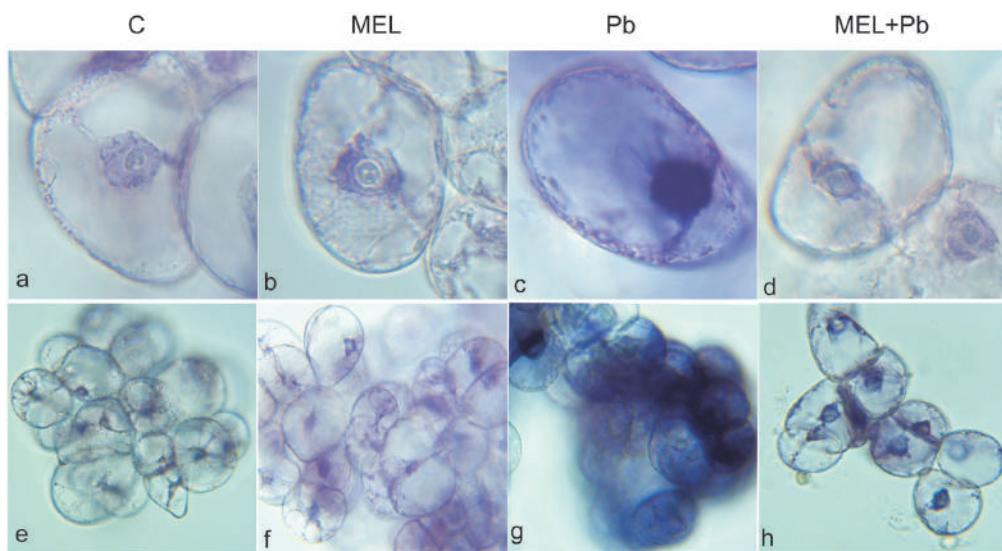
**FIGURE 5 |** Expression of the cytochrome c protein. **(A)** the 4th day – 4 h after lead administration and **(B)** the 6th day – 2 days after lead administration. Lysates from untreated BY-2 cells (C), cells grown on the medium supplemented with melatonin (MEL) and cells exposed to lead without and with melatonin treatment (Pb and MEL + Pb, respectively).

of our investigation was to check melatonin influence on heavy metal-induced cell death in tobacco suspension cultures. There is rather little information on the mechanisms by which melatonin prevents plant cells from dying after Pb exposure.

Presented studies were preceded by analyses of influence the different melatonin concentrations as well as Pb, on BY-2 suspension cells (data not shown). The range of tested melatonin concentrations was 100–1000 nM, whereas Pb 0.5–50  $\mu$ M. The concentration of Pb applied in presented experiments was chosen on the basis of measurement of LC50 on the 7th day of BY-2 cell cultivation. Our preliminary studies revealed that exogenous melatonin has ambiguous effects on BY-2 cells: it is an effective biostimulator when applied in concentration below 300 nM, but in excessive doses (above 300 nM) it significantly decreased both BY-2 cell proliferation and viability. This agrees with the previous publications of our team, where we also observed different effects of melatonin treatments dependent on the dosage used (Posmyk et al., 2008, 2009; Janas and Posmyk, 2013). This indicates, that melatonin, despite its potentially positive properties (e.g., antioxidative), can not be considered as being always protective since high concentration may have harmful side effects. The choice of dosage is crucial for the positive effects to be realized; in BY-2 cells melatonin was protective in the range 100–300 nM.

Our preliminary experiments led us to determine the optimal melatonin dose as 200 nM that stimulated proliferation of BY-2 cells and nearly completely reversed effects of Pb-stress. Thus, initially cell proliferation and viability during lag, log and stationary phases of growth both under optimal (C and MEL) as well as under heavy metal stress condition (Pb and MEL + Pb) were determined. The positive effects of preincubation of BY-2 cells with melatonin were visible during and after Pb-stress which suggests that melatonin at a concentration 200 nM fortifies cells against potentially stress conditions even before they appear. Proliferation of cells pretreated with melatonin but exposed to Pb (MEL + Pb) were only slightly worse than of the unstressed cells (C and MEL) whereas in the Pb-stressed cells the level of proliferation was more than 50% lower in comparison to the control. The protective role of melatonin against cell death was clearly visible in the cell mortality analyses during Pb-stress. The number of dead cells in the Pb-treated cells increased drastically while unexpectedly in the MEL + Pb variant BY-2 cell viability was about 80% higher than in the Pb samples (Figure 1B). The melatonin ability to prevent cell death was confirmed by the studies of fluorescence intensity of nuclear chromatin stained with AO/EB. In our experiments morphological changes in the nuclei of the Pb exposed cells, i.e., chromatin condensation, green-yellow and yellow color of nuclei, was shown (Figures 2, 3).

Plant cells that undergo PCD exhibit many of the same morphological characteristics as cells undergoing PCD in mammals and *Caenorhabditis elegans* including intensified formation of vesicles, cytoplasmic condensation, nuclear condensation, DNA fragmentation and chromatin condensation leading to DNA laddering as well as translocation of Cyt c (Balk et al., 2003; Lord and Gunawardena, 2012). Our results showed that in BY-2 cells DNA fragmentation appeared already at 4 h after Pb application and it persisted until the 2nd day after Pb application. In electrophoregrams of DNA samples isolated from the Pb-exposed cells extensive oligonucleosomal



**FIGURE 6 |** Superoxide anions detected by NBT staining in tobacco BY-2 cells. The cells were stained 4 h after lead administration. C, BY-2 cells cultured on LS medium – the control variant; MEL, BY-2 cells cultured on LS medium with 200 nM melatonin added from the beginning of the culture; Pb, BY-2 cells cultured on LS medium with 15  $\mu$ M  $Pb^{2+}$  added on the 4th day of culture and MEL + Pb, BY-2 cells cultured on LS medium with melatonin added from the start of the culture and with  $Pb^{2+}$  added on the 4th day of culture. BY-2 cells zoomed  $\times 1000$  (a–d) and  $\times 100$  (e–h).

fragmentation caused by this heavy metal was observed, which was not noted in untreated cells (C and MEL). In the MEL + Pb treated cells melatonin completely blocked DNA laddering. Our results are in line with the studies of Lei et al. (2004) who showed that melatonin increased tolerance to cold in carrot suspension cells and protected their DNA against damage. Similar beneficial effects caused by exogenous melatonin were observed by Posmyk et al. (2008) in red cabbage seedlings subjected to copper stress and in cucumber seedlings subjected to chilling stress (Posmyk et al., 2009).

**TABLE 1 |** Melatonin concentration (ngMEL/gFW) in homogenates of BY-2 cells in crucial points of conducted experiments.

Day of culture → Variant	1	4 <sup>1)</sup>	7
C	0.00 ± 0.00	0.72 a ± 0.01	0.94 a ± 0.05*
MEL	6.70 b ± 0.24	15.18 c ± 2.10	34.92 d ± 4.00
Pb	–	–	0.65 a ± 0.24**
MEL + Pb	–	–	41.31 e ± 3.28

HPLC-MS measurements was performed: 1st day – during lag phase; 4th day after passaging – during log phase, <sup>1)</sup>this day was also chosen as the start of Pb-stress; and 7th day – during stationary phase of growth (variants C and MEL) and when Pb-stress symptoms should be detectable (variants Pb and MEL + Pb). Experimental BY-2 cell were cultured according to following variants: control (C) – cells cultured on full LS medium, (MEL) – cell cultured on melatonin-supplemented medium, (Pb) – cells cultured with addition of lead alone and with pretreatment with melatonin (MEL + Pb). The results are expressed as mean values of 3–4 measurements ± SD. Two-way ANOVA and Duncan's post hoc test were performed. The small letters next to the values show statistical significance  $p < 0.0001$ . Melatonin ANOVA results: Variant (C, MEL, Pb, MEL + Pb)  $F_{(3,24)} = 440$ ,  $p < 0.0001$ ; Time (1, 4, 7)  $F_{(2,24)} = 317$ ,  $p < 0.0001$ ; and interaction Variant × Time  $F_{(6,24)} = 100$ ,  $p < 0.0001$ . In a case of 7<sup>th</sup> day C and Pb variant additionally Student's t-test were performed and the statistically significant difference between this two values was determined at  $p < 0.05$  (marked by stars).

The data presented suggest that pre-incubation of BY-2 cells with melatonin limits the toxicity of Pb and protects cells against death. We used the plant model system to pinpoint the PCD phase when melatonin may act. It has been reported that release of Cyt c from mitochondrial intermembrane space to cytosol is a conserved pathway of PCD and it has been noted in many systems (Martínez-Fábregas et al., 2013). In plants, this issue has been poorly investigated and the mechanisms of Cyt c release, its role in determining cell death are still controversial. Vacca et al. (2006) reported that heat shock triggered Cyt c translocation but Cyt c was degraded en route to cell death. Moreover, Cyt c release is linked to ROS burst and strictly depends on ROS production, and it identifies the early phase of cell death. However, our Western blot analyses did not reveal translocation of Cyt c from mitochondria to cytosol 4 h after the heavy metal stress rather this occurred much later (the 6th day of culture, the 2nd day after lead administration). Bolduc and Brisson (2002) as well as Kawai-Yamada et al. (2004) reported that oxidative metabolism leading to generation of ROS was one of the earliest events in PCD induced by biotic or abiotic stress in tobacco plants. Thus, this suggests that high levels of ROS mediate the signaling cascade for defensive gene induction, e.g., *hsp*, *lea*, *cor* (Vincour and Altman, 2005). In our studies, ROS production and DNA laddering were the early features of PCD and translocation of Cyt c was independent of them. We observed DNA fragmentation before Cyt c translocation, which seems to support the hypothesis of Collazo et al. (2006) that NO/ROS ratio may induce a set of defense responses including cleavage of an inhibitor of caspase-activated DNase (ICAD) (Krishnamurthy et al., 2000; Collazo et al., 2006). Although the presence of caspases in plants is debated, cysteine protease activity has been reported to be

induced in plant systems undergoing cell death (Lam and del Pozo, 2000; Bozhkov et al., 2004). These proteases might function in a plant proteolytic network leading to disconnection of ICAD from DNase (CAD) and fragmentation of DNA. Moreover, it would confirm our observations that in MEL + Pb cells, despite Pb application, DNA was not cleaved since this indoleamine is a highly effective antioxidant and it blocked caspase-like signaling leading to activation of CAD. Furthermore, our results revealed accumulation of superoxide radical in Pb exposed cells at the 4th hour after Pb treatment with only a slight detection in the control (C) and melatonin treated cells (MEL, MEL + Pb) (**Figure 6**). Thus, our finding are in line with the data of Vacca et al. (2006) who showed that ROS scavenging inhibited Cyt c release. Jou et al. (2004) documented that in rat brain astrocytes melatonin inhibited opening of mitochondrial permeability transition pore (MPT) and blocked MPT-dependent Cyt c release.

To test whether tobacco cells synthesize endogenous melatonin, and/or are capable of active absorption exogenous melatonin from the environment, the contents of this indoleamine in cell lysates were determined at the crucial points of the experiments, i.e., lag, log and stationary phase of growth.

Our results indicated that tobacco BY-2 cells are able to synthesize small amounts of this indoleamine depending on the phase of growth (its endogenous level increased slightly during experiment) as well as to absorb it actively from the medium (**Table 1**). It is known that biosynthesis and metabolism of this indoleamine are affected and modified by environmental conditions (i.e., stresses), and melatonin levels change during plant ontogenesis (Okazaki and Ezura, 2009). Elevated melatonin synthesis is often combined with the plant defense strategy because generally it was noticed that various plant species rich in melatonin had greater capacity for stress tolerance (Park et al., 2013; Bajwa et al., 2014; Zhang et al., 2015).

Availability of exogenous melatonin allowed BY-2 cells to take up its large quantities throughout the culture period (**Table 1**). Similar results were observed by Kołodziejczyk et al. (2015) in the case of cucumber and corn seeds which were primed with exogenous melatonin – they absorbed quantities

of this indoleamine proportional to its concentration applied during priming (Kołodziejczyk et al., 2015). Interestingly, under unfavorable conditions, Pb-stressed cells (MEL + Pb) absorbed melatonin near 20% more intensively in comparison to the unstressed melatonin treated cells (MEL). Although, it is probably not a natural defense strategy of *Nicotiana tabacum* cells since endogenous melatonin content did not measurably increase in BY-2 cells under Pb stress conditions (**Table 1**). These results suggest that BY-2 cells readily absorb and use the accessed exogenous melatonin to counteract stress-induced damage. Survival of plants in polluted environments largely depends on their ability to sequester and/or detoxify toxic substances such as Pb. Tan et al. (2007) also found that melatonin was effective in preventing the death of pea plants grown in soil contaminated with copper.

We have shown that although tobacco is not a plant rich in endogenous melatonin, it is able to use it from an exogenous source as a potential effective factor for improving its stress defenses. This fact once again confirms the practical use of melatonin as a plant biostimulator (Janas and Posmyk, 2013; Kołodziejczyk and Posmyk, 2016; Nawaz et al., 2016). Moreover, we have presented novel findings concerning a decrease of mitochondrial Cyt c release together with limited DNA degradation, which suggest that the protection mechanism of melatonin is not only *via* limitation of secondary oxidative stress, but also *via* counteraction against PCD. In conclusion, melatonin has multiple actions as a factor fortifying cells against potential, harmful conditions.

## AUTHOR CONTRIBUTIONS

AK: work conception, all experiments concerning *Nicotiana tabacum* BY-2 suspension cells realization, data acquisition and analysis, drafting of the manuscript. RR: research consultation/discussion, manuscript revision: language and editorial corrections. MP: methodological consultant, statistical calculations, data analysis and interpretation, manuscript revision.

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# Exogenous Melatonin Confers Salt Stress Tolerance to Watermelon by Improving Photosynthesis and Redox Homeostasis

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Melatonin, a pleiotropic signal molecule, has been shown to play important roles in the regulation of plant growth, development, and responses to environmental stresses. Since a few species have been investigated to unveil the effect of exogenous melatonin on salt stress, the underlying mechanism of melatonin-mediated salt stress tolerance in other plant species still remains largely unknown. In this study, the effects of melatonin on leaf photosynthesis and redox homeostasis in watermelon were examined under salt stress (300 mM NaCl) along with different doses of melatonin (50, 150, and 500 µM) pretreatment. NaCl stress inhibited photosynthesis and increased accumulation of reactive oxygen species and membrane damage in leaves of watermelon seedlings. However, pretreatment with melatonin on roots alleviated NaCl-induced decrease in photosynthetic rate and oxidative stress in a dose-dependent manner. The protection of photosynthesis by melatonin was closely associated with the inhibition of stomatal closure and improved light energy absorption and electron transport in photosystem II, while the reduction of oxidative stress by melatonin was attributed to the improved redox homeostasis coupled with the enhanced activities of antioxidant enzymes. This study unraveled crucial role of melatonin in salt stress mitigation and thus can be implicated in the management of salinity in watermelon cultivation.

**Keywords:** melatonin, photosynthesis, redox homeostasis, salt stress, watermelon

## INTRODUCTION

Since plants cannot relocate, they have to endure multiple biotic and abiotic stresses throughout their life cycle. Among these stresses, soil salinity is one of the most important environmental hazards that inhibit plant growth and development, causing significant yield losses, particularly in arid and semi-arid areas (Evelin et al., 2009; Porcel et al., 2012). Salinity adversely affects plant physiology through multiple mechanisms. Firstly, increased accumulation of sodium ions ( $\text{Na}^+$ ) cause damage to cellular organelles, inhibit protein synthesis and enzyme activities, and uncouple photosynthesis and respiration; secondly, salinity decreases nutrient uptake and/or transport to the shoot, resulting in a nutrient imbalance; and thirdly, salinity decreases soil osmotic potentials and

hinders water uptake by roots, leading to a physiological drought in the plant (Ruiz-Lozano et al., 2012).

Photosynthesis, the most important physico-chemical process accountable for the energy production in higher plants, is very sensitive to salt stress (Meloni et al., 2003). During salt stress, the intercellular CO<sub>2</sub> concentration in leaf is decreased due to stomatal closure. In addition, salt stress reduces consumption of NADPH by the Calvin cycle, inhibits chlorophyll synthesis and Rubisco activity, and disrupts the photosynthetic electron transport. Notably, salinity-induced inhibition of the photosynthetic electron transport results in excessive accumulation of toxic reactive oxygen species (ROS) such as O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub>, and •OH and disruption of cellular redox homeostasis. Over accumulation of ROS promotes degradation of chlorophyll and reduces photochemical efficiency of photosystem II (PSII) forming a vicious cycle (Woo et al., 2004; Allakhverdiev et al., 2008). Moreover, as strong oxidant, ROS at high concentration can damage membranes through lipid peroxidation, break DNA strand, and inactivate various vital enzymes (Cheng and Song, 2006).

Melatonin (*N*-acetyl-5-methoxytryptamine), a pleiotropic and highly conserved molecule, is ubiquitous throughout the animal and plant kingdoms (Hardestrand et al., 2011). Since the discovery of melatonin in vascular plants in 1995 (Dubbels et al., 1995; Hattori et al., 1995), numerous subsequent studies have demonstrated its important roles in regulating plant growth, development, and defense against various environmental stresses (Arnao and Hernández-Ruiz, 2014, 2015; Zhang et al., 2015; Nawaz et al., 2016). The beneficial role of melatonin in stress mitigation is broadly attributable to higher photosynthesis, improvement of cellular redox homeostasis and alleviation of oxidative stress, and regulation of the expression of stress-responsive genes involved in signal transduction (Li et al., 2012; Bajwa et al., 2014; Zhang and Zhang, 2014). Recently, several studies have shown that salt stress can increase melatonin content in roots (Arnao and Hernández-Ruiz, 2009; Mukherjee et al., 2014) and exogenous application of melatonin enhances salt stress tolerance in *Malus hupehensis* and *Helianthus annuus* (Li et al., 2012; Mukherjee et al., 2014). However, it is still unclear whether such response of melatonin against salt stress is universal for other plant species. Furthermore, the mechanism of melatonin-mediated salt stress tolerance is obscure.

Watermelon (*Citrullus lanatus* L.), one of the economically important crops in the world, is a high water-consuming plant and is very sensitive to salt stress (Yetişir and Uygur, 2009). In the present study, we tried to understand the regulatory mechanism controlling melatonin-mediated salt stress tolerance in watermelon in terms of photosynthesis and redox homeostasis. The response of photosynthetic process including stomatal movement, energy absorption, photosynthetic electron transport, and CO<sub>2</sub> assimilation and the response of redox homeostasis including ROS-generation and -scavenging under salt stress were investigated. This study provides a novel physiological basis for further dissection of the regulatory mechanism of melatonin-mediated salt stress tolerance in watermelon.

## MATERIALS AND METHODS

### Plant Material and Treatments

Seeds of watermelon (*Citrullus lanatus* L. cv. 04-1-2) were provided by the Watermelon and Melon Research Group at Northwest A&F University, Yangling, China. Seeds were surface sterilized with 5% sodium hypochlorite for 5 min, pre-soaked at 25°C sterile water for 10 h and then placed on moist filter paper in Petri dish in the dark at 30°C for germination. Germinated seeds were sown in plastic pots (10 cm × 7 cm × 8.5 cm) filled with a mixture of 2:1 (v/v) autoclaved sand and commercial peat-based compost (Shaanxi Yufeng Seed Industry Co., Ltd., Yangling, China). The seedlings were pre-cultured under natural light in a greenhouse at Northwest A&F University, Yangling, China (34°28'N, 108°06'E) where the relative humidity was 65–95%, and the temperature was 28–35°C/16–20°C (day/night). All seedlings were uniformly watered daily and fertilized weekly with 1/2 strength Hoagland's solution.

Seedlings at the three-leaf stage were treated with 0, 50, 150, or 500 µM melatonin (80 mL per plant) on roots for 6 days (once every 2 days). The melatonin (Sigma-Aldrich, St. Louis, MO, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol/water (v/v) = 1/10000]. After melatonin pretreatment, plants were irrigated with 300 mM NaCl (80 mL per plant). Seven days later, samples of leaf (the second fully expanded leaf beneath the growing point) were harvested after measuring the gas exchange and chlorophyll fluorescence parameters. Harvested samples were rapidly frozen in liquid nitrogen and stored at –80°C until the biochemical assay.

### Gas Exchange and Chlorophyll Content Measurements

The gas exchange of attached leaves was measured using an infrared gas analyzer, Li-Cor-6400 (Li-Cor Inc., Lincoln, NE, USA) equipped with an LED red/blue light source (6400-02B). The photosynthetic photon flux density (PPFD) was set at 1000 µmol m<sup>-2</sup> s<sup>-1</sup> and the cuvette air flow rate was 500 ml min<sup>-1</sup>. Net photosynthetic rate (Pn) and stomatal conductance (Gs) were recorded simultaneously.

Photosynthetic pigments from leaf were extracted in 80% acetone and the contents of chlorophyll a and chlorophyll b were determined according to the method of Lichtenthaler and Wellburn (1983).

### Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence parameters were measured in leaves with a Portable Chlorophyll Fluorometer (PAM2500; Heinz Walz, Effeltrich, Germany) after a 30 min dark-adaptation. The maximum photochemical efficiency of PSII (Fv/Fm), actual photochemical efficiency of PSII [Y(II)], photochemical quenching (qP), and non-photochemical quenching [Y(NPQ)] were calculated according to Pfundel et al. (2008).

## Analysis of H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>·-</sup>, Malondialdehyde, and Relative Electrolyte Leakage

H<sub>2</sub>O<sub>2</sub> content was measured according to the method of Willekens et al. (1997) with a slight modification. Briefly, 0.3 g of leaf samples was homogenized with 3 mL of 1 M HClO<sub>4</sub> at 4°C, and the homogenate was centrifuged at 6,000 × g for 5 min at 4°C. pH of the supernatant was adjusted to 6.0–7.0 with 4 M KOH and centrifuged at 12,000 × g for 5 min at 4°C. Afterward, the supernatant was passed through an AG1x8 pre-packed column (Bio-Rad, Hercules, CA, USA) and H<sub>2</sub>O<sub>2</sub> was eluted with 4 mL double-distilled H<sub>2</sub>O. The sample (800 μL) was mixed with 400 μL reaction buffer containing 4 mM 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) and 100 mM potassium acetate at pH 4.4, 400 μL deionized water and 0.25 U of horseradish peroxidase (HRP). H<sub>2</sub>O<sub>2</sub> content was measured at OD<sub>412</sub>.

Superoxide production was quantified according to the method of Elstner and Heupel (1976) with a slight modification. 0.5 g of leaf sample was homogenized with 3 mL of 65 mM potassium phosphate buffer (pH 7.8). After centrifugation, 1 mL of the supernatant was mixed with 0.9 mL of 65 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride. After incubation at 25°C for 20 min, 17 mM sulfanilamide and 7 mM α-naphthylamine were added to the incubation mixture and reaction at 25°C for 20 min. Then, ethyl ether in the same volume was added and centrifuged at 1,500 × g for 5 min. The absorbance was recorded at 530 nm. Sodium nitrite was used as a standard solution to calculate the production rate of superoxide.

Malondialdehyde (MDA) as an end product of lipid peroxidation was measured according to the method of Hodges et al. (1999). Leaf sample (0.3 g) was homogenized in 5 mL of 10% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 3,000 × g for 10 min. Then, 4 mL of 20% TCA containing 0.65% (w/v) TBA was added to 1 mL of supernatant. The mixture was heated at 95°C for 25 min and immediately cooled to stop the reaction. After centrifugation at 3,000 × g for 10 min, the absorbance of the supernatant was recorded at 440, 532, and 600 nm.

The relative electric leakage (REL) was measured and calculated as described previously by Zhou and Leul (1998). The second fully expanded leaf beneath the growing point (1.0 g) were cut into 0.5-cm circles using a punch and placed in a 50-mL test tube containing 25-mL deionized water. Afterward, the test tubes were vacuumed for 10 min, the leaf samples were immersed and vibrated 20 min, and then measured the conductivity of the solution (C<sub>1</sub>) using a conductivity meter (DDS-2307). Then samples were boiled for 10 min and the conductivity (C<sub>2</sub>) was measured again when the solution was cooled to room temperature. REL was calculated as C<sub>1</sub>/C<sub>2</sub> × 100%.

## AsA and GSH Determination

Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were determined according to Rao et al. (1995) by an enzymatic recycling method. Leaf sample (0.3 g) was homogenized in 2 mL of 5% metaphosphoric acid containing

2 mM EDTA and centrifuged at 4°C for 15 min at 12,000 × g. For the total glutathione assay, 0.1 mL of the supernatant was added to a reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM 5,5'-dithio-bis (2-nitrobenzoic acid). The reaction was started by adding 3 U of GR and was monitored by measuring the changes in absorbance at 412 nm for 1 min. For the GSSG assay, GSH was masked by adding 20 μL of 2-vinylpyridine for 1 h at 25°C. The GSH concentration was obtained by subtracting the GSSG concentration from the total concentration.

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) were measured following the method of Law et al. (1983). Leaf sample (0.3 g) was homogenized in cold 6% (w/v) TCA. For total ascorbate (AsA+DHA) content assay, the extract was incubated with 150 mM phosphate buffer solution (pH 7.4) and 10 mM DTT for 20 min to reduce all DHA to AsA and then 100 μL of 0.5% (w/v) N-ethylmaleimide (NEM) was added to remove excess DTT. For AsA content assay, 200 μL deionized H<sub>2</sub>O was substituted for DTT and NEM. Then the reaction mixtures were added 400 μL 10% (w/v) TCA, 400 μL 44% phosphoric acid (v/v), 400 μL 70% (v/v) α'-dipyridyl in ethanol, and 200 μL 3% (w/v) FeCl<sub>3</sub>. The reaction mixtures were then incubated at 37°C for 60 min in a water bath and the absorbance was recorded at 525 nm. The DHA concentration was obtained by subtracting the AsA concentration from the total concentration.

## Antioxidant Enzyme Extraction and Activity Assays

Antioxidant enzyme activities were assayed in leaves by using spectrophotometric methods. For extraction of enzymes, frozen leaf sample (0.3 g) was ground with 3 mL ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM AsA, and 2% PVP. The homogenates were centrifuged at 4°C for 20 min at 12,000 × g, and the resulting supernatants were used for the determination of enzymatic activity. Protein contents were determined following the method of Bradford and Williams (1976).

Superoxide dismutase (SOD) activity was assayed according to the method of Stewart and Bewley (1980) based on photochemical reduction of nitro blue tetrazolium (NBT). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction rate of NBT as monitored at 560 nm. According to the procedure described by Cakmak and Marschner (1992), catalase (CAT) activity was measured in a reaction mixture containing 25 mM phosphate buffer (pH 7.0), 10 mM H<sub>2</sub>O<sub>2</sub>, and the enzyme extract. A decline in 240 nm was monitored. Ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) activities were measured according to the method of Nakano and Asada (1981). The reaction mixture for APX contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM AsA, 1 mM H<sub>2</sub>O<sub>2</sub>, and enzyme extract. The reaction was initiated by adding H<sub>2</sub>O<sub>2</sub> and the decrease in absorbance at 290 nm was monitored for 1 min. The reaction solution for DHAR contained 50 mM phosphate buffer, pH 7.0, 2.5 mM GSH, 0.1 mM EDTA, 0.2 mM dehydroascorbate (DHA), and enzyme extract. The reaction was initiated by adding DHA and the change in absorbance at 265 nm was monitored.

for 1 min. Monodehydroascorbate reductase (MDHAR) activity was assayed by monitoring the decrease in absorbance at 340 nm owing to reduced nicotinamide adenine dinucleotide (NADH) oxidation (Arrigoni et al., 1981). The assay solution contained 50 mM HEPES-KOH, pH 7.6, 2.5 mM AsA, 0.1 mM NADH, 0.5 U AsA oxidase, and 100  $\mu$ L enzyme extract. The reaction was initiated by adding AsA oxidase.

## Statistical Analysis

The experiment was a completely randomized design with three replicates. Each replicate contained at least 10 plants. Analysis of variance (ANOVA) was used to test for significance, and significant differences ( $P < 0.05$ ) between treatments were determined using Tukey's test.

## RESULTS

### The Effects of Melatonin on Photosynthesis in Watermelon under Salt Stress

As shown in **Figure 1A**, NaCl treatment resulted in a decreased  $P_n$  and  $G_s$  in watermelon. However, pretreatment with various concentrations (50–500  $\mu$ M) of melatonin obviously alleviated salt stress-induced reduction in leaf  $P_n$  and  $G_s$ , whereas 150  $\mu$ M melatonin appeared to be the most effective concentration in alleviating salt stress. For instance, after imposition of salt stress,  $P_n$  and  $G_s$  in the watermelon plants pretreated with 150  $\mu$ M melatonin were reduced by 35.9 and 67.3%, respectively, which were fairly lower than those in the control plants, accounting for 76.7 and 88.9%, respectively. Both the higher and lower doses of melatonin were less effective in improving photosynthesis under salt stress. Similarly, chlorophyll a and chlorophyll b contents were decreased by NaCl stress, however, these decreases were alleviated by pretreatment with melatonin (**Figure 1B**).

Leaf  $F_v/F_m$  remained almost unchanged under NaCl treatment alone or combined with melatonin pretreatment (**Figure 2**).  $Y(II)$  and  $qP$  were significantly decreased by NaCl stress. NaCl-induced decreases in  $Y(II)$  were alleviated by pretreatment with melatonin at 150 and 500  $\mu$ M, more remarkably with the former one, while the decreases in  $qP$  were alleviated by pretreatment with melatonin at 50 and 150  $\mu$ M, especially with the latter one.  $Y(II)$  and  $qP$  in the plants with 150  $\mu$ M melatonin pretreatment were 45.4 and 27.2% higher than those in the control plants, after NaCl stress. However,  $Y(NPQ)$  which represents heat dissipation in PSII was induced by NaCl treatment, but this induction was attenuated by pretreatment with melatonin at 150  $\mu$ M.

### The Effects of Melatonin on Oxidative Stress in Watermelon under Salt Stress

As shown in **Figure 3A**,  $O_2^{\bullet-}$  generation and  $H_2O_2$  content were increased by NaCl stress. However, melatonin pretreatment significantly reduced NaCl-induced accumulation of  $O_2^{\bullet-}$  and  $H_2O_2$ . The optimum concentrations of melatonin for

alleviating NaCl-induced accumulation of  $O_2^{\bullet-}$  and  $H_2O_2$  were 50 and 150  $\mu$ M, respectively. Similarly, both MDA and REL, which reflect damage to cell membrane, were significantly increased by NaCl stress in control plants, and these increases were attenuated by pretreatment with melatonin at 50, 150, or 500  $\mu$ M (**Figure 3B**). For instance, mean values of  $O_2^{\bullet-}$ ,  $H_2O_2$ , MDA, and REL in the plants, pretreated with 150  $\mu$ M melatonin followed by NaCl stress, were increased by 31.4, 110.4, 54.2, and 11.0%, respectively, far less than those in the control plants, accounting for 60.8, 348.9, 214.6, and 47.2%, respectively, after NaCl stress alone.

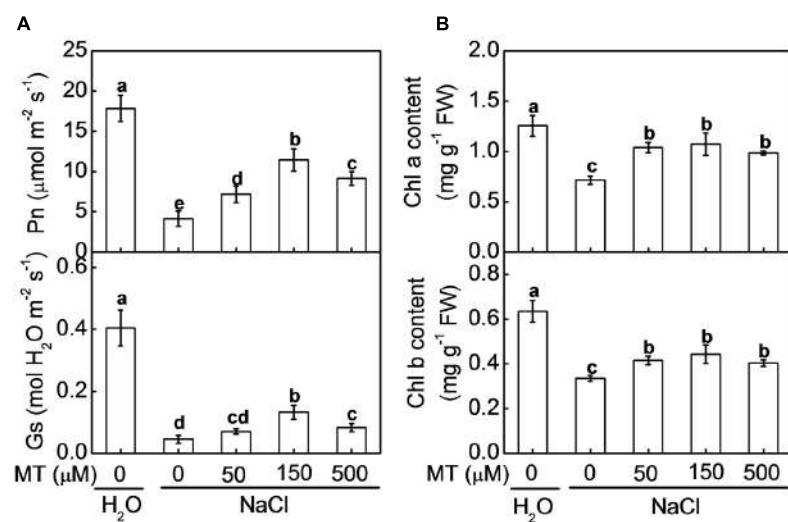
### The Effects of Melatonin on Antioxidant System in Watermelon under Salt Stress

Through evolution, plants have developed a set of antioxidant systems to remove excessive ROS that are harmful to plant cells. In control plants, the contents of GSH and AsA were decreased, but the contents of GSSG and dehydroascorbate (DHA) were increased by NaCl stress (**Figure 4**). As a result, the ratios of GSH/GSSG and AsA/DHA in control plants were dramatically reduced by NaCl stress. However, pretreatment with melatonin increased GSH and AsA contents but decreased GSSG and DHA contents under salt stress. Eventually, the ratio of GSH/GSSG and AsA/DHA were significantly higher in the plants pretreated with melatonin than those in control plants after NaCl stress. Moreover, the highest ratios of GSH/GSSG (26.5) and AsA/DHA (6.1) were found in the plants with 150  $\mu$ M melatonin pretreatment after NaCl stress.

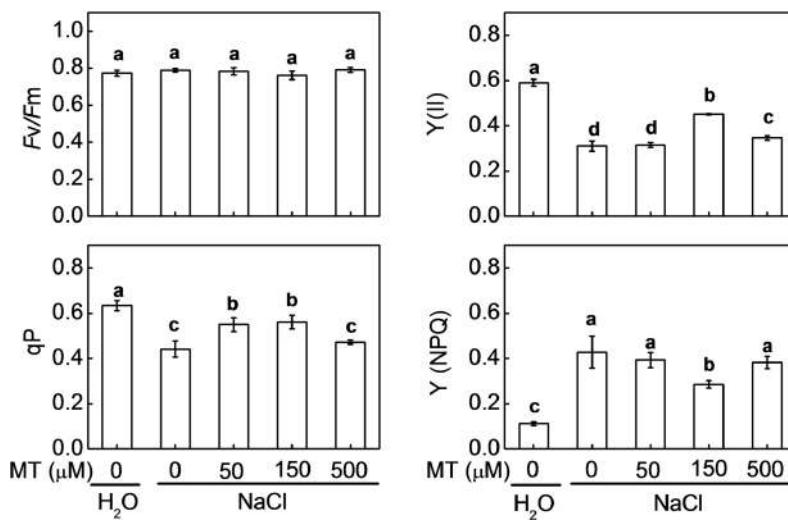
The activities of major antioxidant enzymes such as SOD, CAT, APX, DHAR, and MDHAR were significantly decreased by NaCl stress in control plants (**Figure 5**). However, NaCl-induced decreases in CAT and APX were only alleviated by pretreatment with 150  $\mu$ M melatonin, the decreases in SOD and DHAR were alleviated by pretreatment with 50, 150, or 500  $\mu$ M melatonin, and the decrease in MDHAR was alleviated by pretreatment with 150 or 500  $\mu$ M melatonin. Moreover, after NaCl treatment, the highest activities of CAT, APX, and MDHAR were recorded in the plants with 150  $\mu$ M melatonin pretreatment, while the highest activities of SOD and DHAR were recorded in the plants with 500 and 50  $\mu$ M melatonin pretreatment, respectively.

## DISCUSSION

In recent years, melatonin has emerged as a research focus in plant science. Previous studies have shown that exogenous melatonin enhances salt stress tolerance in some plant species including *M. hupehensis* and *Glycine max* (Li et al., 2012; Mukherjee et al., 2014). Nevertheless, our knowledge regarding the mechanisms involved in melatonin-mediated tolerance to salt stress still remains fragmentary. In this study, we examined the effects of melatonin on photosynthesis and redox state in watermelon under NaCl stress. Our results indicate that application of melatonin on roots enhances watermelon tolerance



**FIGURE 1 | Changes in (A) the net photosynthetic rate ( $\text{Pn}$ ) and stomatal conductance ( $\text{Gs}$ ) and (B) contents of chlorophyll a and chlorophyll b in watermelon plants as influenced by salt stress alone or combined with melatonin pretreatment. All data were determined on the seventh day after NaCl treatment. The bars (means  $\pm$  SD,  $n = 3$ ) labeled with different letters are significantly different at  $P < 0.05$  according to Tukey's test. MT, melatonin.**



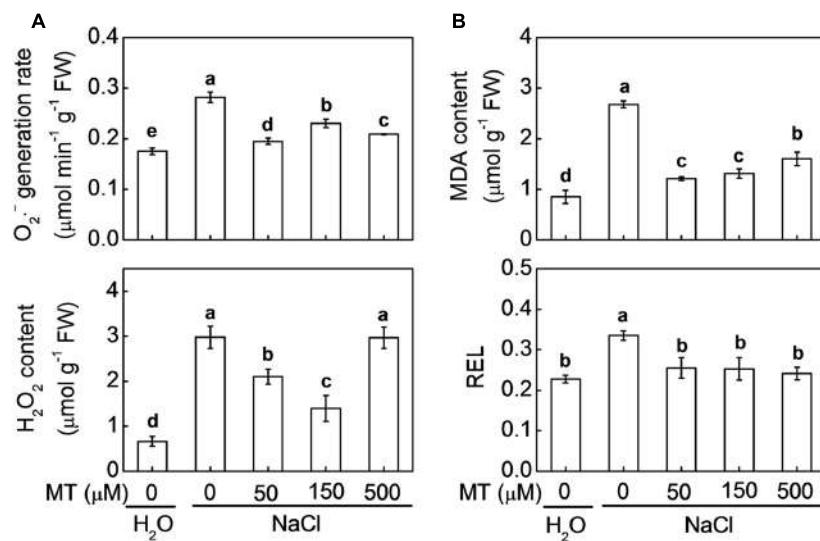
**FIGURE 2 | Changes in chlorophyll fluorescence parameters in watermelon plants as influenced by salt stress alone or combined with melatonin pretreatment.** Data are the means  $\pm$  SD of three replicates. Means denoted by the same letter did not differ significantly according to Tukey's test ( $P < 0.05$ ). MT, melatonin.

to salt stress by improving photosynthesis and cellular redox homeostasis in leaves.

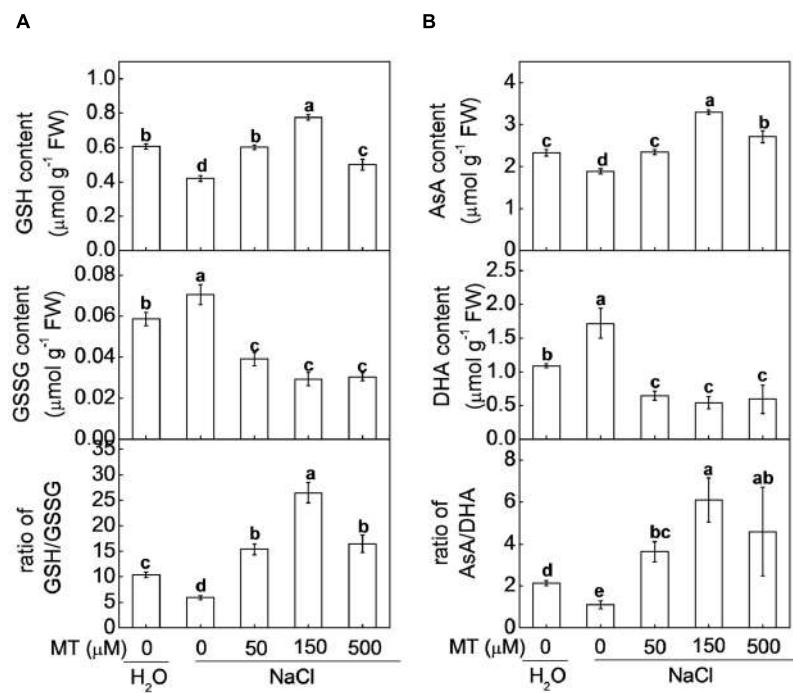
### Exogenous Melatonin Alleviated NaCl-Induced Inhibition in Photosynthesis

Photosynthesis is one of the key physiological processes highly sensitive to salt stress due to its detrimental effect on bioenergetic processes of photosynthesis (Takahashi and Murata, 2008). In the current study, NaCl stress inhibited photosynthesis and biomass accumulation in watermelon seedlings. However, in

agreement with earlier study (Li et al., 2012), pretreatment with melatonin alleviated NaCl-induced inhibition in photosynthesis and biomass production and this role of melatonin was dose-dependent (Figure 1 and Supplementary Figure S1). Melatonin with 500 or 50  $\mu\text{M}$  concentration was less effective in improving photosynthesis and biomass accumulation under salt stress compared with the 150  $\mu\text{M}$  concentration of melatonin. Under salt stress, one important response of plants is to close their stomata to minimize water loss, which is accompanied by notable decreases in  $\text{Gs}$  and consequently, stomatal limitation of photosynthesis (Brugnoli and Lauteri, 1991; Meloni et al., 2003). However, application of melatonin with optimal dose



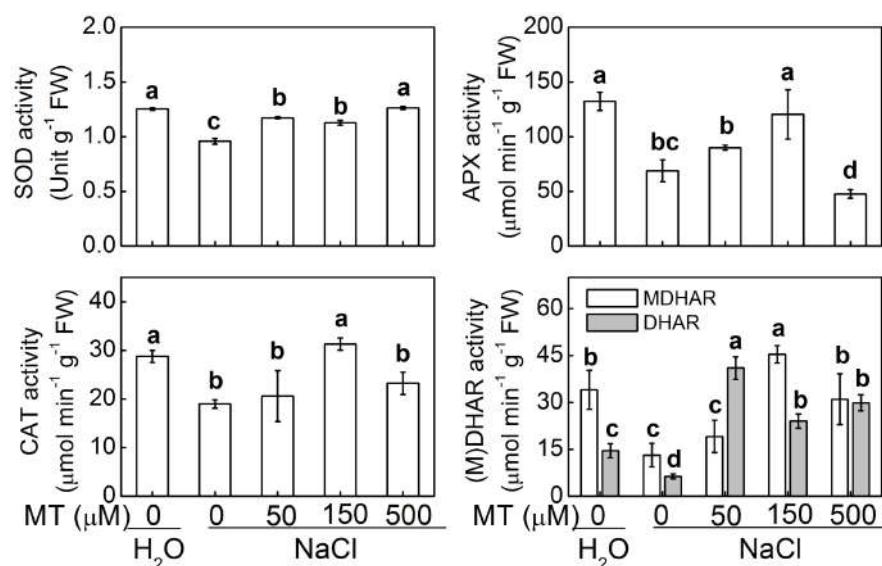
**FIGURE 3 | (A)** Accumulation of  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$  and **(B)** lipid peroxidation reflected by malondialdehyde (MDA) and relative electrolytic leakage (REL) in response to salt stress alone or combined with melatonin pretreatment. The bars (means  $\pm$  SD,  $n = 3$ ) labeled with different letters are significantly different at  $P < 0.05$  according to Tukey's test. MT, melatonin.



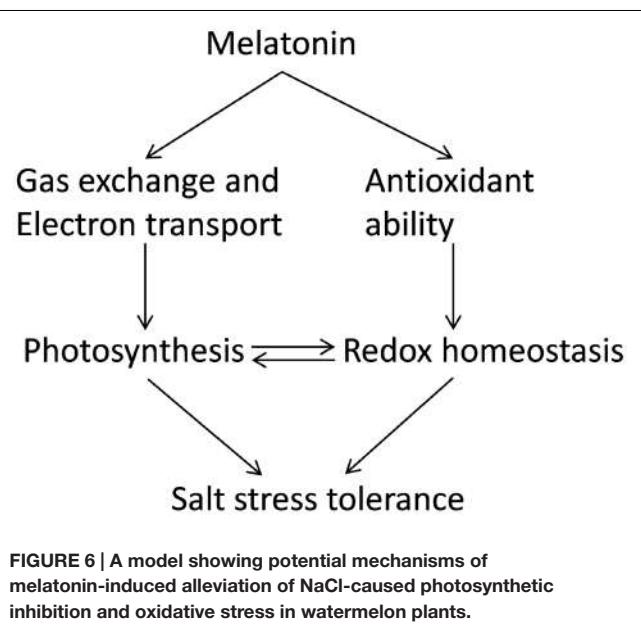
**FIGURE 4 | (A)** Glutathione and **(B)** ascorbate homeostasis in watermelon plants as influenced by salt stress alone or combined with melatonin pretreatment. Data are the means  $\pm$  SD of three replicates. Means denoted by the same letter did not differ significantly according to Tukey's test ( $P < 0.05$ ). MT, melatonin; GSH, reduced glutathione; GSSG, oxidized glutathione; AsA, ascorbic acid; DHA, dehydroascorbic acid.

can improve the functions of stomata, by enabling plants to reopen their stomata under osmotic stresses such as salt or drought stress (Li et al., 2014; Ye et al., 2016). Consistently, we also observed Gs was significantly decreased by NaCl stress, however,

this decrease was alleviated by exogenous melatonin in a dose-dependent manner. Thus, alleviation of stomatal limitation by melatonin contributed to enhancement in photosynthesis under salt stress.



**FIGURE 5 |** The activities of antioxidant enzymes in response to salt stress alone or combined with melatonin pretreatment. The bars (means  $\pm$  SD,  $n = 3$ ) labeled with different letters are significantly different at  $P < 0.05$  according to Tukey's test. MT, melatonin; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase.



**FIGURE 6 |** A model showing potential mechanisms of melatonin-induced alleviation of NaCl-caused photosynthetic inhibition and oxidative stress in watermelon plants.

Photon flux is absorbed by the antenna pigments that excite chlorophyll. Part of the excitation energy is converted to redox energy via electron transport and leads to ultimate CO<sub>2</sub> fixation, and the other is dissipated as heat and fluorescence emission (Strasser and Strasser, 1995). Decreases of chlorophyll contents and Y(II) and qP indicated that light energy absorption and electron transport in PS II were restricted by salt stress (Figure 2). Additionally, higher Y(NPQ) in salt-stressed plants also indicated that excitation energy was excessive for the capacity of electron transport. However, these decreases of chlorophyll contents,

Y(II), and qP were alleviated by pretreatment with melatonin. Taken together, melatonin alleviated NaCl-induced inhibition in photosynthesis by regulating both stomatal and non-stomatal factors.

### Exogenous Melatonin Improved Cellular Redox Homeostasis under Salt Stress

Salt stress-induced decrease of PSII activity results in imbalance between the generation and utilization of electrons. To dissipate excess light energy, excess electron is transported to molecular oxygen and thus generating ROS such as O<sub>2</sub><sup>•-</sup>, <sup>1</sup>O<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, and •OH and other oxidants in the presence of respective reaction partners. Excess ROS can block the electron transport by inducing protein degradation and affecting the repair process of PSII, forming a vicious cycle (Allakhverdiev et al., 2008). Moreover, ROS could move to thylakoid and cell membranes, where they trigger levels of membrane lipid peroxidation and membrane permeability (Sharma et al., 2012). We found that salt stress-induced accumulation of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> was consistent with the increase in MDA and REL, indicating that excess ROS might be responsible for the NaCl-induced membrane damage (Figure 3). Melatonin is a well-documented antioxidant and plays important roles in alleviating environmental stress-induced oxidative stress by scavenging most ROS or RNS (reactive nitrogen species) directly or indirectly in plants. In our previous study (Li et al., 2016), exogenous application of melatonin alleviated methyl viologen-induced photooxidative stress. In this study, we also found that salt stress-induced accumulation of ROS and ROS-caused membrane damage were alleviated by exogenous melatonin.

Strong evidence has demonstrated that melatonin is unable to directly scavenge O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> (Fowler et al., 2003;

Bonnefont-Rousselot et al., 2011) and thus the regulation of redox homeostasis by melatonin results from its ability to induce antioxidant systems including antioxidant enzymes and non-enzymatic antioxidants. In plant cells,  $O_2^{•-}$  is rapidly converted to  $H_2O_2$  by the action of SOD, while  $H_2O_2$  can be scavenged by an AsA and/or a GSH regenerating cycle and CAT (Noctor and Foyer, 1998). It is reported that exogenous melatonin increased AsA and GSH levels and redox status via upregulating activities of some key enzymes and alleviated dark- or methyl viologen-induced ROS accumulation and subsequent oxidative stress (Wang et al., 2012; Li et al., 2016). Consistently, we found GSH and AsA levels and redox status and the activities of some key enzymes (APX, DHAR, and MDAHR) involved in AsA-GSH cycle were significantly increased by melatonin at certain concentration (mostly by 150  $\mu$ M) under salt stress (Figures 4, 5). GSH is an essential co-substrate and reductant is required for regeneration of AsA (Foyer and Noctor, 2011) and cellular glutathione homeostasis has long been considered as a key element of signaling cascades, transducing information on environmental stress to their respective targets (May et al., 1998). In addition, Li et al. (2012) reported that melatonin-induced the activities of some antioxidant enzymes such as CAT under salt stress, which is well in agreement with our current study where melatonin promoted activities of SOD and CAT under salt stress (Figure 5). These results indicated that melatonin could improve cellular redox homeostasis by activating the entire antioxidant system in plants to protect cells from salt stress-induced oxidative stress.

To sum up, we have demonstrated that melatonin enhanced salt stress tolerance in watermelon seedlings in a dose-dependent manner. Under salt stress, melatonin increased photosynthesis by regulating stomatal movement and improving light energy absorption and electron transport in PSII. In addition, melatonin pretreatment improved redox homeostasis by inducing the

activities of antioxidant enzymes and redox status of GSH/GSSG and AsA/DHA and subsequently reduced oxidative stress. Increased photosynthesis alleviated disruption of cellular redox homeostasis, while improved redox homeostasis contributed to keeping higher photosynthesis, forming a virtuous cycle (Figure 6).

## AUTHOR CONTRIBUTIONS

HL and XZ designed research; HL, JC, HC, ZW, and XG performed research; HL, JC, XG, ZW, CW, JM, YZ, and JY analyzed data; HL, JC, and XZ wrote and revised the paper.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2017.00295/full#supplementary-material>

**FIGURE S1 | Changes in biomass production in watermelon plants as influenced by salt stress alone or combined with melatonin pretreatment.** All data were determined on the seventh day after NaCl treatment. The bars (means  $\pm$  SD,  $n = 5$ ) labeled with different letters are significantly different at  $P < 0.05$  according to Tukey's test. MT, melatonin.

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# Melatonin Regulates Root Architecture by Modulating Auxin Response in Rice

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It has been suggested that melatonin acts as an important regulator in controlling root growth and development, but the underlying molecular mechanism driving this relationship remains undetermined. In this study, we demonstrated that melatonin acts as a potent molecule to govern root architecture in rice. Treatments with melatonin significantly inhibited embryonic root growth, and promoted lateral root formation and development. Genome-wide expression profiling by RNA-sequencing revealed auxin-related genes were significantly activated under melatonin treatment. Moreover, several transcription factors and candidate *cis*-regulatory elements involved in root growth and developments, as well as auxin-related processes, were over-represented in both co-up and -down differentially expressed genes, suggesting that melatonin-mediated root growth occurs in an auxin signal pathway-dependent manner. Further, gravitropic response analysis determined that melatonin affects auxin-regulated processes in rice root. These data show that melatonin shapes root architecture by directly or indirectly activating the auxin signaling pathway.

**Keywords:** root growth and development, root architecture, melatonin, auxin, transcriptome, rice

## INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) is highly conserved, biologically active molecule, presents in all eukaryotic organisms including fungi, mosses, plants, and animals (Tan et al., 1993; Reiter et al., 2014; Schippers and Nichols, 2014). It is best known as a neurohormone that controls circadian physiology and seasonal behavior in animals (Dollins et al., 1994; Karasek, 2004; Tosches et al., 2014). Recently, numerous studies revealed that melatonin is widely distributed in the plant kingdom, acting in many morphological and physiological processes (Hattori et al., 1995; Chen et al., 2003; Iriti, 2009; Hardeland et al., 2012; Zuo et al., 2014; Vigentini et al., 2015). Like animals, it shows daily rhythmic fluctuations in its production and function in plants as a cellular protectant against free radicals and oxidation (Mercolini et al., 2012; Zhao et al., 2013; Liang et al., 2015). Exogenously applied or endogenously induced melatonin enhances plant resistance to drought (Wang et al., 2013b; Zuo et al., 2014), salt (Li et al., 2012), cold (Kang et al., 2010; Bajwa et al., 2014), and oxidative stresses (Hardeland et al., 1993; Park et al., 2013), and also delays leaf senescence (Byeon et al., 2012; Wang et al., 2013a; Liang et al., 2015; Shi et al., 2015a). Beyond that, the changes in melatonin levels during seed germination, as well as flower and fruit development

indicate specific, mediatory roles in plant growth and development (Zhang et al., 2013; Byeon and Back, 2014b; Wei et al., 2015). Strikingly, it has been suggested that melatonin functions in root development including primary root growth and lateral root formation (Park and Back, 2012; Zhang et al., 2013, 2014).

Roots are essential to plants for many physiological functions, such as anchoring and mechanical support, water and nutrient uptake, and in some cases carbohydrate storage (Hochholdinger et al., 2004; De Smet et al., 2012; Gao et al., 2014). They also serve as the primary interface to sense and respond to unfavorable soil environments, enabling plants to overcome stress challenges (Raven and Edwards, 2001; Malamy, 2005). Thus, a well-developed root system is extremely critical for maintaining vegetative growth, improving crop yield, and optimizing agricultural land use. The architecture of root systems is controlled by a number of endogenous factors and also influenced by several external factors, especially environmental stimuli, such as the availability of water and nutrients (Osmont et al., 2007; Petricka et al., 2012; Gao et al., 2014).

Auxin is characterized as a “root-forming phytohormone” that plays a central role in shaping the root architecture (Xie et al., 2000; Overvoorde et al., 2010). The predominant form of auxin in plants is indole-3-acetic acid (IAA) (Wang et al., 2015), and genetic and biochemical studies of biosynthetic and signaling genes clearly demonstrated that IAA is a key component of endogenous factors that regulate root growth and development (Overvoorde et al., 2010). In plants, melatonin has many similarities with IAA, since both are indole-compounds and share a common biosynthetic route through the compound tryptamine in the tryptophan (Trp)-dependent IAA biosynthetic pathway (Murch et al., 2000; Tan et al., 2016). Arnao and Hernandez-Ruiz thus proposed that melatonin may have auxin-like functions in the regulation of plant growth and development (Arnao and Hernandez-Ruiz, 2006). This hypothesis about the properties melatonin has been demonstrated by several recent studies (Hernandez-Ruiz et al., 2004, 2005; Arnao and Hernandez-Ruiz, 2007). For example, similar to IAA, melatonin can promote the growth of shoots in canary grass, wheat, oat (Hernandez-Ruiz et al., 2005), soybean (Wei et al., 2015), and rice (Liang et al., 2015), while it has a distinctly inhibitory growth effect on pre-existing roots (Hernandez-Ruiz et al., 2005). Furthermore, melatonin promotes lupine hypocotyl growth and regeneration in a dose-dependent manner (Hernandez-Ruiz et al., 2004). Melatonin thus acts as an important regulator of root architecture in the same manner as an auxin.

Rice (*Oryza sativa*) has a typical fibrous root system comprised of embryonic roots, crown roots, and lateral roots (Gao et al., 2014). Root architecture is one of the primary morphological traits to respond to inconsistent or unusual developmental cues or unfavorable environmental conditions, and is closely correlated with rice yield (Gao et al., 2014). Melatonin was recently reported to be a crucial regulator of root developmental processes in rice (Park and Back, 2012). However, to date, mechanistic details of how this molecule regulates root growth remain largely undetermined. In this study, we provide insight

into the molecular events associated with the action of melatonin in mediating root growth and development in rice. Genome-wide expression profiling analysis clearly demonstrated that melatonin controls root architecture by modulating auxin response to promote lateral root development and inhibit embryonic root growth. Elucidation of the molecular mechanisms mediated by melatonin will deepen our understanding of the role of this molecule in root growth and development, and further facilitate the application of control over root architecture control for agricultural plants.

## MATERIALS AND METHODS

### Plant Material and Treatment of Melatonin

Rice seedlings of Dongjin (*Oryza sativa* ssp. *Japonica*) were germinated and grown in a growth chamber with a 12-h-light (30°C)/12-h-dark (28°C) photoperiod, with approximately 200 μmol photons/m<sup>2</sup>/sphoton densities and 70% humidity. We previously reported that melatonin delays rice leaf senescence and cell death, and enhances salt stress tolerance by directly or indirectly counteracting the cellular accumulation of H<sub>2</sub>O<sub>2</sub> (Liang et al., 2015). We also found that a low concentration of melatonin (<20 μM) increased shoot growth, while a high concentration (>20 μM) can mitigate its growth-promoting effect or even have an inhibitory effect (Liang et al., 2015). Therefore, different concentrations of melatonin (0, 10, 20, and 50 μmol/L) were added to the hydroponic cultures when seminal roots of seedlings had reached 2–3 cm. Seminal root length and crown root number were recorded every day. Lateral root length was represented by mean length of the longest three lateral roots about 2 cm from the root tip. Lateral root number was represented by the number of all lateral roots in 1–2 cm region from the root tip. These two parameters were recorded 5-days after treatment. All data were recorded using 30 seedlings. For all experiments, the overall data were statistically analyzed in the SPSS 20 program (SPSS Inc., Chicago, IL, USA). LSD and Tukey's *post-hoc* test were used for testing the differences in growth and root developmental responses during different melatonin treatments.

### RNA Sequencing and Data Analysis

RNAs extracted from roots of 2-week-old seedlings treated with water (M0), 10 μmol melatonin (M10), or 20 μmol melatonin (M20) were used for RNA sequencing. For direct comparison, three libraries, M0, M10, and M20, with different melatonin concentrations for each treatment, were prepared in the same manner and run side by side by BGI Company (Shenzhen, China) on Illumina Hiseq 2000 platform. Differentially expressed genes (DEGs) were analyzed by the Cufflinks software with the fragments per kilo-base per million reads (FPKM) measurement: FPKM = 109 C/NL, where “C” is the number of mapped fragments for a certain gene, “N” is the total reads mapped to the entire genome, and “L” is exon length of a certain gene.

To perform clustering analysis, the expression abundance of each gene was calculated after a pseudo-count of 1 was added to the raw FPKM value for each gene, with the application of log<sub>2</sub> transformation and z-score normalization by the following

formula:

$$V' = \frac{\log_2(V + 1)}{\sum_{i=1}^n \log_2(v_i + 1)} \quad (1)$$

where  $V = (v_1, \dots, v_n)$  is the original raw FPKM abundance estimation of the transcript and  $V'$  is the new normalized density vector. The Silhouette function was used to select an optimized number of clusters. As a result, eight clusters were obtained using K-means clustering analysis (Ranzani et al., 2015). GO enrichment analysis was performed using in-house perl scripts with known gene function annotations downloaded from PlantGSEA and the BinGO plugin provided in Cytoscape (Yi et al., 2013). To find known *cis*-regulatory motifs within up-regulated and down-regulated genes, the promoter region (1000 bp upstream from the transcription start site) of each gene and the entire genome was scanned with known motifs extracted from both AGRIS and PLACE (Higo et al., 1999; Palaniswamy et al., 2006). The significance level was calculated using Fisher's exact test based on the hypergeometric distribution hypothesis. Transcription factor families were downloaded from the Plant Transcription Factor Database (PlantTFDB; Jin et al., 2014).

## RNA Extraction, cDNA Preparation, and Gene Expression Analysis

Twenty plants were collected for RNA isolation. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed using the ReverTra Ace qPCR RT Master mix with gDNA Remove Kit (Toyobo, Osaka, Japan). For quantitative real-time PCR (qRT-PCR), SYBR Green I was added to the reaction mix and run on a Chromo4 real-time PCR detection system according to the manufacturer's instructions (CFX96; Bio-Rad, California, USA). The data were analyzed with Opticon monitor software (Bio-Rad). Rice ACTIN1 was used as an internal control. The primers used for qRT-PCR are listed in **Supplementary Table 8**. Values are mean  $\pm$  SD. of three biological repeats. Student's *t*-test was used for statistical analysis. Asterisks indicate statistically significant differences compared with wild type (\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ).

## Root Gravitropism Assay

To assess the root gravitropic response, wild-type seedlings were grown vertically until the length of roots reached 3–4 cm and arranged parallel on filter paper infiltrated with 0, 10, 20, and 50 melatonin, respectively. Then seedlings were gravistimulated with 90° rotation. After 24 h, the root curvature of seedlings with different treatments was quantified and compared. This experiment was performed with a population of more than 30 seedlings per treatment.

## RESULTS

### Melatonin Participated in Shaping Rice Root Architecture

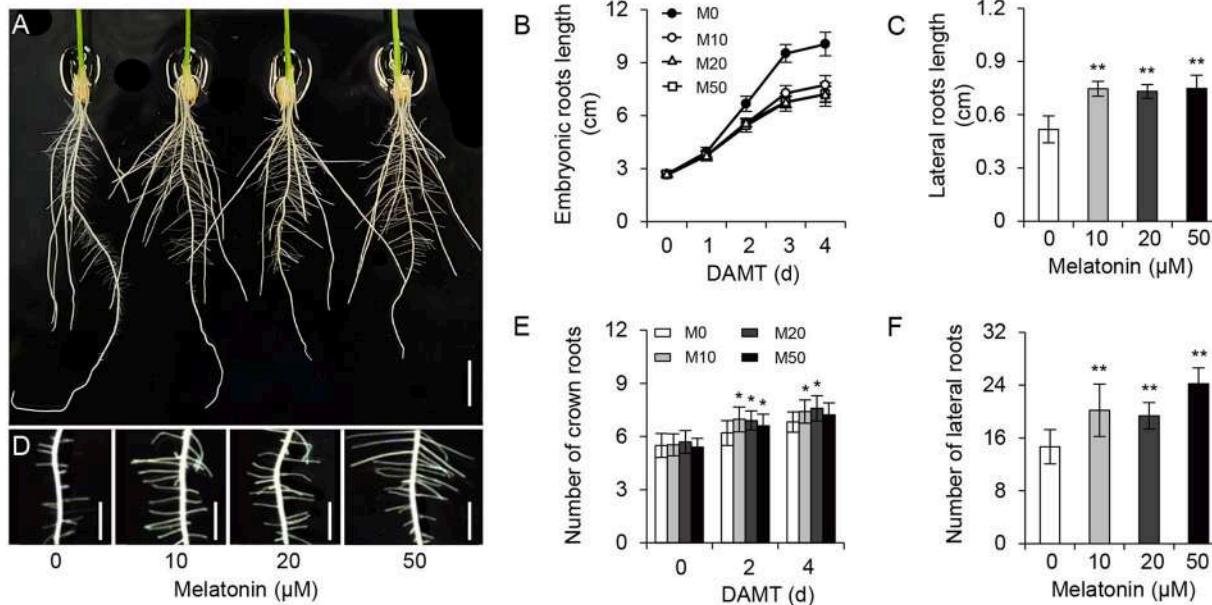
To examine the effect of melatonin on rice root architecture, we treated rice seedlings with a concentration gradient of melatonin. Visual observation and statistical analysis showed

that the embryonic root lengths were significantly decreased with both low and high concentrations of melatonin application, compared with the control treatment (M0; **Figures 1A,B** and **Supplementary Figure 1**). Likewise, the average length of crown roots also clearly inhibited with treatment (**Figure 1A**). In contrast to the decreased length of embryonic and crown roots, the plants treated with melatonin had longer lateral roots on average than the M0 control at 5-days after treatment (**Figures 1C,D**). Moreover, M10-, M20-, and M50-treated plants showed more crown roots (**Figures 1A,E**) and lateral roots than the M0 control (**Figures 1D,F**). These data strongly suggest that melatonin plays an important role in root architecture.

## RNA-Seq Analysis of a Melatonin-Treated Rice Transcriptome

To explore the morphological effects of melatonin on root growth and development in rice, we performed RNA-seq using M0-, M10-, and M20-treated roots (**Supplementary Table 1**). Compared with transcripts of non-treated samples (M0), 796 differentially expressed genes (DEGs), with 2-fold or higher changes, were identified in the M10-treated samples, while 1211 DEGs were identified in M20-treated roots (**Figure 2A** and **Supplementary Table 2**). In both M10 and M20 plants, up-regulated genes outnumbered down-regulated genes approximately 4.7- and 5.4-fold in each sample, respectively (**Figure 2A**). Notably, 314 of the up-regulated and 51 of the down-regulated DEGs overlapped between M10- and M20-treated samples (**Figure 2A**). Furthermore, Quantitative real-time (qRT)-PCR was performed to validate these DEGs. Forty-four genes with different changes of expression levels under 10 or 20  $\mu$ mol/L melatonin treatments were randomly selected for qRT-PCR analysis (**Supplementary Table 2**). As shown in **Figure 2B**, the regression slope for RNA-seq vs. qRT-PCR is close to 1, suggesting a high positive correlation between RNA-seq data and qRT-PCR data, thus demonstrating the credibility of the RNA-seq data.

To test the efficacy of melatonin we assign genes to functional categories and grouped the DEGs from M10 and M20 using the K-Means clustering algorithm. The 1819 (90.6%) DEGs were mainly clustered into eight groups (K1–K8; **Figure 2C** and **Supplementary Table 3**). Most of the bins exhibited enrichment for specific clusters of expressed genes. Cluster 1, 2, 3, and 4 showed patterns of up-regulation, while cluster 5 and 8 exhibited down-regulated gene expression profiles. In cluster 6, genes with induced expression in M10 samples are inhibited in M20 samples, whereas genes in cluster 7 were down-regulated in M0 but up-regulated in M20. Genes in cluster K1 and K8 include those for response to stimuli, hormone-mediated signaling pathways, protein amino acid phosphorylation, defense response, and metabolic processes. Genes in clusters K2, K3, and K6 include those encoding nutrition absorption and transport. Significantly, genes encoding enzymes for response to auxin stimulus, auxin-mediated signaling pathway, and auxin metabolic processes are greatly enriched in cluster K1, 6, and 8, K1 and 8, K1 and 7, respectively. These observations imply that melatonin regulates the expression of these genes to govern root development in rice.



**FIGURE 1 | Melatonin effects on root growth and development in rice. (A)** Phenotypes of rice root architecture with 4-days melatonin treatment. Scale bar = 1 cm. **(B)** Embryonic root length of control and plants cultivated under different concentrations of melatonin. **(C)** Lateral root length of control and plants treated with 10, 20, and 50  $\mu$ M melatonin for 5 days. **(D)** Phenotypes of lateral roots after 5-days melatonin treatment. Scale bar = 3.6 mm. **(E,F)** number of crown root **(E)** and lateral root **(F)** for 5-days of 0, 10, 20, and 50  $\mu$ M melatonin treatment. DAMT, days after melatonin treatment. Values are mean  $\pm$  SD of 20 measurements. M0, samples treated with water. M10, samples treated with 10  $\mu$ M melatonin. M20, samples treated with 20  $\mu$ M melatonin. M50, samples treated with 50  $\mu$ M melatonin.

\* $P \leq 0.05$ , \*\* $P \leq 0.01$ . Student t-test was used to generate P-value.

To further clarify the plant gene expression responses to melatonin, we used BinGO to construct gene ontology (GO) term networks for DEGs of M10 and M20 plants, as well as overlapping DEGs between M10 and M20. As expected, GO terms correlated with response to auxin stimulus were highlighted in the biological process category among the three groups. Genes encoding ATPase activity were clearly over-represented in the molecular function category. Eighteen genes annotated as response to auxin stimuli and auxin signaling pathway, were dominant as the main categories of biological process (Figure 3 and Supplementary Table 4). The array of genes associated with auxin-related processes suggests that melatonin might regulate root architecture by affecting auxin signaling in rice.

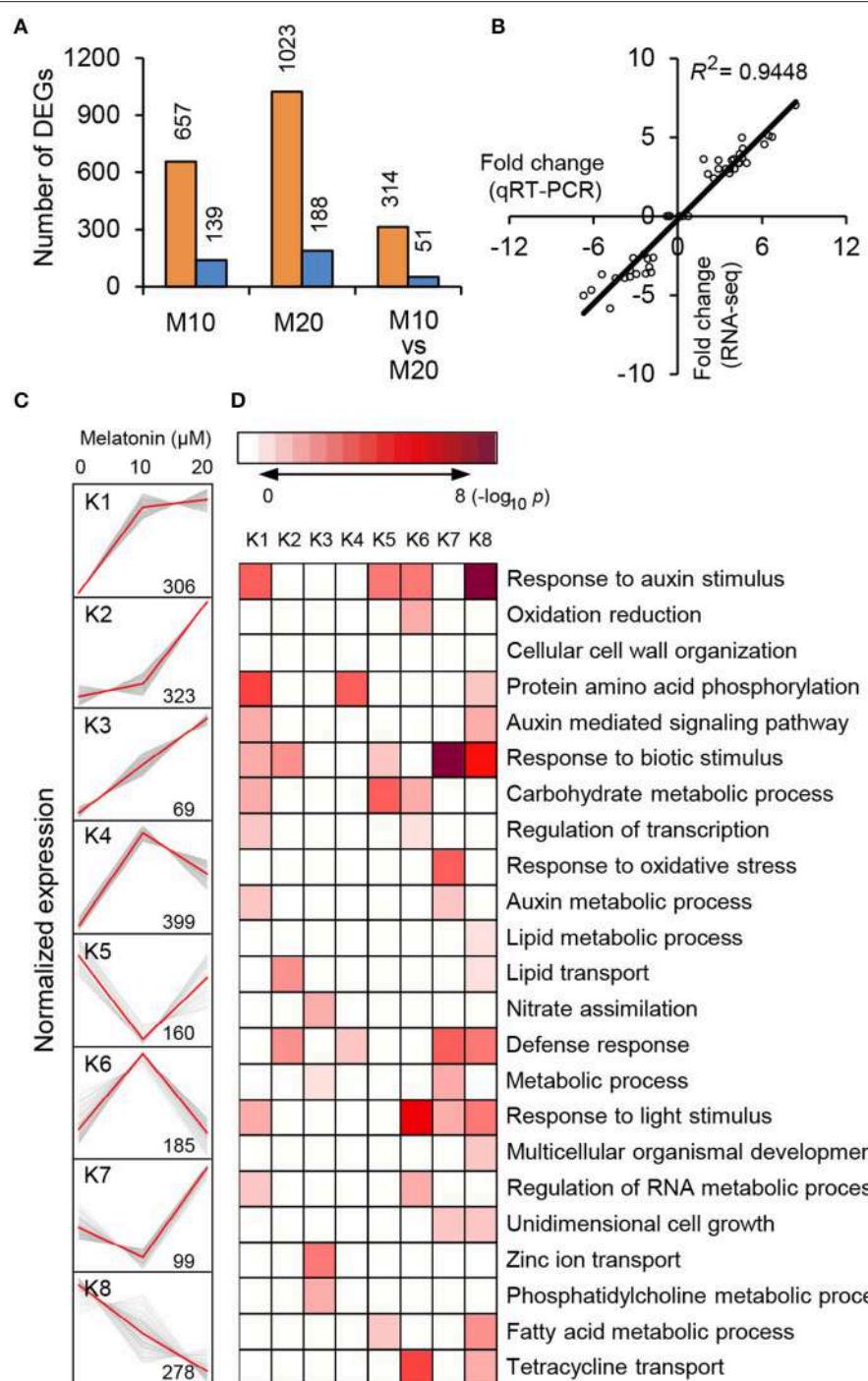
## Expression Pattern of Transcription Factors

Transcriptional activators and repressors have vital roles in regulating gene expression surrounding both the melatonin-mediated biological processes and shaping the plant root architecture (Gao et al., 2014; Liang et al., 2015). A total of 120 TFs belonging to 24 families, were up- or down-regulated in the M10 and M20 samples compared with M0 control (Figure 4A and Supplementary Table 5). The expression of roughly 25% (30) of TFs were significantly activated in both M10 and M20, including six WRKY, four NAC, three MYB, three bHLH, three LBD, two C3H, two HD-ZIP, one SRS, one B3, one HSF, one DBB, one C2H2, one ARF, and 1 ERF, while 14 (11.7%) TFs were inhibited (Figures 4A,B). Intriguingly, the expression of

many auxin-induced TFs (<http://ricexpro.dna.affrc.go.jp/>), such as *Os01g61080* (WRKY), *Os03g21710* (WRKY), *Os05g09020* (WRKY), *Os05g46020* (WRKY), *Os09g25070* (WRKY), *Os11g29870* (WRKY), *Os05g34830* (NAC), *Os07g112340* (NAC), *Os09g32260* (NAC), and *Os01g09990* (bHLH), were significantly upregulated in the both M10 and M20 samples (Figures 4B,C), whereas the expression of several auxin-inhibited TFs, including *Os04g43560* (NAC), *Os10g42130* (NAC), *Os02g43940* (ERF), *Os02g51280* (TCP), *Os04g23910* (MIKC), *Os02g53690* (GRF), and *Os06g17410* (DOF), were down-regulated in both samples (Figures 4B,C). These data further confirmed the relationship between melatonin signaling and auxin response in rice. Notably, among 44 co-up- or co-down-regulated TFs, 21 genes, such as *Os03g21710* (WRKY), *Os09g25070* (WRKY), *Os11g29870* (WRKY), *Os04g43560* (NAC), *Os09g32260* (NAC), *Os01g09990* (bHLH), *Os04g23910* (MIKC), and *Os02g51280* (TCP), were specifically or primarily expressed in roots (Figures 4B-D and Supplementary Table 5), implying that these TFs may be potential key regulators of melatonin signaling pathway. Taken together, these results provide strongly evidence that melatonin acts as an important regulator of root development in a partially auxin-dependent auxin manner in rice.

## Identification of Melatonin-Associated Motifs in Co-expressed Genes

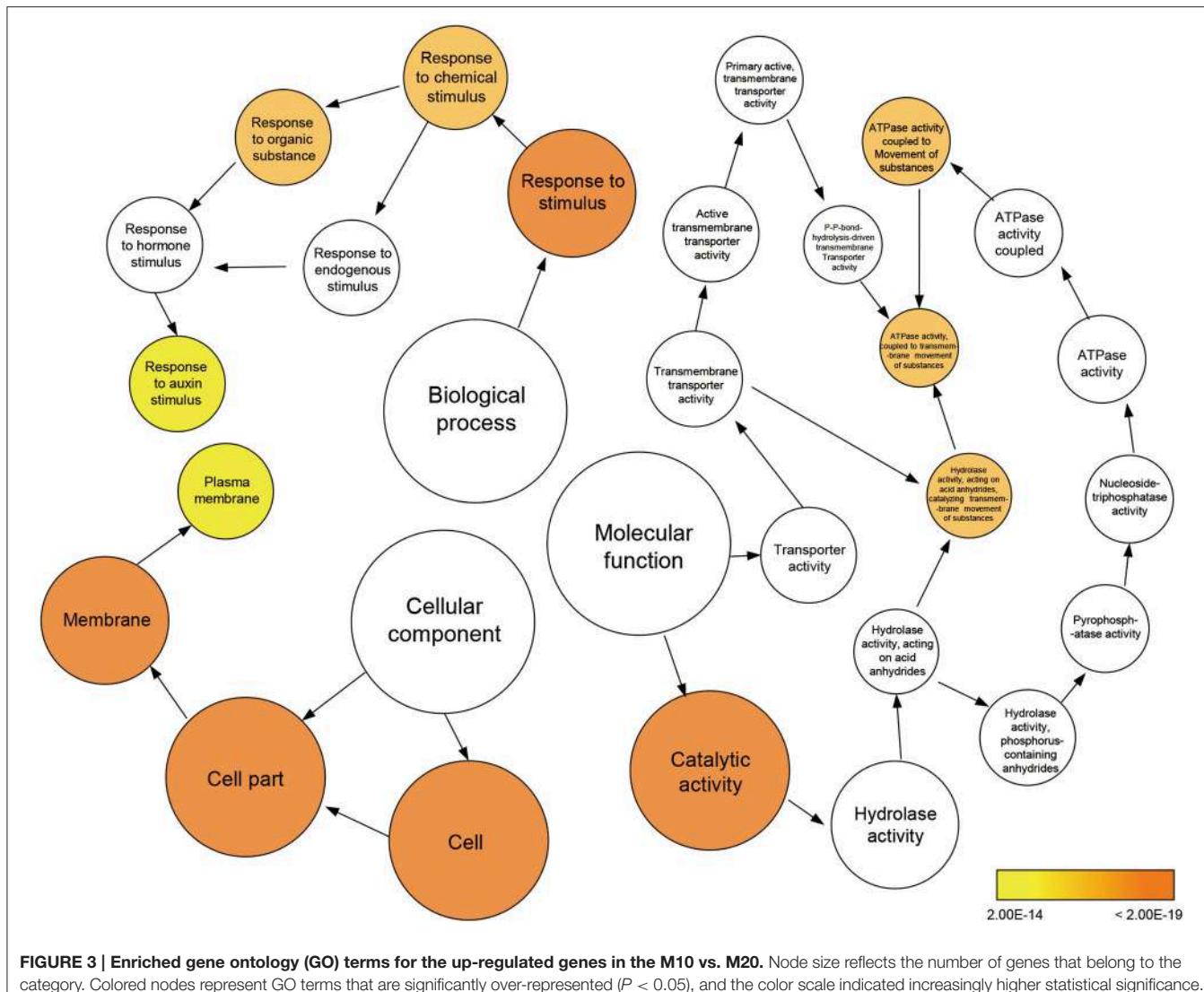
Given that genes with similar expression profile may contain a conserved *cis*-element in their promoters, we screened the 1000 bp sequences upstream of the transcriptional start site of



**FIGURE 2 | RNA-seq analysis of melatonin-treated transcriptome. (A)** Overlapping DEG numbers between M0/M10 and M0/M20. **(B)** Correlation of RNA-seq (y-axis) and qRT-PCR data (x-axis). The correlation assay was carried out for 36 DEGs with  $\log_2$  ratios  $>1.0$  or  $<-1.0$ . **(C)** K-mean clustering showing the expression pattern of the DEGs of M10 and M20 transcriptome in rice roots. Eight clusters were identified with 10 and 20  $\mu\text{M}$  melatonin treatment from 2007 differentially expressed genes. **(D)** Functional category enrichment among the eight major clusters.

co-expressed genes or co-inhibited genes by AGRIS and PLACE. We then submitted the candidate motifs to the motif searching program to identify statistically over-represented regulatory motifs. Fifty-eight and twenty-one candidate *cis*-elements

were identified in co-up and co-down DEGs, respectively (Supplementary Table 6). Significantly, three known conserved motifs, including an ARF binding site motif (motif1, TGTCTC), a root tip meristem-related element (motif3, TATTCT), and



**FIGURE 3 |** Enriched gene ontology (GO) terms for the up-regulated genes in the M10 vs. M20. Node size reflects the number of genes that belong to the category. Colored nodes represent GO terms that are significantly over-represented ( $P < 0.05$ ), and the color scale indicated increasingly higher statistical significance.

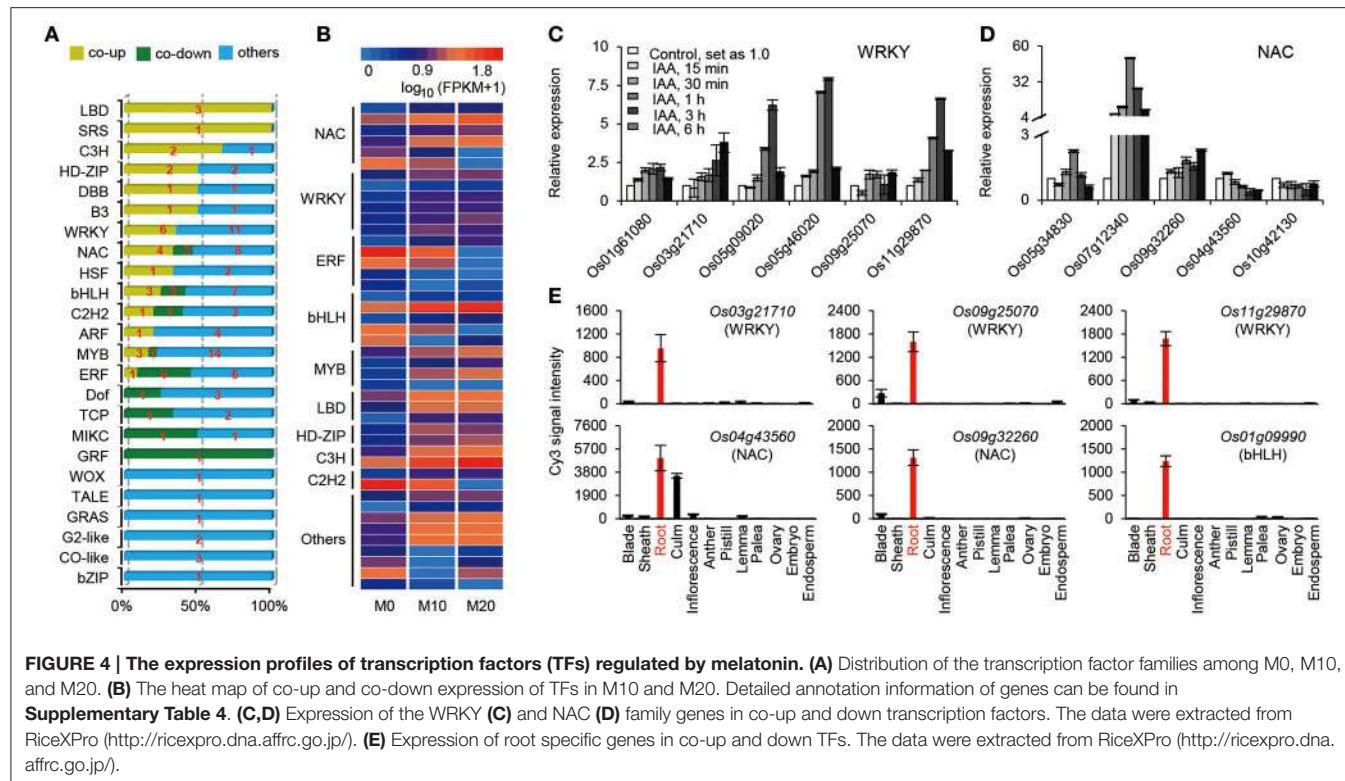
a root-specific element (RSE, motif4, ATATT), were found to be enriched in promoters of 79, 95, and 266 co-expressed DEGs (Figure 5A and Supplementary Table 7). Similarly, a conserved sequence, TACACAT (motif2), required for auxin responsiveness, and the RSE motif, were identified in 17.6% (9) and 90.2% (46) of co-inhibited DEGs, respectively (Figure 5A). These data further demonstrate that melatonin functions in roots architecture in an auxin-related manner. In addition, a conserved W-box (motif5, TTGAC and motif6, TGACT), the core binding site of WRKY TFs, was enriched in co-up DEGs (Figure 5A and Supplementary Table 7), which correspond well with the GO results showing that WRKY families were the most highly enriched of all TFs in the co-up TFs (Figures 4B,C).

Further analysis of the seven clusters of genes containing motif 1–6 showed that the co-up-regulated genes harboring promoter motifs 1, 3, 4, 5, or 6 were commonly enriched among GO terms such as response to auxin stimulus, oxidation reduction, and regulation of transcription, while the co-down genes with

motifs 2 and 4 are enriched in GO term pathways related to the asparagine biosynthetic process and nitrate assimilation (Figure 5B). Genes encoding enzymes for the auxin-mediated signaling pathway, metabolic process, meristem initiation, maintenance of meristem identity, and regulation of vegetative meristem growth are greatly enriched in motif3 and motif4 clusters, which contain *cis*-elements related to root development (Figure 5B). Several GO terms associated with auxin response and root development showed significant enrichment in genes carrying motifs5 and motif6, suggesting that the WRKY TFs may also participate in melatonin-mediated growth response in rice.

## Melatonin Affects Auxin-Regulated Processes in Rice

The transcriptional response data brought to light the possibility that melatonin regulates root architecture in an auxin dependent interaction. To further explore this hypothesis, we retrieved genes involved in the auxin biosynthesis and signaling pathways



in co-transcribed DEGs, based on previous reports, to analyze their expression patterns (**Supplementary Table 2**). Strikingly, we found that the expression of several classes of auxin-related genes, including five Aux/IAA members, four *OsGH3* members, one *ARF*, and one SAUR gene involved in the auxin signaling pathway were all notably up-regulated both in M10 and M20, compared with M0 control (**Figure 6A**). The activated transcripts for these genes were further confirmed by qRT-PCR (**Figure 6B**). However, no remarkable change was observed in the genes involved in IAA biosynthesis and metabolism pathways. This result confirms that melatonin regulates root development in rice, probably acting in a manner dependent on auxin signaling.

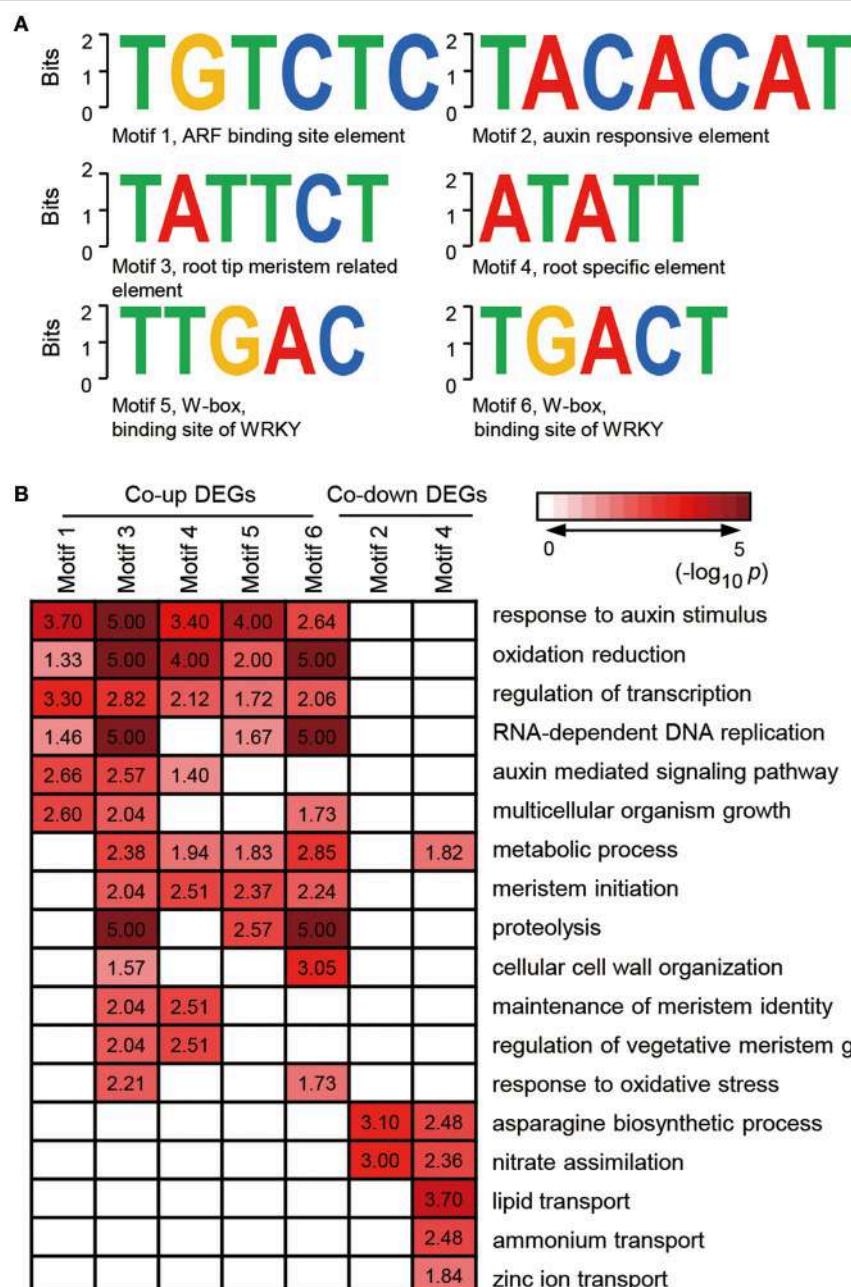
## Melatonin Regulates Root Architecture by Modulating Auxin Signaling

Gravitropic responses have been widely used as a reporter for auxin homeostasis or signal transduction in plants. We examined the root gravitropic response in M10 and M20 plants by measuring the curvature after gravistimulation at 90° to the vertical for 24 h. About 67.9% of M0 roots had root tip angles of 30° to 60°, while 72.8, 66.7, and 66.7% of M10, M20, and M50 roots, respectively, were observed to have root tip angles of 61–90% (**Figures 7A,B**). The average root tip angles of the M10 were 55.9°, whereas M10, M20, and M50 roots had respective average angles 71.9°, 66.5°, and 66.4° (**Figure 7C**). The difference in root tip angles between treatments and control clearly demonstrated that melatonin mediates root growth and development processes through effects on the auxin signaling pathway.

## DISCUSSION

Melatonin is a ubiquitous and physiological compound and is proposed to be an important regulator in controlling root development (Arnao and Hernandez-Ruiz, 2007; Park and Back, 2012; Zhang et al., 2013). However, both the mechanistic evidence of melatonin's regulatory role in root architecture and the molecular interactions driving melatonin-mediated root development remain largely unknown. In our study, embryonic root and crown root elongation were inhibited significantly by exogenous melatonin treatment, while the number and length of lateral roots were distinctly increased both in M10 and M20 plants, compared with the M0 control (**Figure 1**). The role of melatonin in orchestrating rice root architecture was highly similar with the most well-characterized auxin-associated phenotypes, such as increased length of epidermal-derived root hairs, inhibited growth of pre-existing primary roots, and increased number of lateral roots (Overvoorde et al., 2010). As demonstrated by the enhanced gravitropic response in M10, M20, and M50 (**Figure 7**), melatonin may have a function similar to auxin in root developmental regulation, and consistent with auxin-related processes.

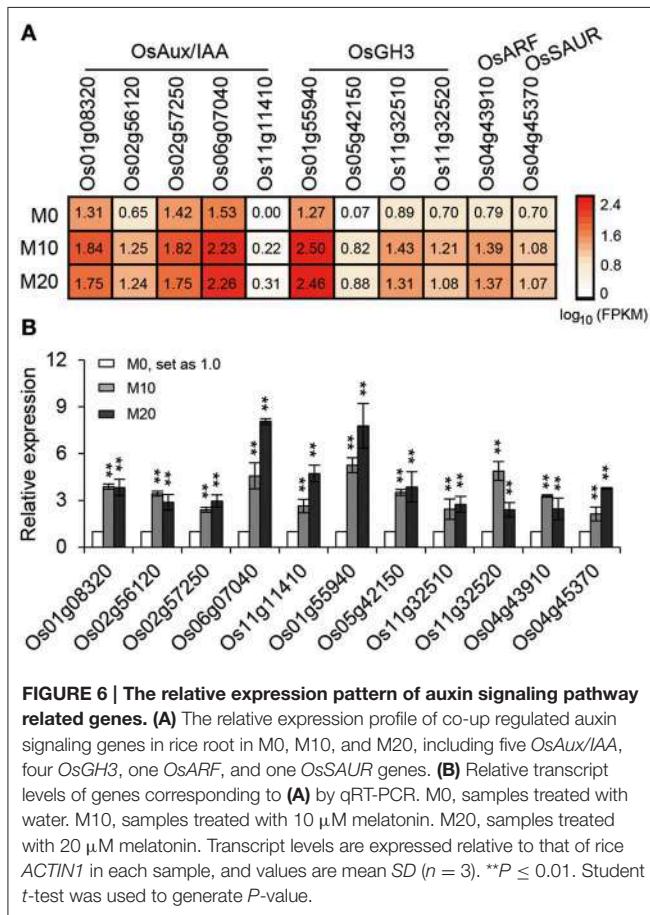
Auxins are known to be critical phytohormones involved in regulating root development. A number of auxin-related mutants, such as *Arabidopsis* *crownless root1* (*crl1/adventitious rootless1*) (*crl1*) (Inukai et al., 2005; Liu et al., 2005), *crl4/gnom1* (Liu et al., 2009), and *root enhancer1* (*ren1-D*) (Gao et al., 2014), show abnormalities in root growth and development. Several lines of evidence in our RNA-seq data



**FIGURE 5 |** Discovered candidate motifs from co-up and co-down DEGs and enriched analysis of genes with the motifs binding sites. **(A)** Candidate cis-elements identified by ELEMENT from co-up and co-down DEGs. **(B)** GO annotation enrichment analysis for the genes containing motif1 to motif6. DEGs, differentially expressed genes.

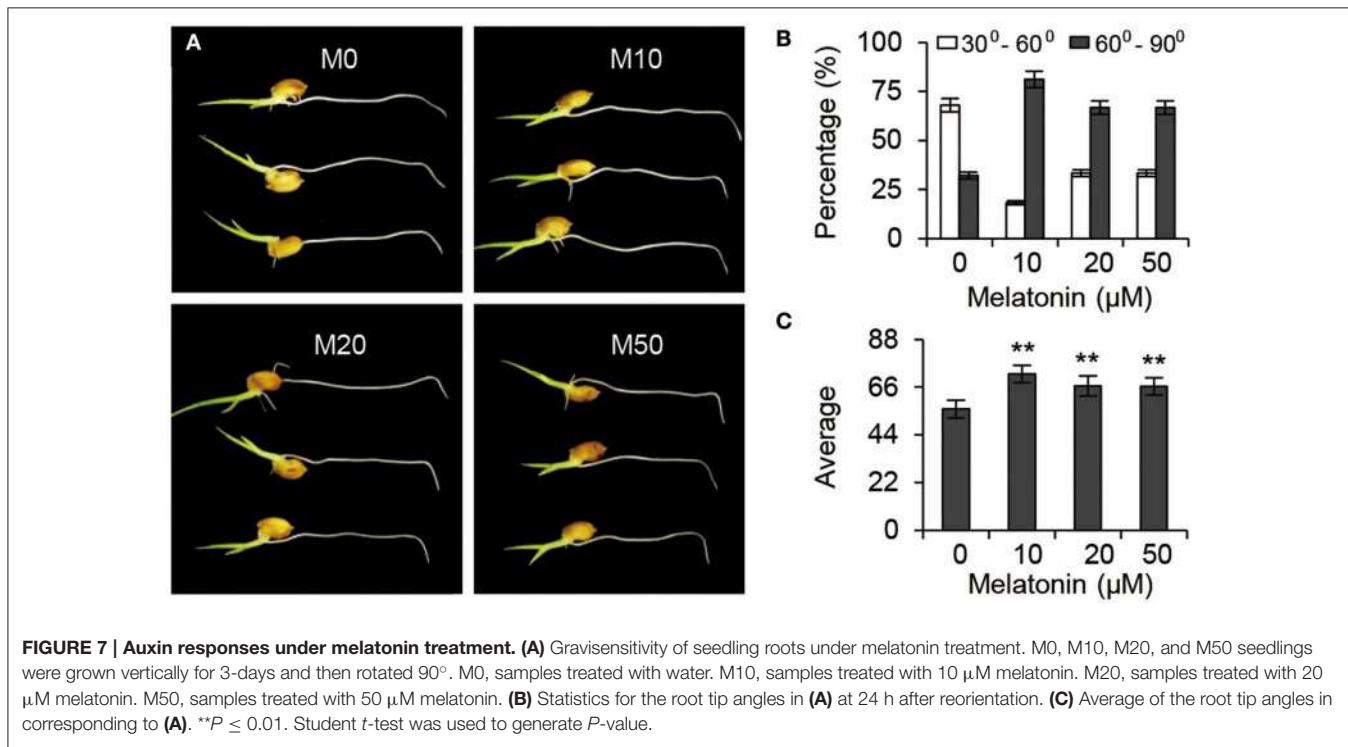
support the idea melatonin shared function with auxin. First, a large proportion of DEGs were determined to be involved in the response to auxin stimulus and the auxin mediated signaling pathway during melatonin treatment (Figures 2, 3). Second, based on our enrichment analysis, several classes of auxin responsive genes, including five *OsAux/IAA*, four *OsGH3*, one *OsARF*, and one *OsSAUR*, were significantly up-regulated in both M10 and M20 compared with M0 control (Figure 6 and Supplementary Table 2). The ARF,

IAA/Aux, GH3, SAUR genes are the most important auxin signaling and response gene families in plants. The regulation of post-embryonic root growth and lateral root formation is closely controlled by auxin signaling. For example, gain-of-function mutations *iaa1/axr5* (Yang et al., 2004), *iaa3/shy2* (Tian and Reed, 1999), *iaa14/sl* (Fukaki et al., 2002), *iaa18/crane* (Uehara et al., 2008), *iaa19/msg2* (Tatematsu et al., 2004), and *iaa28* (Rogg et al., 2001), exhibited an obviously altered capacity to form lateral roots. A recent discovery showed



**FIGURE 6 | The relative expression pattern of auxin signaling pathway related genes. (A)** The relative expression profile of co-up regulated auxin signaling genes in rice root in M0, M10, and M20, including five OsAux/IAA, four OsGH3, one OsARF, and one OsSAUR genes. **(B)** Relative transcript levels of genes corresponding to **(A)** by qRT-PCR. M0, samples treated with water. M10, samples treated with 10  $\mu$ M melatonin. M20, samples treated with 20  $\mu$ M melatonin. Transcript levels are expressed relative to that of rice ACTIN1 in each sample, and values are mean SD ( $n = 3$ ). \*\* $P \leq 0.01$ . Student t-test was used to generate P-value.

that melatonin inhibits the transcripts of an AUX/IAA gene, *IAA17*, to delay natural leaf senescence in *Arabidopsis* (Shi et al., 2015a). Several OsAux/IAA genes, including *Os01g08320* (*OsIAA1*), *Os02g56120* (*OsIAA9*), *Os02g57250* (*OsIAA10*), *Os06g07040* (*OsIAA20*), and *Os11g11410* (*OsIAA27*), were significantly upregulated in our analysis, demonstrating that activity in plant development may be through an auxin signaling pathway. However, some reports suggest that melatonin does not regulate AXR3/IAA17 nor activate auxin-inducible gene expression in root development in *Arabidopsis* (Pelagio-Flores et al., 2012). The differences may be caused by the differential expression profiles of OsAux/IAA genes. In plants, the Aux/IAA members have tissue-specific expression patterns, and distinct functions in auxin-mediated growth and development. Alternatively, this discrepancy also might be due to different mechanisms for melatonin regulation of root development between species. Comparative transcriptome analysis revealed that there are the differences in gene expression and hormone signaling pathways during root development in different plant species. Third, the expression of many auxin-related TFs (<http://ricexpro.dna.affrc.go.jp>), including WRKY, NAC, MYB, bHLH, HD-ZIP, and ERF, exhibited consistently up- and down-regulated expression profiles under both M10 and M20 treatment, respectively (**Figure 4**). Fourth, two conserved auxin-related cis-elements, an ARF binding site motif (TGTCTC) and an auxin-response element (TACACAT), were identified in 25.2% (27) co-up and 17.6% (9) co-down DEGs, respectively (**Figure 5**). Based on these results, we can reasonably speculate that melatonin-controlled root growth and developmental regulation is closely



**FIGURE 7 | Auxin responses under melatonin treatment. (A)** Gravisensitivity of seedling roots under melatonin treatment. M0, M10, M20, and M50 seedlings were grown vertically for 3-days and then rotated 90°. M0, samples treated with water. M10, samples treated with 10  $\mu$ M melatonin. M20, samples treated with 20  $\mu$ M melatonin. M50, samples treated with 50  $\mu$ M melatonin. **(B)** Statistics for the root tip angles in **(A)** at 24 h after reorientation. **(C)** Average of the root tip angles in corresponding to **(A)**. \*\* $P \leq 0.01$ . Student t-test was used to generate P-value.

associated with the activation of auxin response pathway in rice.

In addition, we observed enrichment of several up-regulated genes containing “root tip meristems” and “root-specific element (RSE)” promoter motifs associated with GO terms categories meristem initiation, oxidation reduction, RNA-dependent DNA replication, and proteolysis. Significant enrichment was also observed in down-regulated genes having the “RSE” *cis*-element annotated for nitrate assimilation, lipid and zinc ion transport, and the asparagine biosynthetic process. These results further supported our hypothesis that melatonin is an important mediator for shaping root architecture via modulation of auxin response in rice.

In spite of recent progress in elucidating the biological function of melatonin, understanding of the molecular mechanisms of melatonin-mediated root growth and development is still at a beginning stage. Currently, a limited number of genes for melatonin biosynthesis, degradation, and signaling pathways have been identified using a reverse genetic approach (Kang et al., 2013; Zhao et al., 2013; Byeon and Back, 2014a; Lee et al., 2014; Zuo et al., 2014; Shi et al., 2015a,b). Given that it is difficult to identify melatonin-related genes using a forward genetic approach so far, the identification of critical components controlling melatonin biosynthesis, degradation, and signaling, needs to be accomplished through several different approaches. Our studies suggest that a systems biology approach, especially combining different “omics” methods and CRISPR/Cas9, should accelerate the identification of key genes underlying melatonin biosynthesis, degradation, and signaling. On the other hand, analyses that measure changes in melatonin content and signaling effects in mutants for auxin biosynthesis, degradation, and signaling would also serve as an important means to uncover the relationship between melatonin and auxin.

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Chengzhen Liang and CC conceived and designed the experiments; Chengzhen Liang, AL, WL, and RZ performed laboratory experiments; Chengzhen Liang, AL, HY, and Chengzhi Liang performed data analysis and interpretation; Chengzhen Liang, AL, SG, RZ, and CC wrote the paper.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2017.00134/full#supplementary-material>

**Supplementary Table 1 | Statistics of clean reads in RNA sequencing.**

**Supplementary Table 2 | Differentially expressed genes.**

**Supplementary Table 3 | Genes in K-mean clustering of eight clusters.**

**Supplementary Table 4 | GO annotation enrichment analysis.**

**Supplementary Table 5 | Transcription factors.**

**Supplementary Table 6 | Candidate cis-elements discovered from co-up and -down genes.**

**Supplementary Table 7 | Candidate Cis-elements discovered by ELEMENT from co-up and co-down genes.**

**Supplementary Table 8 | Primers used for qRT-PCR in this study.**

**Supplementary Figure 1 | Melatonin effect on rice root growth in greenhouse. (A)** Phenotypes of 2-week-old plants with different concentrations of melatonin. **(B)** Root length corresponding to **(A)**.

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# Endophytic Bacterium *Pseudomonas fluorescens* RG11 May Transform Tryptophan to Melatonin and Promote Endogenous Melatonin Levels in the Roots of Four Grape Cultivars

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Endophytes have been verified to synthesize melatonin *in vitro* and promote abiotic stress-induced production of endogenous melatonin in grape (*Vitis vinifera* L.) roots. This study aimed to further characterize the biotransformation of tryptophan to melatonin in the endophytic bacterium *Pseudomonas fluorescens* RG11 and to investigate its capacity for enhancing endogenous melatonin levels in the roots of different grape cultivars. Using ultra performance liquid chromatography-tandem mass spectrometry combined with <sup>15</sup>N double-labeled *L*-tryptophan as the precursor for melatonin, we detected isotope-labeled 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, and melatonin, but tryptamine was not detected during the *in vitro* incubation of *P. fluorescens* RG11. Furthermore, the production capacity of these four compounds peaked during the exponential growth phase. RG11 colonization increased the endogenous levels of 5-hydroxytryptophan, *N*-acetylserotonin, and melatonin, but reduced those of tryptamine and serotonin, in the roots of the Red Globe grape cultivar under salt stress conditions. Quantitative real-time PCR revealed that RG11 reduced the transcription of grapevine tryptophan decarboxylase and serotonin *N*-acetyltransferase genes when compared to the un-inoculated control. These results correlated with decreased reactive oxygen species bursts and cell damage, which were alleviated by RG11 colonization under salt stress conditions. Additionally, RG11 promoted plant growth and enhanced the levels of endogenous melatonin in different grape cultivars. Intraspecific variation in the levels of melatonin precursors was found among four grape cultivars, and the associated root crude extracts appeared to significantly induce RG11 melatonin biosynthesis *in vitro*. Overall, this study provides useful information that enhances the existing knowledge of a potential melatonin synthesis pathway in rhizobacteria, and it reveals plant-rhizobacterium interactions that affect melatonin biosynthesis in plants subjected to abiotic stress conditions.

**Keywords:** melatonin, plant growth-promoting bacteria, root crude extracts, salt stress, grapevine

## INTRODUCTION

During their evolution, most land plants developed symbiotic associations with microbes, including fungi, bacteria, and actinomycetes that can grow in the roots and other plant tissues (Compant et al., 2011; Hameed et al., 2015; Wang et al., 2016). Numerous plant-associated microbes are beneficial components of plant micro-ecosystems (Chebotar et al., 2015) because they provide protection against phytopathogens (Lindow and Brandl, 2003; Jetiyanon, 2007), enhance mineral nutrient acquisition (Elbeltagy et al., 2001; Johnson, 2008; Richardson et al., 2009), and help plants withstand abiotic stresses (Castiglioni et al., 2008; Zhang et al., 2008; Khan et al., 2015). The most researched role of plant-associated microbes is their potential to regulate plant growth by acting as suppliers of diverse phytohormones, including gibberellins (Bottini et al., 2004; Shahzad et al., 2016), indole acetic acid (Patten and Glick, 1996; Etesami et al., 2014), cytokinins (Ie et al., 2001; Arkhipova et al., 2007), jasmonates and abscisic acid (Forchetti et al., 2007; Belimov et al., 2014; Salomon et al., 2014).

We previously reported that several common endophytic bacteria have the capacity to synthesize the additional plant physiology regulator, melatonin (*N*-acetyl-5-methoxytryptamine), and could promote the abiotic stress-induced production of endogenous melatonin in grape roots (*Vitis vinifera* L.) (Jiao et al., 2016). This indoleamine molecule was first isolated from the bovine pineal gland (Lerner et al., 1958), and is now recognized as a ubiquitous compound among living organisms including humans, animals, plants, bacteria, fungi, and macroalgae (Tilden et al., 1997; Rodriguez-Naranjo et al., 2012; Tan et al., 2012). Melatonin was first identified in edible plants in 1995 (Dubbelz et al., 1995; Hattori et al., 1995), and it was subsequently identified in hundreds of plant species (Burkhardt et al., 2001; Reiter et al., 2005; Okazaki and Ezura, 2009; Murch et al., 2010). Exogenous melatonin can act as a phytoregulator of seed germination (Zhang et al., 2013, 2014), flowering (Kolář et al., 2003), fruit ripening, anthocyanin accumulation (Sun et al., 2016), root system architecture (Pelagio-Flores et al., 2012), chlorophyll preservation, and leaf senescence (Zhang et al., 2016). It is also a powerful antioxidant that directly decreases the levels of ROS or indirectly modulates antioxidant enzyme activities (Posmyk et al., 2008, 2009; Arnao and Hernández-Ruiz, 2009; Nawaz et al., 2016). Abiotic stressors can elevate the levels of endogenous melatonin in plants (Arnao and Hernández-Ruiz, 2013a,b, 2016; Shi et al., 2015), so stress-induced ROS may trigger melatonin accumulation (Arnao and Hernández-Ruiz, 2015).

Because melatonin has multiple functions, more information regarding the melatonin-producing mechanism in endophytic bacteria and bacterial interactions with plants via melatonin is needed. Unlike exogenous melatonin application, melatonin-producing endophytes might have long-term effects on endogenous melatonin levels in plants once they enter plant

tissues. In vertebrates, the biosynthesis of tryptophan to melatonin is well described, and its synthetic pathway involves three main intermediates, 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin, which are catalyzed by tryptophan hydroxylase, aromatic amino acid decarboxylase, and animal serotonin *N*-acetyltransferase, respectively (Tan et al., 2015). However, this pathway in plants differs markedly from that in vertebrates (Tan et al., 2014) in that plants initially decarboxylate tryptophan to form tryptamine with tryptophan decarboxylase, and hydroxylate tryptamine is subsequently used to form serotonin with tryptamine 5-hydroxylase (Byeon and Back, 2013; Byeon et al., 2014b). It is speculated that the melatonin synthetic machinery in eukaryotes was inherited from bacteria as a result of endosymbiosis (Tan et al., 2013); however, the melatonin synthetic mechanism in bacteria remains unclear. Additionally, we observed that the levels of endogenous melatonin in grape roots were enhanced by the colonization of melatonin-producing endophytic bacteria under abiotic stress conditions (Jiao et al., 2016), but the internal and external elements that could influence melatonin production need further study.

Among the abiotic stresses, salt stress is a major problem for agricultural lands located near coastal regions. In addition to the use of traditional breeding and plant genetic engineering, the use of melatonin-producing endophytes might be useful for the development of strategies that facilitate plant growth under salt stress conditions (Mayak et al., 2004). Here, we used the melatonin-producing endophytic bacterium *Pseudomonas fluorescens* RG11 to characterize the bacterial melatonin synthetic pathway using <sup>15</sup>N double-labeled *L*-tryptophan as the melatonin precursor. The roots of four grape cultivars were inoculated with RG11 in order to: (i) investigate changes in ROS accumulation levels, endogenous melatonin levels, and related gene expression; and (ii) detect differences in root colonization, growth promotion, and endogenous melatonin levels among cultivars subjected to salt stress conditions. Finally, we compared the levels of melatonin intermediates in the roots of grape cultivars and further determined whether the root crude extracts could influence melatonin biosynthesis in RG11 *in vitro*.

## MATERIALS AND METHODS

### Microorganism and Culture Conditions

The *P. fluorescens* RG11 strain, which was previously isolated from the roots of the *V. vinifera* Red Globe grape cultivar, was used in this study. The root surfaces were sterilized to ensure that bacteria were isolated from internal tissues. A preliminary test showed that RG11 was able to produce melatonin *in vitro* and *P. fluorescens* was identified using morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Krieg and Holt, 1984) and using a 16S rRNA sequencing analysis. The *P. fluorescens* 16S rDNA gene sequence (GenBank accession no. KY172955) was amplified using the universal bacterial primer pairs 27F/1492R in accordance with previously described conditions (Bai et al., 2002), and a neighbor-joining dendrogram

**Abbreviations:** H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; MDA, malondialdehyde; MS, Murashige and Skoog; ROS, reactive oxygen species; TCA, trichloroacetic acid; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry.

generated by the program MEGA 6.06 indicated close similarity to *P. fluorescens* (**Figure 1**).

The RG11 strain was maintained at 4°C on nutrient agar slants (3 g L<sup>-1</sup> beef extract, 10 g L<sup>-1</sup> tryptone, 5 g L<sup>-1</sup> NaCl, and 20 g L<sup>-1</sup> agar; pH 7.4). To prepare inoculum cultures, cells from one colony were transferred to 50 mL nutrient broth medium and cultivated for 12–16 h at 30°C with an agitation of 150 rpm to a final OD<sub>600</sub> of approximately 1.0. The cultures were then centrifuged at 6,000 × g for 10 min, re-suspended, and standardized to 1 × 10<sup>8</sup> cells mL<sup>-1</sup> in 0.9% sterilized saline solution using a Petroff-Hausser counting chamber.

## Measurement of the Ability of RG11 to Secrete Melatonin and Its Intermediates Using <sup>15</sup>N Double-Labeled *L*-Tryptophan

To characterize melatonin biosynthesis in RG11, 1 mL of standardized bacterial inoculum was added to individual 100-mL brown bottles that contained 50 mL of nutrient broth (3 g L<sup>-1</sup> beef extract, 10 g L<sup>-1</sup> tryptone, and 5 g L<sup>-1</sup> NaCl; pH 7.4) with 200 mg L<sup>-1</sup> <sup>15</sup>N double-labeled *L*-tryptophan. The cultures were incubated in a rotary shaker at 28°C with a rotational speed of 150 rpm in the dark under aerobic conditions. Samples were collected every 6 h for viable cell counts using the plate counting method and appropriate dilutions. A 1-mL aliquot of the bacterial culture was centrifuged, and the supernatant was diluted 1:1 with methanol. The resulting mixture was passed through a 0.22-μm filter, and was then used for the detection and quantification of <sup>15</sup>N-tryptamine, <sup>15</sup>N-5-hydroxytryptophan, <sup>15</sup>N-serotonin, <sup>15</sup>N-N-acetylserotonin, and <sup>15</sup>N-melatonin using UPLC-MS/MS. The concentrations of these compounds were determined using an eight-point calibration curve of non-isotopic labeling standards as described by Jiao et al. (2016). The experiment was repeated in triplicate.

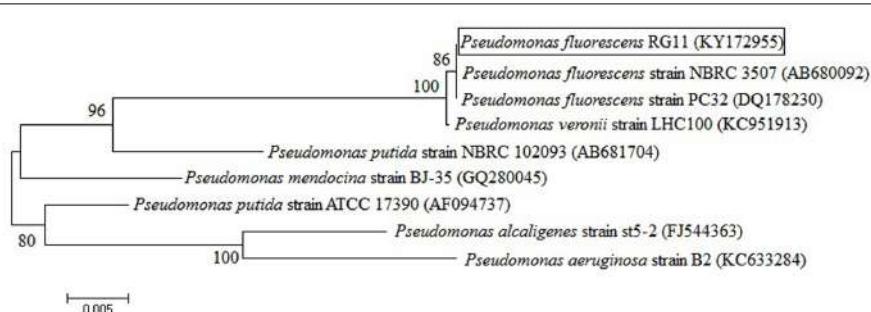
## Greenhouse Experiment and Microbial Inoculation

*Vitis vinifera* ‘Red Globe grape’ plantlets, obtained from an *in vitro* culture system, were grown in sterilized 500-mL glass bottles filled with 1/2 MS modified basal salt mixture (PhytoTechnology Laboratories, Shawnee Mission, KS, USA),

which contained 3% sucrose, 0.6% agar, and 0.2 mg L<sup>-1</sup> indolebutyric acid. Plantlets were grown at 28°C with a 16-h light/8-h dark cycle for 6 weeks. Plantlets of approximately 8 cm in height and with six leaves were selected and standardized to four 5-cm adventitious roots using sterilized scissors. The roots of some plantlets were immersed in the RG11 bacterial inoculum for 1 min (E+), whereas others were immersed in 0.9% sterile saline solution (E-). All plantlets were transferred to 500-mL culture bottles that contained 150 g sterile nutrient soil (Pindstrup, Ryomgaard, Denmark) and 40 mL nutrient-rich water that was prepared from 1/2 MS. The bottles were randomly placed in a controlled chamber at 26°C with 70% humidity under a 16-h light/8-h dark cycle, and plantlets were irrigated with 5 mL distilled water every 2 days.

After 20 days, 12 plantlets from each group (E+ and E-) were randomly selected for root length, root fresh weight (FW), lateral root number, plant height, and chlorophyll content measurements. The chlorophyll content of fully expanded leaves was analyzed using a chlorophyll ELISA kit, according to the manufacturer’s instructions (Ivyuan, Beijing, China). Both E+ and E- groups were divided into two subgroups (at least 84 plantlets each), and were watered either normally or with 20 mL of 80 mM NaCl solution. Roots were collected from 12 randomly selected plantlets from each subgroup between 9:00 AM and 10:00 AM daily. The roots were ground into powder in liquid nitrogen in individual mortars, and 0.5 g samples were extracted with 2 mL methanol as previously described (Boccalandro et al., 2011). The extracts were mixed with 2 mL ultrapure water, centrifuged, passed through a 0.22-μm filter, and stored in amber vials for the detection and quantification of 5-hydroxytryptophan, tryptamine, serotonin, N-acetylserotonin, and melatonin with UPLC-MS/MS. Other samples were used for the determination of MDA content and H<sub>2</sub>O<sub>2</sub> accumulation.

To investigate whether the grape genotype could influence bacterial colonization and melatonin production, RG11 was used to inoculate the roots of three additional grape cultivars, including Riesling, Chardonnay, and Cabernet Sauvignon. We used the procedure described above with the following two modifications: (i) after measuring the growth attributes of each plantlet, the whole root was collected to assess bacterial



**FIGURE 1 |** Neighbor-joining dendrogram based on 16S rRNA gene sequences that were available in the GenBank database. The tree was constructed using the MEGA 6.06 package. Bootstrap values were based on 1,000 replications and are listed as percentages at the nodes. The scale bar indicates genetic distance.

colonization ability; and (ii) after exposure to 80 mM NaCl stress for 4 days, the plantlet roots were sampled between 9:00 AM and 10:00 AM for subsequent UPLC-MS/MS analyses. To assess bacterial colonization ability, roots were surface-sterilized with 70% ethanol for 3 min, soaked in sodium hypochlorite (3% available chlorine) for 2 min, and rinsed three times with sterile water. Then, 0.5 g of the root samples were ground and homogenized in 1.5 mL ice-cold phosphate-buffer saline solution using sterile quartz sand in individual mortars. The homogenates were serially diluted, spread, and incubated on Luria–Bertani agar at 30°C for 2–3 days to determine viable bacterial counts.

## Measurement of RG11 Melatonin Production *In vitro* with Crude Root Extracts

After exposure to 80 mM NaCl stress for 4 days, 10 g of root samples from E– Red Globe, Riesling, Chardonnay, and Cabernet Sauvignon plantlets were collected, washed three times with sterile H<sub>2</sub>O, ground, and homogenized in 20 mL sterile H<sub>2</sub>O using sterile quartz sand in individual mortars. The homogenates were centrifuged at 6000 × g for 10 min, and the supernatants were filtered through 0.22-μm membrane filters (Millipore, Billerica, MA, USA). The extraction sterility was tested by plating 100 μL of extract on nutrient agar and incubating at 37°C for 24 h. A 0.25-mL sample from each standardized bacterial culture ( $1 \times 10^8$  cells mL<sup>-1</sup>) was used to inoculate 25 mL nutrient broth containing 200 mg L<sup>-1</sup> *L*-tryptophan and 20% (v/v) sterile root extract. The cultures were incubated as described above and sampled every 6 h to measure melatonin production.

## Determination of MDA Content and H<sub>2</sub>O<sub>2</sub> Accumulation

Fresh root samples were ground into a powder in liquid nitrogen, and H<sub>2</sub>O<sub>2</sub> accumulation was quantified using a hydrogen peroxide assay kit (Beyotime, Shanghai, China), according to the manufacturer's instructions. In brief, 0.1 g of root powder was homogenized in 1 mL phosphate buffer (50 mM; pH 6.0) at 4°C. The supernatant (50 μL) was mixed with 100 μL of test solution and placed at 30°C for 30 min. The absorbance was measured with a spectrometer at 560 nm and calibrated to a standard curve generated using known H<sub>2</sub>O<sub>2</sub> concentrations.

In addition, 0.5 g of root powder was extracted with 2 mL of 10% (w/v) TCA to determine the MDA content of the grape roots as previously described (Zhao et al., 2013) with some modifications. After centrifugation at 8,000 × g for 10 min, 0.2 mL of the supernatant was added to the same volume of 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA, and was then heated at 100°C for 20 min. Reactions were stopped on ice. After centrifugation at 8,000 × g for 10 min, the absorbance was measured with a spectrometer at 440 nm, 532 nm, and 600 nm, and the MDA content (nmol g<sup>-1</sup>) was calculated as follows: [6.45 × (A532 – A600) – 0.56 × A450] × V/W, where V (mL) is the volume of the tissue extract, and W (g) is the FW.

## Quantitative Analysis of Melatonin and Its Intermediates Using UPLC-MS/MS

All standard reference materials were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 15N double-labeled *L*-tryptophan was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The reagents, including methanol and formic acid (HPLC grade), were purchased from Merck (Darmstadt, Germany). Stock solutions were prepared by dissolving 10 mg of each standard in 1 mL of methanol under dim light conditions, and the stocks were then stored at –80°C to prevent degradation. Fresh working solutions were prepared in a 50:50 (v/v) solution of methanol:water.

Quantitative detection was conducted using a triple quadrupole UPLC-MS/MS (Agilent, Santa Clara, CA, USA). In the solvent system, eluent A was composed of Milli-Q water containing 0.1% (v/v) formic acid, and eluent B was methanol. Analyte separation was accomplished using an Agilent ZORBAX Eclipse XDB-C18 Rapid Resolution HT column (1.8 μm, 3.0 mm × 50 mm) at 42°C with linear elution gradient protocols of 0–6 min for 5–55% B and 6–15 min for 55–100% B at a flow rate of 0.2 mL min<sup>-1</sup>. Then, 100% B was kept constant for 2 min, and the column was re-equilibrated for 5 min using the initial solvent composition. The injection volume was 1 μL. Quantitation was determined using the multiple reactions monitoring mode under unit mass-resolution conditions (*L*-tryptophan  $m/z^+$  205→188; <sup>15</sup>N-*L*-tryptophan  $m/z^+$  207→189; tryptamine  $m/z^+$  161→144; <sup>15</sup>N-tryptamine  $m/z^+$  163→145; 5-hydroxytryptophan  $m/z^+$  221→204; <sup>15</sup>N-5-hydroxytryptophan  $m/z^+$  223→205; serotonin  $m/z^+$  177→160; <sup>15</sup>N-serotonin  $m/z^+$  179→161; *N*-acetylserotonin  $m/z^+$  219→160; <sup>15</sup>N-*N*-acetylserotonin  $m/z^+$  220→161; melatonin  $m/z^+$  233→174; and <sup>15</sup>N-melatonin  $m/z^+$  235→175).

## RNA Isolation and Quantitative Real-Time PCR Analysis

The expression levels of the grapevine tryptophan decarboxylase gene (*VvTDC1*) and the serotonin *N*-acetyltransferase gene (*VvSNAT*) in grape roots, previously predicted by Byeon et al. (2014a) and Jiao et al. (2016), were analyzed using quantitative real-time PCR (qRT-PCR). Root tissues were washed three times with sterile H<sub>2</sub>O, and RNA was extracted using the cetyl trimethylammonium bromide method (Reid et al., 2006). The RNA quantity was determined by measuring the A<sub>260</sub> and A<sub>280</sub> (NanoDrop® ND-1000; Thermo Scientific, Wilmington, DE, USA). RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). qRT-PCR was conducted using a Roche 480 light cycler system with SYBR Fast qPCR Mix (TaKaRa) under the following conditions: 95°C for 30 s; followed by 40 cycles at 95°C for 5 s, 60°C for 10 s, and 72°C for 15 s. The melting curve analysis was performed at 95°C for 5 s, 60°C for 1 min, 95°C continuously, and then 50°C for 30 s. The primers used in this study are listed in Table 1. *EF1-α* was used as an internal reference to calculate the relative expression of each gene. All qRT-PCR reactions were performed in triplicate.

**TABLE 1 | Gene-specific primers used for quantitative real-time PCR.**

Genes name	Description	GenBank no.	Forward primers (5' to 3')	Reverse primers (5' to 3')
VvSNAT	Serotonin N-acetyltransferase	XM_002266325	GCCCGTGTACATCAGATCA	TTTGATGCCCTGGGTCAG
VvTDC1	Putative tryptophan decarboxylase-1	XM_010654123	CTGCCAGATTCCGCACCTAA	TCCGCGCAGGAGAAGTAATC
EF-1 $\alpha$	Elongation factor-1 $\alpha$	XM_002284888	GAACGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA

## Statistical Analysis

A correlation analysis based on a simple linear regression was performed on the assayed variables at the 95% confidence level between the relative expression of *VvTDC1* and *VvSNAT* and the MDA content. Data were expressed as the average of 3–12 replicates  $\pm$  standard deviation. One-way analysis of variance (ANOVA) was performed in conjunction with Tukey's test to detect melatonin intermediates in the E $-$  roots of the four grape cultivars, and Student's *t*-test was used to identify significant differences ( $P < 0.05$ ) in bacterial colonization between treatments. All statistical analyses were performed using SPSS 19.0 (IBM, Armonk, NY, USA).

## RESULTS

### Characterization of Melatonin Biosynthesis in *P. fluorescens* RG11

The different melatonin synthetic pathways in animals and plants are presented in **Figure 2A**, and the chemical structures and chromatograms of six unlabeled standards (40–60 ng mL $^{-1}$ ) and their corresponding  $^{15}\text{N}$ -metabolites are presented in **Figures 2B,C**. If tryptophan hydroxylase and tryptophan decarboxylase (or enzymes with similar activity) were present in *P. fluorescens* RG11,  $^{15}\text{N}$ -tryptophan could be converted to  $^{15}\text{N}$ -5-hydroxytryptophan ( $m/z^+ 223 \rightarrow 205$ ) and  $^{15}\text{N}$ -tryptamine ( $m/z^+ 163 \rightarrow 145$ ). A peak was obtained at  $m/z^+ 223 \rightarrow 205$  with an identical retention time as that of the unlabeled 5-hydroxytryptophan standard, indicating that the compound was  $^{15}\text{N}$ -5-hydroxytryptophan. However, after RG11 was incubated with  $^{15}\text{N}$ -tryptophan, no peak was detected at the correct retention time for  $^{15}\text{N}$ -tryptamine at any time point of  $m/z^+ 163 \rightarrow 145$  (**Figure 2C**). These findings suggest that 5-hydroxytryptophan might be a key intermediate in the melatonin biosynthesis pathway of *P. fluorescens* RG11. Similarly, the  $^{15}\text{N}$ -serotonin,  $^{15}\text{N}$ -*N*-acetylserotonin, and  $^{15}\text{N}$ -melatonin were all detected and confirmed using the reference compounds.

All of the  $^{15}\text{N}$ -metabolites were detectable 6 h post-incubation, and their levels showed a progressive increase over time (**Figure 3**).  $^{15}\text{N}$ -5-hydroxytryptophan,  $^{15}\text{N}$ -serotonin, and  $^{15}\text{N}$ -*N*-acetylserotonin were found at relatively higher concentrations that reached maximum values of  $18.06 \pm 1.14$  ng mL $^{-1}$ ,  $8.28 \pm 0.65$  ng mL $^{-1}$ , and  $8.66 \pm 0.82$  ng mL $^{-1}$  at 36 h post-incubation, respectively. The concentrations of  $^{15}\text{N}$ -melatonin reached a maximum value of  $1.32 \pm 0.12$  ng mL $^{-1}$  at 30 h post-incubation and declined slightly thereafter. When the results were expressed in ng 10 $^{-11}$  viable cells, the production capacity for all metabolites peaked at 6 h post-incubation (cell number:  $9.06 \log$

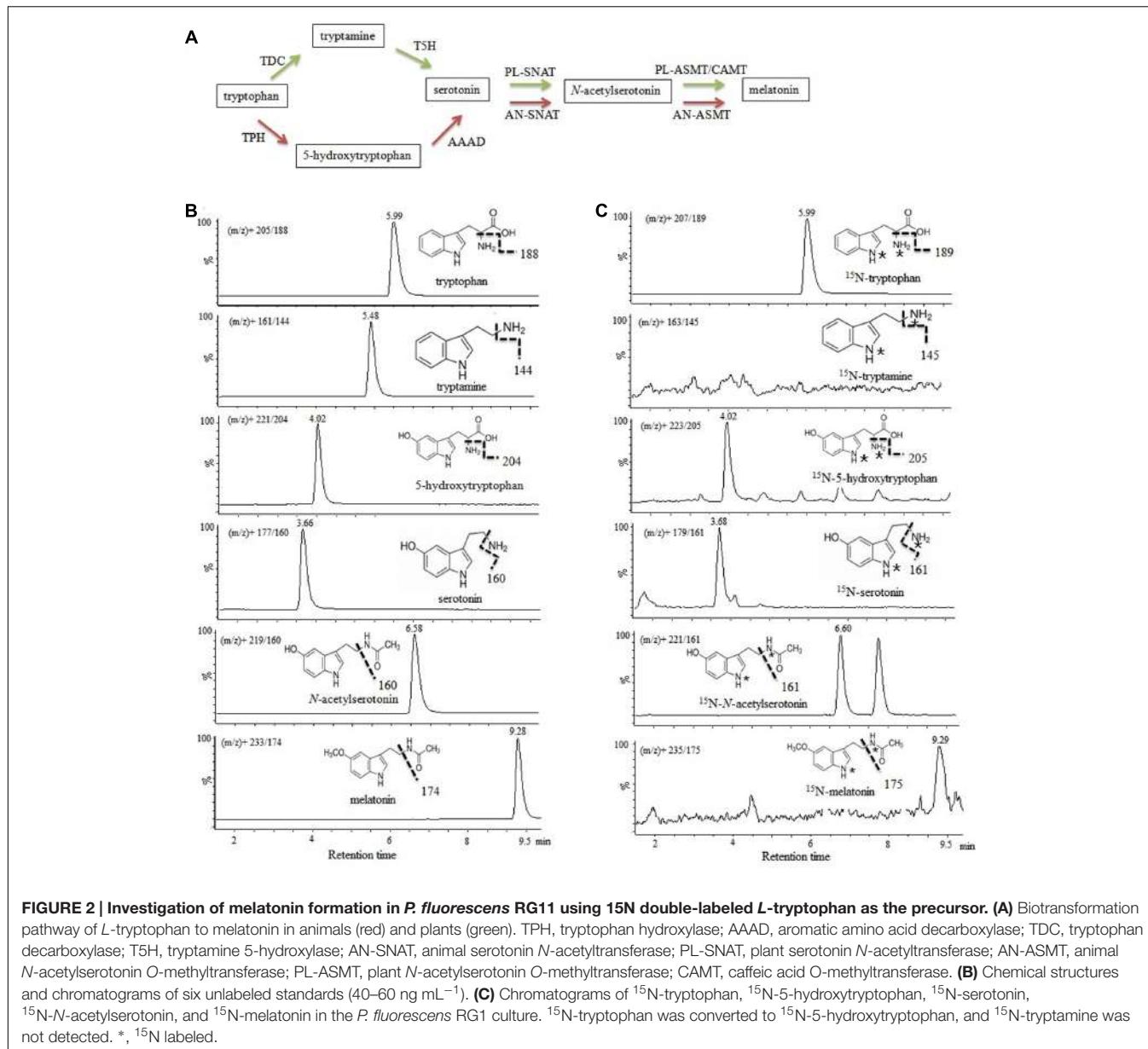
10 CFU mL $^{-1}$ ) and subsequently declined with the increasing cell density (final cell number:  $11.52 \log 10$  CFU mL $^{-1}$ ).

### Changes in Endogenous Melatonin Levels and Its Intermediates in the Roots of E $+$ or E $-$ Red Globe Plantlets under Salt Stress Conditions

The effects of *P. fluorescens* RG11 on the levels of endogenous melatonin and its intermediates in the roots of Red Globe plantlets under salt stress are shown in **Figure 4**. The results show that the production of melatonin and its intermediates first increased in the roots of both E $+$  and E $-$  plantlets and then decreased over time. The levels of 5-hydroxytryptophan, *N*-acetylserotonin, and melatonin in the roots of E $+$  plantlets were higher than those in the roots of E $-$  plantlets, and the opposite trend was observed for levels of tryptamine and serotonin. The roots of E $+$  plantlets produced approximately 19.92–26.01% higher melatonin than those of E $-$  plantlets between day 2 and day 6 of the salt stress treatment. The maximum value of melatonin in the roots of E $+$  plantlets ( $401.31 \pm 37.78$  pg g $^{-1}$  FW) was found on day 4 of the salt stress treatment, whereas the maximum melatonin value of the roots of E $-$  plantlets ( $326.66 \pm 23.40$  pg g $^{-1}$  FW) was observed on day 5 of the salt stress treatment. Similar trends were also observed for 5-hydroxytryptophan and *N*-acetylserotonin, since their levels in the roots of E $+$  plantlets were 13.21–24.69% and 12.40–23.84% higher, respectively, compared with those in the roots of E $-$  plantlets. These results indicate that the biosynthesis of melatonin in RG11-colonized plants responded earlier to salt stress, and the melatonin levels increased much more than E $-$  plants. Conversely, the levels of tryptamine and serotonin in the roots of E $+$  plantlets were lower than those in the roots of E $-$  plants, with a rate of decline of 12.10–21.98% and 6.23–15.54% between day 2 and day 6 of the salt stress treatment, respectively.

### Effect of *P. fluorescens* RG11 on Salt Stress-Induced Oxidative Damage and the Regulation of Melatonin-Related Genes in the Roots of E $+$ and E $-$ Plantlets

The levels of MDA and H<sub>2</sub>O<sub>2</sub> were similar in the roots of E $+$  and E $-$  plantlets, but strongly increased after the salt stress treatment in all plantlets (**Figure 5**). However, E $+$  plantlets had levels that were 15.07–20.76% and 14.29–27.59% ( $P < 0.05$ ) lower in MDA and H<sub>2</sub>O<sub>2</sub>, respectively, compared to E $-$  plantlets between day 2 and day 6 of the salt stress treatment.



**FIGURE 2 |** Investigation of melatonin formation in *P. fluorescens* RG11 using  $^{15}\text{N}$  double-labeled L-tryptophan as the precursor. **(A)** Biotransformation pathway of L-tryptophan to melatonin in animals (red) and plants (green). TPH, tryptophan hydroxylase; AAAD, aromatic amino acid decarboxylase; TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxylase; AN-SNAT, animal serotonin N-acetyltransferase; PL-SNAT, plant serotonin N-acetyltransferase; AN-ASMT, animal N-acetylserotonin O-methyltransferase; PL-ASMT, plant N-acetylserotonin O-methyltransferase; CAMT, caffeic acid O-methyltransferase. **(B)** Chemical structures and chromatograms of six unlabeled standards ( $40\text{--}60 \text{ ng mL}^{-1}$ ). **(C)** Chromatograms of  $^{15}\text{N}$ -tryptophan,  $^{15}\text{N}$ -5-hydroxytryptophan,  $^{15}\text{N}$ -serotonin,  $^{15}\text{N}$ -N-acetylserotonin, and  $^{15}\text{N}$ -melatonin in the *P. fluorescens* RG11 culture.  $^{15}\text{N}$ -tryptophan was converted to  $^{15}\text{N}$ -5-hydroxytryptophan, and  $^{15}\text{N}$ -tryptamine was not detected. \*,  $^{15}\text{N}$  labeled.

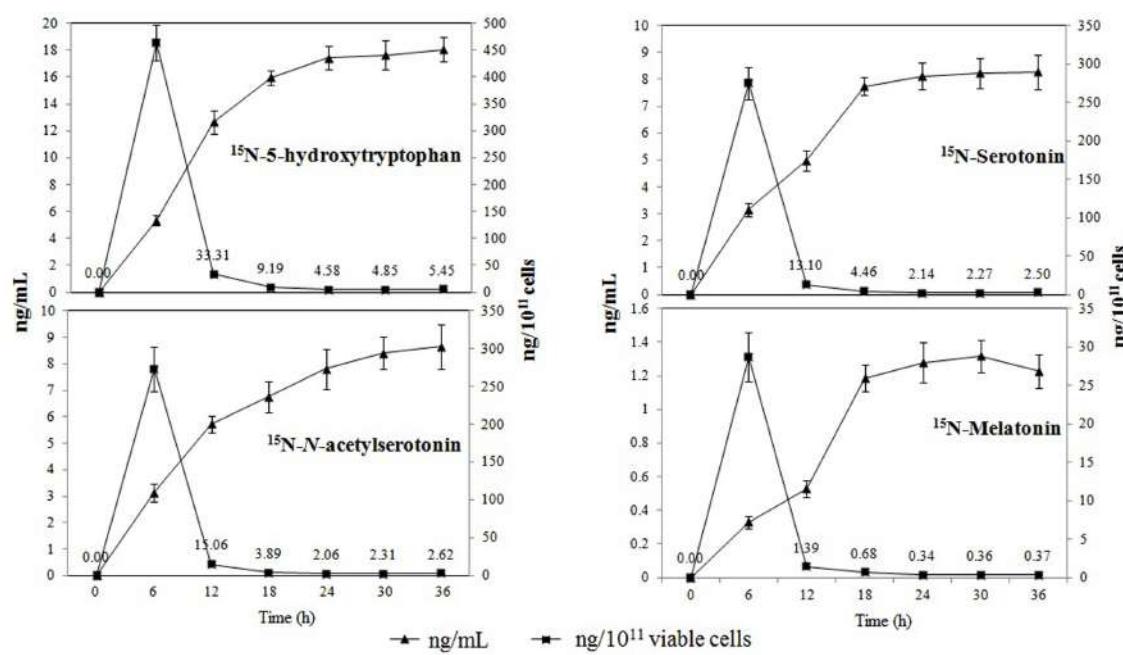
The expression profiles of *VvTDC1* and *VvSNAT* in the roots of salt-treated plantlets are shown in **Figure 6A**. The qRT-PCR analysis demonstrated that the expression of *VvTDC1* and *VvSNAT* first increased in the roots of all plantlets, and then decreased on day 5 or day 6 of the salt stress treatment. Changes in the expression of *VvTDC1* and *VvSNAT* showed that the response of E+ plantlets to salt stress was slower and weaker compared to that of E- plantlets. However, the levels of endogenous melatonin in the roots of E+ plantlets were higher than those in the roots of E- plantlets.

Regression analyses showed a higher linear correlation coefficient between the relative expression of *VvTDC1* and the MDA content ( $R^2 = 0.9759$  and 0.9404) in the roots of E+ and E- plantlets than that of *VvSNAT* ( $R^2 = 0.7574$  and 0.6853; **Figure 6B**). The results indicate that the melatonin synthesis

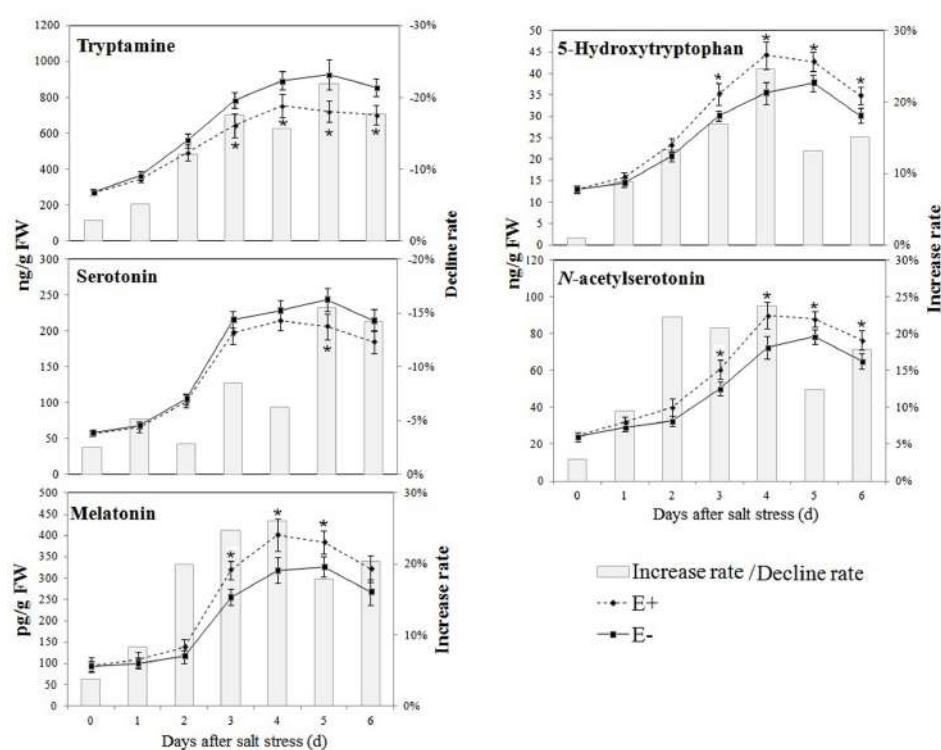
genes, especially *VvTDC1*, might be induced by salt stress, and that the transcript levels were highly correlated with stress-induced oxidative damage.

## Effects of *P. fluorescens* RG11 Colonization on Plant Growth and Melatonin Biosynthesis in the Roots of Different Grape Cultivars under Salt Stress Conditions

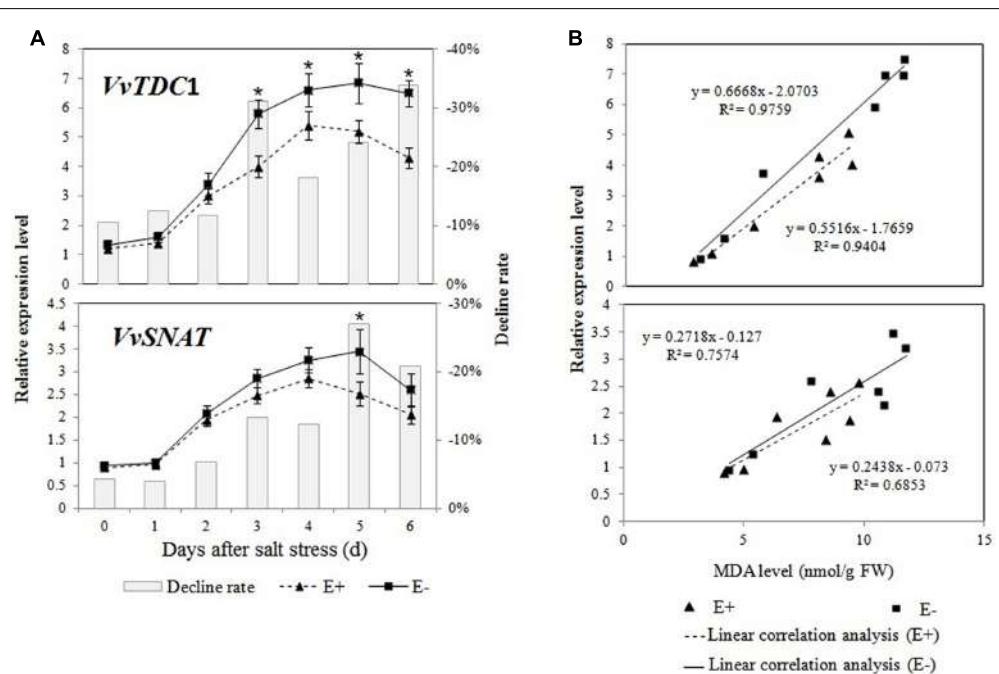
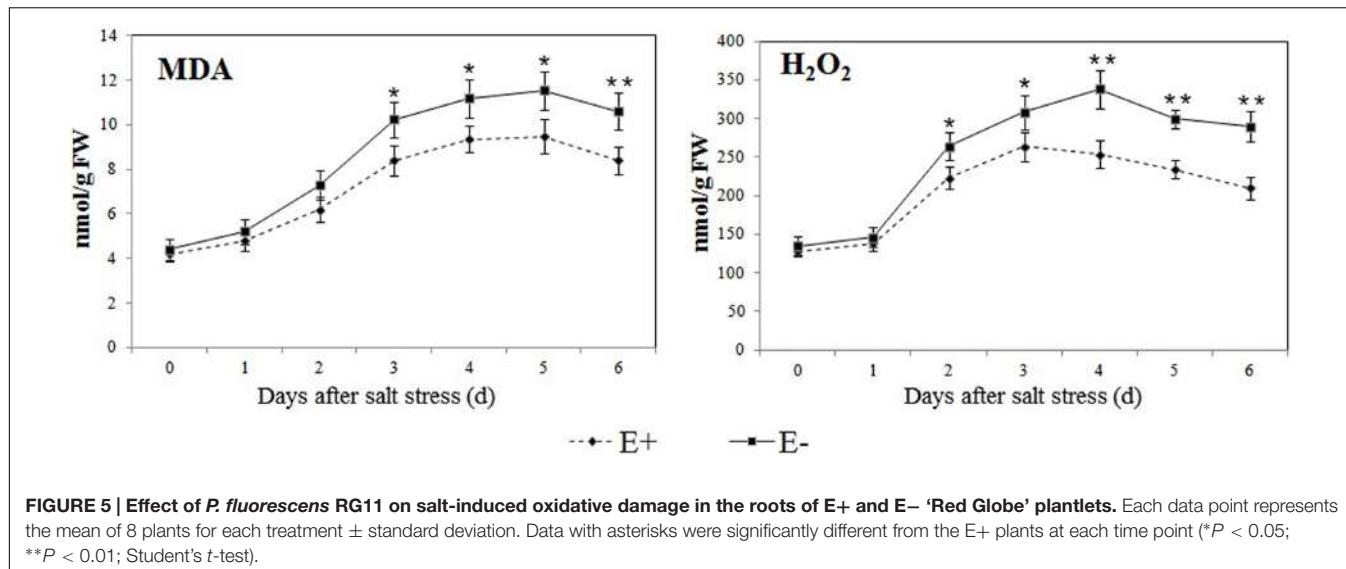
Different grape hosts were used to assess the root colonization capacity of RG11, as well as its ability to promote plant growth. Root colonization of the inoculated endophyte is considered a prerequisite for successful growth promotion. We found that at 20 days post-inoculation, this strain successfully colonized the



**FIGURE 3 | Production of <sup>15</sup>N-metabolites in the melatonin biosynthesis pathway of *P. fluorescens* RG11 in ng mL<sup>-1</sup> (triangle) and ng 10<sup>-11</sup> cells (square). Each data point represents the mean ± standard deviation (n = 3).**



**FIGURE 4 | Effect of *P. fluorescens* RG11 on the levels of endogenous melatonin and its intermediates in the roots of Red Globe plantlets under salt stress conditions.** E+, plants treated with RG11; E-, plants not treated with RG11. Changes in metabolite levels were calculated as follows: (E+/E-) - 1. Each data point represents the mean of 12 plants for each treatment ± standard deviation. Data with asterisks were significantly different from the E- plants at each time point (\*P < 0.05; Student's t-test).



**FIGURE 6 | Effects of *P. fluorescens* RG11 on the relative expression of *VvTDC1* and *VvSNAT* in the roots of E+ and E- Red Globe plantlets under salt stress (A).** The results of a linear correlation analysis between the MDA content and the relative expression of *VvTDC1* and *VvSNAT* (B). Changes in metabolite levels were calculated as follows: (E+/E-) – 1. Each data point represents the mean of 12 plants in each treatment  $\pm$  standard deviation. Data with asterisks are significantly different from the E+ plants at each time point (\* $P < 0.05$ ; Student's *t*-test).

roots of all grape cultivars, and it was able to establish endophytic populations within the different cultivars with a density of approximately 5.44–6.05 Log 10 CFU g<sup>-1</sup> FW.

Further, colonization with RG11 beneficially improved the growth of the grape plants as observed in the significant increase of growth attributes, although the extent of improvement varied between grape varieties (Table 2; Figure 7A). The increased ratios of root FW, total root length, and the number of lateral

roots in relation to control plants ranged from approximately 25.98–38.85%, 18.51–44.41%, and 41.51–63.64%, respectively, and the highest ratio was found in the Red Globe cultivar. On the other hand, Cabernet Sauvignon displayed the highest increase in the ratio of plant height as well as in the chlorophyll content in leaves as compared to the other three varieties. Thus, it could be inferred that RG11 possesses a fairly broad plant growth promoting ability in different grape cultivars.

**TABLE 2 | Effects of *P. fluorescens* RG11 colonization on different growth attributes of Red Globe, Riesling, Chardonnay, and Cabernet Sauvignon grape cultivars 20 days post-inoculation (*n* = 12).**

		Cabernet Sauvignon	Riesling	Chardonnay	Red Globe
Cell viability (Log 10 CFU/g FW)		5.44 ± 0.12	5.65 ± 0.25	5.76 ± 0.14	5.48 ± 0.18
Root fresh weight (g)	E+	3.26 ± 0.11**	2.77 ± 0.10*	2.88 ± 0.12**	3.86 ± 0.15**
	E-	2.54 ± 0.06	2.04 ± 0.08	2.26 ± 0.10	2.78 ± 0.14
	Increase ratio (%)	28.35	25.98	27.32	38.85 <sup>a</sup>
Total root length (cm)	E+	345.40 ± 32**	254.25 ± 22*	325.64 ± 27**	416.56 ± 36**
	E-	258.25 ± 25	214.54 ± 18	258.53 ± 15	288.45 ± 24
	Increase ratio (%)	33.59	18.51	25.91	44.41 <sup>a</sup>
Lateral root number	E+	12.4 ± 1.8*	10.6 ± 0.74**	15 ± 2.20*	14.4 ± 1.5**
	E-	8.1 ± 0.76	7.4 ± 0.65	10.6 ± 1.22	8.8 ± 0.66
	Increase ratio (%)	53.09	43.249	41.51	63.64 <sup>a</sup>
Plant height (cm)	E+	12.06 ± 1.04*	9.46 ± 0.82*	8.48 ± 0.55*	11.46 ± 0.96*
	E-	9.44 ± 0.66	8.04 ± 0.60	7.46 ± 0.42	9.42 ± 0.54
	Increase ratio (%)	27.75 <sup>a</sup>	17.67	13.67	21.66
Chlorophyll content (mg/g)	E+	1.55 ± 0.16*	1.08 ± 0.08*	1.14 ± 0.12	1.34 ± 0.15
	E-	1.29 ± 0.11	0.95 ± 0.10	1.02 ± 0.05	1.16 ± 0.13
	Increase ratio (%)	20.53 <sup>a</sup>	14.16	11.76	15.52

<sup>a</sup>The highest ratio of increase in growth attributes. E+, plants treated with RG11; E-, plants not treated with RG11. Data with asterisks were significantly different from the E- plants within each variety (\*P < 0.05; \*\*P < 0.01; Student's t-test).

The maximum difference in the levels of melatonin in E+ and E- Red Globe plantlets was detected at approximately day 4 after the start of the salt stress treatment (Figure 4), so the roots of E+ and E- plantlets were collected at this time for UPLC-MS/MS (Figure 7B). No significant differences were identified in the levels of melatonin between E+ and E- plantlets within each cultivar. However, all plants responded to salt stress by synthesizing melatonin, and *P. fluorescens* RG11 colonization increased the up-regulation of endogenous melatonin levels in the E+ plantlets of all cultivars as compared to E- plantlets. The levels of melatonin were higher (61.11%) in the roots of E+ Cabernet Sauvignon plantlets than in the roots of E- plantlets. Similar trends were observed in Red Globe, Riesling, and Chardonnay, although the increase was relatively lower. Therefore, *P. fluorescens* RG11 colonization might enhance the synthesis of melatonin in the roots of grape plantlets, especially in Cabernet Sauvignon, under salt stress conditions.

## Root Extraction Stimulated the Melatonin Biosynthesis of *P. fluorescens* RG11 *In vitro*

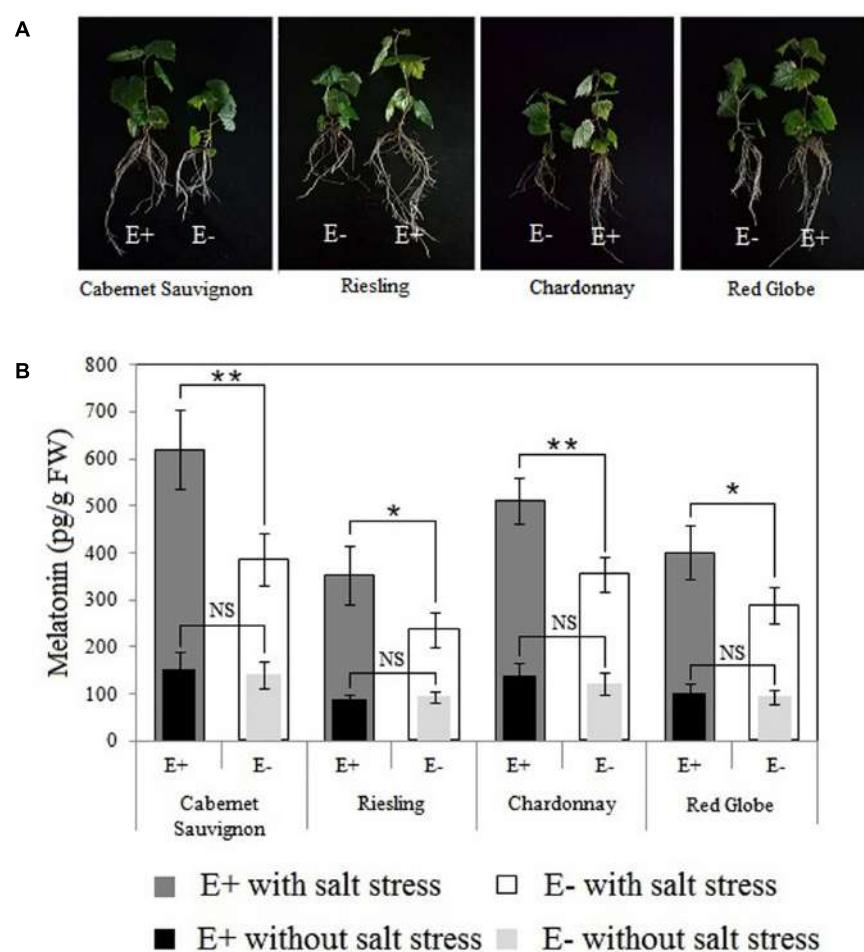
To further understand the relatively higher increase in melatonin production by RG11 colonization in Cabernet Sauvignon than in the other three varieties, we analyzed the levels of melatonin biosynthesis intermediates in the E- roots of the four grape cultivars (Figure 8A). At day 4 of the salt stress treatment, the levels of melatonin intermediates in the E- roots were different among the four grape cultivars: 894.55–1459.84 ng g<sup>-1</sup> FW for tryptamine, 35.48–61.23 ng<sup>-1</sup> FW for 5-hydroxytryptophan, 228.47–406.27 ng<sup>-1</sup> FW for serotonin, and 82.87–126.65 ng<sup>-1</sup> FW for N-acetylserotonin. Cabernet Sauvignon had the highest levels of tryptamine, 5-hydroxytryptophan, serotonin, and

N-acetylserotonin, and these respective levels were 10.42–63.19%, 4.10–72.58%, 33.11–78.07%, and 15.42–52.83% higher than those in the roots of Chardonnay, Riesling, and Red Globe. These results suggest that genetic traits of the cultivars may noticeably influence the levels of melatonin intermediates in grapes.

The addition of root extracts collected from different cultivars significantly enhanced the melatonin synthesis of RG11 *in vitro* compared to the control (Figure 8B). The melatonin levels initially increased, but then slightly decreased in all cultures. The highest melatonin levels were detected after 24 h post-inoculation in control cultures and 30 h post-inoculation in cultures with root extracts. At 30 h post-inoculation, the levels of melatonin in cultures with root extracts from Red Globe, Riesling, Chardonnay, and Cabernet Sauvignon were higher by 5.75-fold, 10.11-fold, 8.19-fold, and 13.42-fold, respectively, as compared to the control (1.14 ± 0.16 ng mL<sup>-1</sup>). Noticeably, the root extracts of Cabernet Sauvignon had the strongest capacity to promote the melatonin synthesis of RG11 *in vitro*.

## DISCUSSION

In a previous study, we characterized the potential melatonin synthesis in the endophytic bacterium *Bacillus amyloliquefaciens* SB-9 by detecting melatonin intermediates using L-tryptophan as a precursor (Jiao et al., 2016). Other melatonin intermediates, such as tryptamine and serotonin, were also found in small quantities in the untagged nutrient broth (data not reported), suggesting that beef extract or tryptone may possess these components. In the present study, we used <sup>15</sup>N double-labeled L-tryptophan as the precursor of melatonin to investigate the biotransformation of <sup>15</sup>N-tryptophan to <sup>15</sup>N-melatonin in *P. fluorescens* RG11 in order to avoid

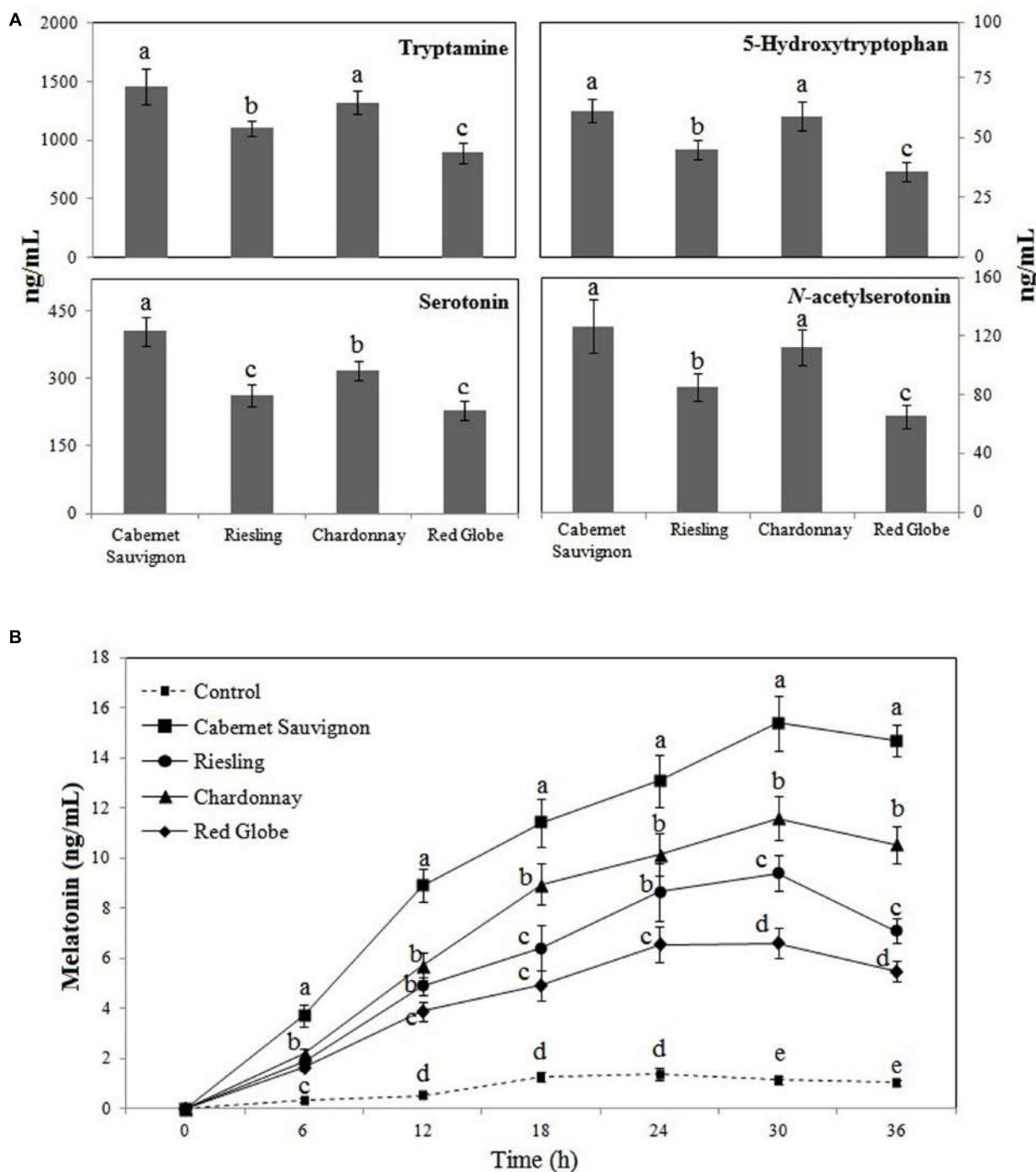


**FIGURE 7 |** Effects of *P. fluorescens* RG11 colonization on morphological differences between E+ and E- plantlets (A) and the endogenous melatonin levels in the roots of the four grape cultivars under salt stress conditions (B). E+, plants treated with the RG11; E-, plants not treated with the RG11. Each data point represents the mean of 12 plants for each treatment  $\pm$  standard deviation. Data with asterisks indicate significant differences ( $*P < 0.05$ ;  $**P < 0.01$ ; Student's *t*-test). NS, not significant based on Student's *t*-test.

the interference of intermediates that possibly existed in the nutrient broth. Additionally, this process allowed us to monitor the flow of isotopic tryptophan in the melatonin synthesis pathway. Although the genes involved in bacterial melatonin biosynthesis were not investigated in this study, to our knowledge, this is the first report on the flow of the carbon skeleton from tryptophan to melatonin in bacteria.

Isotopic tryptamine was not detected in the *P. fluorescens* RG11 culture, but the concentration of isotopic 5-hydroxytryptophan increased throughout the incubation period (Figure 2), indicating that the carbon skeleton of isotopic tryptophan was incorporated into 5-hydroxytryptophan rather than tryptamine by tryptophan hydroxylase (TPH, EC 1.14.16.4) or other enzymes with similar activity. However, the TPH genes, which catalyze the conversion of tryptophan into 5-hydroxytryptophan, were not found in the genome sequences of *P. fluorescens* deposited in GenBank. TPH belongs to the class

of pterin-dependent aromatic amino acid hydroxylases (AAAHs) that include other two subgroups: phenylalanine hydroxylases (PAH, phenylalanine 4-monoxygenase, EC 1.14.16.1) and tyrosine hydroxylases (TH, EC 1.14.16.2) (Kappock and Caradonna, 1996). We found AAAHs are widely distributed in bacteria, but so far, the most of them have been identified as PAH. Some bacterial PAH homolog genes in *Chromobacterium violaceum* (Kino et al., 2009) and *P. aeruginosa* (Zhao and Jensen, 1994) have been reported to catalyze the conversion of tryptophan into 5-hydroxytryptophan. We, therefore, speculated that PAH might be responsible for the formation of 5-hydroxytryptophan, which is the first step of melatonin biosynthesis in *P. fluorescens* RG11. In several *Pseudomonas* species, including *P. fluorescens*, PAH has been confirmed to catalyze tryptophan hydroxylation (Lin et al., 2014). Further studies using knockout mutants of the PAH gene are needed to determine the correlation between PAH and bacterial melatonin production.



**FIGURE 8 |** The levels of tryptamine, 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin in E– roots of Red Globe, Riesling, Chardonnay, and Cabernet Sauvignon grape cultivars under salt stress conditions (A) and the effects of root extracts on *P. fluorescens* RG11 melatonin synthesis *in vitro* (B). Each data point represents the mean  $\pm$  standard deviation ( $n = 3$ ). Different letters indicate significant difference based on Tukey's multiple comparison test ( $P < 0.05$ );  $n = 3$ .

Our data also show that the concentration of isotopic melatonin and its intermediates progressively increased between 0 and 30 h post-incubation; however, the production capacity peaked at 6 h and then sharply declined in the growth phase

of *P. fluorescens* RG11 (Figure 3). These results are consistent with our previous report on *B. amyloliquefaciens* SB-9 (Jiao et al., 2016). The studies of melatonin in other bacteria are currently limited, but melatonin in yeast was found to be mainly produced

during the exponential growth phase (Rodríguez-Naranjo et al., 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2016). Tan et al. (2015) hypothesized that melatonin primarily functions as a free radical scavenger and antioxidant in unicellular organisms, and other functions were acquired during the evolution of multicellular organisms. Consistent with what has been demonstrated in yeast, our results also indicate that melatonin could serve as a bacterial growth-signaling molecule or as protection against ROS in the medium to facilitate early adaptability.

Previous studies showed *P. fluorescens* strains induced systemic resistance in plants (Ramamoorthy et al., 2001; Wang et al., 2005; Saravanakumar and Samiyappan, 2007) and decreased oxidative damage by enhancing plant antioxidant enzyme activities and/or increasing the production of phenolic compounds or other antioxidants (Torres et al., 2012). The results of the present study concur with those of previous reports, and we found that after exposure to salt stress, oxidative damage in the roots of E+ Red Globe plantlets was decreased by RG11 colonization (Figure 5). Thus, it would be expected that the levels of melatonin and its intermediates in the roots of E+ plantlets would be lower than those in the roots of E- plantlets, because the transcript levels of melatonin synthesis genes, *VvTDC1* and *VvSNAT*, were relatively lower than those in E- plantlets (Figure 6A). The upregulation of these genes is positively correlated with ROS levels caused by abiotic stress (Li et al., 2014). However, we observed that only tryptamine and serotonin followed this trend, whereas the levels of melatonin and other intermediates were higher in the roots of E+ plantlets (Figures 4 and 7). The higher endogenous 5-hydroxytryptophan, N-acetylserotonin, and melatonin levels in the roots of these plantlets resulted from RG11 colonization, which might compensate for the production of these compounds via a supplemental bacterial melatonin biosynthesis pathway, a possible exchange of metabolites between the plant and endophyte, or additional promoting factors produced by the endophyte. In fact, in the present study, RG11 was able to secrete all of these compounds *in vivo*, with the exception of tryptamine. Nevertheless, we still have no direct evidence that the enhanced levels of endogenous melatonin were derived from production by endophytic bacteria.

Previous studies also showed that *P. fluorescens* promotes plant growth by affecting various traits, including nitrogen fixation, phosphorus solubilization, production of 1-aminocyclopropane-1-carboxylate deaminase, or induction of physical and chemical (gibberellins and auxin) changes (Jaleel et al., 2007; Saravanakumar and Samiyappan, 2007; Etesami et al., 2014). We found that *P. fluorescens* RG11 exhibited a fairly broad plant growth-promoting ability (Table 2; Figure 7A). Although we cannot be sure whether *P. fluorescens* RG11 simultaneously possesses the traits listed above, we believe that the growth-promoting ability observed in different grapes may be derived from the bacteria's combined effects. Moreover, the increased melatonin levels from RG11 colonization in E+ roots might partly contribute to growth promotion, since melatonin has been reported to stimulate plant growth in several plants, even at low concentrations (Chen et al., 2009; Park and Back, 2012; Bajwa et al., 2014; Wei et al., 2014; Arnao and Hernández-Ruiz, 2016).

Additionally, *P. fluorescens* RG11 enhanced the production of endogenous melatonin in the roots of different grape cultivars under salt stress conditions, especially that of Cabernet Sauvignon (Figure 7B). The root tissue fluid is the natural source of nutrients for endophytes that produce phytohormones that induce a physiological response in the host plant (Kamilova et al., 2006). Therefore, RG11 might utilize melatonin precursors produced by root tissues to expedite its own melatonin biosynthesis inside the roots, and the intraspecific variation of these metabolite levels or other promoters between the roots of the four grape cultivars could be responsible for the relatively high differences in the melatonin levels of E+ plants. The roots of Cabernet Sauvignon exhibited the highest concentrations of melatonin precursors, and its root crude extracts significantly induced RG11 melatonin biosynthesis *in vitro* compared to the control and other grape cultivars (Figure 8).

## CONCLUSION

The results of this study revealed a potential melatonin synthesis pathway in bacteria and functions for the enhancement of endogenous melatonin in plants, which are crucial for understanding plant-rhizobacteria interactions and improving the application of melatonin-producing endophytes in agriculture. The successful utilization of melatonin-synthesizing bacteria in agriculture requires a thorough understanding of the mechanisms that increase the levels of endogenous melatonin in plants. However, further studies are needed to verify the functions of melatonin-related genes in endophytic bacteria.

## AUTHOR CONTRIBUTIONS

CL, YM, and JJ conceived the study; YM and JJ performed the experiments, analyzed the data, and wrote the manuscript; JJ analyzed the UPLC-MS/MS data; XF, YZ, JJ, and HS provided suggestions and revised the manuscript. All authors approved the final manuscript and agreed to be accountable for all aspects of the work, thus ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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# Melatonin Application to *Pisum sativum* L. Seeds Positively Influences the Function of the Photosynthetic Apparatus in Growing Seedlings during Paraquat-Induced Oxidative Stress

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Melatonin, due to its pleiotropic effects plays an important role improving tolerance to stresses. Plants increase endogenous melatonin synthesis when faced with harsh environments as well as exogenously applied melatonin limits stress injuries. Presented work demonstrated that single melatonin application into the seeds during pre-sowing priming improved oxidative stress tolerance of growing seedlings exposed to paraquat (PQ). PQ is a powerful herbicide which blocks the process of photosynthesis under light conditions due to free radicals excess production, when  $O_2$  is rapidly converted to  $O_2^{\bullet-}$  and subsequently to other reactive oxygen species. The parameters of chlorophyll fluorescence [ $F_v/F_m$ ,  $F_v/F_o$ , Rfd,  $\Phi_{PSII}$ , qP, and non-photochemical quenching (NPQ)] in all variants of pea leaves (derived from control non-treated seeds – C, and those hydroprimed with water – H, and hydroprimed with melatonin water solution 50 or 200  $\mu$ M – H-MEL50 and H-MEL200, respectively) were analyzed as a tool for photosynthetic efficacy testing. Moreover stability of the photosynthetic pigments (chlorophylls a, b, and carotenoids) was also monitored under oxidative stress conditions. The results suggest that melatonin applied into the seed significantly enhances oxidative stress tolerance in growing seedlings. This beneficial effect was reflected in reduced accumulation of  $O_2^{\bullet-}$  in leaf tissues, preservation of photosynthetic pigments, improved functioning of the photosynthetic apparatus and higher water content in the tissues during PQ-mediated stress. Our findings provide evidence for the physiological role of this molecule and serve as a platform for its possible applications in agricultural or related areas of research.

**Keywords:** carotenoids, chlorophyll fluorescence, hydropriming, melatonin, oxidative stress, paraquat, *Pisum sativum* L..

## INTRODUCTION

Melatonin, due to its pleiotropic effects (Reiter et al., 2010; Vriend and Reiter, 2015) can play important roles in improving plant tolerance to adverse conditions. Plants increase endogenous melatonin production when faced with harsh environments (Arnao and Hernández-Ruiz, 2014; Zhang et al., 2015); moreover, exogenously applied melatonin limits stress injuries in plants (Janas and Posmyk, 2013; Bajwa et al., 2014; Meng et al., 2014; Kołodziejczyk and Posmyk, 2016). Melatonin also regulates other physiological processes in plants including seed germination, growth promotion, photoperiodic responses, flower development, root system architecture and senescence delay (Murch and Saxena, 2002; Hernández-Ruiz et al., 2005; Janas and Posmyk, 2013; Wang et al., 2013a,b; Byeon and Back, 2014; Chan and Shi, 2015; Wei et al., 2015). Many of the positive melatonin-induced effects in plants are correlated with its strong anti-oxidative properties, since many processes depend on homeostasis in cell redox-status. Melatonin detoxifies a variety of free radicals (FR) and reactive oxygen species (ROS) (Tan et al., 2014, 2015; Zhang and Zhang, 2014; Manchester et al., 2015). A highly appealing property of this molecule, which distinguishes it from most antioxidants, is that its metabolites also have the ability to scavenge ROS and reactive nitrogen species (RNS). Melatonin generates a free radical scavenging cascade which provides a highly protective defense system; thus, even at low concentrations, melatonin is highly effective in protecting organisms from oxidative stress (Galano et al., 2013; Tan et al., 2014, 2015). Although melatonin acts as a direct free radical scavenger, it also elevated the activities of several antioxidant enzymes which assists in its ability to reduce oxidative damage (Rodriguez et al., 2004; Fischer et al., 2013; Reiter et al., 2015; Bañabusta et al., 2016).

Since high quality of seeds is the basis of crop production, our research has developed effective methods to improve their storage and to protect against harmful factors. In relation to this, exogenous melatonin application into the seeds using different priming methods has proven highly effective (Posmyk et al., 2008, 2009a,b; Janas et al., 2009; Szafranska et al., 2012, 2013, 2014; Kołodziejczyk et al., 2015).

Seed priming is one of the most effective and cost-efficient methods for seed quality improvement and stress tolerance in plants. This technique is based on controlled seed hydration that induces a particular physiological state in plants (initial steps of germination *sensu stricto*); this process allows the application natural and synthetic compounds into the seeds before their germination. The beneficial effects of seed priming on their resistance to environmental stresses is documented (Jisha et al., 2013). Our data indicate that the positive effects of pre-sowing melatonin application by priming relates not only to seed quality (higher germination and vigor under suboptimal conditions) but also to seedling development, plant growth and product yield (Janas et al., 2009; Posmyk et al., 2009a; Szafranska et al., 2012, 2013, 2014).

Reactive oxygen species overproduced under different stresses are harmful factors that cause lipid peroxidation, enzyme disturbances and DNA damage. On the other hand, the crucial role of ROS in plant signal transduction is also known. Thus plant's dilemma is not how to totally eliminate ROS, but how to control them (Considine et al., 2015).

The photosynthetic apparatus is a typical physiological, endogenous source of ROS (Foyer and Shigeoka, 2011). Insufficient energy dissipation during photosynthesis triggers excessive chlorophyll excitation, which initiates a reaction with  $O_2$  to yield singlet oxygen ( $^1O_2$ ). This ROS is responsible for damage to the photosystem and other systems involved in photosynthesis. The formation of ROS in thylakoid membranes can be also initiated through the univalent reduction of  $O_2$  to form the superoxide anion radical ( $O_2^-$ ) at the donor side of photosystem I (PSI) (Tambussi et al., 2004). Taking into account highly effective antioxidant properties of melatonin and its positive role in plant physiology, its potential role in protecting the photosynthetic apparatus is an obvious study to perform.

$O_2^-$  is generated in chloroplasts when leaves are treated with paraquat (PQ), a widely used non-selective herbicide for agricultural crops. PQ is a redox-active molecule, which quickly penetrates through the leaves and blocks photosynthesis by accepting electrons from PSI. This leads the inhibition of ferredoxin reduction resulting in depletion of NADPH and inhibition of  $CO_2$  fixation/assimilation (Moustaka and Moustakas, 2014; Moustaka et al., 2015). The increased efficiency of electron capture by PQ enhances the linear electron transport rate and production of PQ radicals ( $PQ^+$ ) which transfer electrons to  $O_2$  to produce  $O_2^-$  (Moustaka and Moustakas, 2014). Plants tolerate overproduction of ROS only if sufficient antioxidant mechanisms are involved. We have postulated that melatonin application could help plants to tolerate oxidative stress during PQ exposure.

In plants the energy absorbed by chlorophyll *a* is utilized/deactivated in three different means: (a) a major portion is used for non-cyclic (ATP and NADPH synthesis) or cyclic (ATP synthesis) electron transfer in photosynthesis, (b) excess energy is dissipated as heat, (c) energy is emitted as light (fluorescence; Iriel et al., 2014). Measuring chlorophyll fluorescence is a great tool in determining the photosynthetic efficiency. The measurement of fluorescence not only perfectly illustrates the reactions of photosystem II (PSII) under different abiotic stresses, but also distinguishes the type of stress to which a plant is subjected. Among many techniques available to study photosynthesis, pulse amplitude-modulated (PAM) fluorometry is widely used as a rapid, sensitive and non-invasive tool for the estimation of inhibition and damage in PSII electron transfer process (Moustaka et al., 2015).

Powerful herbicides disrupt ROS homeostasis in plant cells adversely affecting the process of photosynthesis. The objective of the current study was to test whether the pre-sowing seed treatment with melatonin positively influenced the parameters of chlorophyll fluorescence in pea leaves under oxidative stress triggered by PQ.

## MATERIALS AND METHODS

### Plant Material

*Pisum sativum* L. seeds provided by TORSSED (Torun, Poland) were hydro-primed with water (H), 50 and 200 µM melatonin/water solutions (H-MEL50, H-MEL200), while non-primed seeds were used as a control (C). To perform seed hydro-priming firstly their initial and final water contents were determined and based on these data the amount of water necessary to achieve appropriate seed moisture content was calculated (Posmyk et al., 2008). The seeds were hydro-primed in closed glass bottles on the STR4 DRIVE rotator (BioCote) at room temp. Portions of water and the aqueous MEL solutions were added at 1-h intervals. This procedure lasted for about 6 h, according to the kinetics of pea seed imbibition at room temperature (these parameters were established experimentally). Next the seeds were air-dried for the subsequent 3 days (time sufficient for the seeds to return to the initial water content) and then used for the experiments.

The seeds were surface sterilized with a fungicide (Thiuram, Organika-Sarzyna, Poland), placed in plastic boxes with cotton wool moistened with distilled water and germinated at 25°C for 3 days. The young seedlings were transplanted into plastic pots filled with sterilized universal soil and perlite (3:1); they were grown for 21 days in a breeding room at constant temperature of 25°C and a fixed photoperiod (16 h light/8 h dark) with light intensity of 7.7–8.4 µmol m<sup>-2</sup> s<sup>-1</sup>.

### Paraquat Treatment

Paraquat (PQ, methyl viologen, 1,1'-dimethyl 4,4'-bipyridinium dichloride), obtained from Sigma-Aldrich (Germany), was used to trigger oxidative stress in tissues. Leaf disks 18 mm in diameter were cut from 24-day-old pea plants. Some leaf disks were immediately used for analysis ( $T_0$ ), and the others were put into Petri dishes filled with 15 ml of 75 µM PQ. They were then placed in a growth chamber (Orbis DATA LOG) with constant light (3.5–3.7 µmol m<sup>-2</sup> s<sup>-1</sup>), at 25°C and incubated for specified time for different analysis: 2, 4, and 6 h for chlorophyll fluorescence parameters analysis and 24 and 48 h for pigment and relative water contents (RWCs). After these times, leaf disks were nitroblue tetrazolium (NBT) stained to identify O<sub>2</sub><sup>•-</sup> generation. Therefore, leaf disks were removed from the Petri dishes, dried on a paper towels and used for further analysis.

### PAM Fluorometry

Chlorophyll fluorescence parameters were obtained with a pulse amplitude modulated (PAM) fluorometer (JUNIOR-PAM, WALZ Germany), using WinControl Windows Software, according to the manufacturer's instruction. The leaf disks previously incubated with PQ 75 µM were transferred into Petri dishes containing distilled water and dark adapted for 30 min before the fluorescence measurements started. To record the chlorophyll fluorescence, the leaf disks, supported with a special clip, were illuminated with a modulated beam of low intensity light (ML, 200–300 mV) to measure the initial fluorescence ( $F_0$ ). Maximal fluorescence ( $F_m$ ) was determined after exposure to a

saturating pulse of white light (SP, 10 000 µmol photons m<sup>-2</sup> s<sup>-1</sup>, 800 ms) to close all reaction centers. From these data, the maximum photochemical quantum yield of PS II ( $F_v/F_m$ , where  $F_v = F_m - F_0$ ) and  $F_v/F_0$  (a value that is proportional to the activity of the water-splitting complex on the donor side of the PSII) were calculated. Subsequently, the samples were exposed to the actinic light (AL, 190 µmol photons m<sup>-2</sup> s<sup>-2</sup>) until a steady-state fluorescence value ( $F_s$ ) was reached, and a new SP was applied to record the maximum fluorescence for light-adapted leaves ( $F'_m$ ). The quantum efficiency of PSII [ $\Phi_{PSII} = (F'_m - F_s)/F'_m$ ] was then obtained. The vitality index (Rfd, chlorophyll fluorescence decrease ratio) which is the indicator of CO<sub>2</sub> fixation was calculated as  $(F_m - F_s)/F_s$  (Lichtenthaler et al., 2005). The photochemical quenching (qP), which quantifies the actual fraction of PSII reaction centers (RCs II) being in the open state was calculated as  $(F'_m - F_s)/(F'_m - F'_0)$  (Genty et al., 1989). The NPQ parameter, which was calculated as  $(F_m - F'_m)/F'_m$ , estimates the NPQ that reflects heat dissipation of excitation energy in the antenna system (Bilger and Björkman, 1990).

All the measurements were performed at room temperature (25°C), in the dark room lit only with dim green light to facilitate work, on the ad-axial face of leaves. Each chlorophyll fluorescence parameter represents mean values from 6 to 7 leaf disks each with 4–5 areas of interest ( $n \sim 25–30$ ).

### Pigments Content

Chlorophyll *a*, *b*, *a+b* and carotenoids were quantified spectrophotometrically. The leaf disks (25 mg) were homogenized in a chilled mortar and pestle with MgCO<sub>3</sub> and 5 ml of 80% acetone and filtered. In the obtained supernatant absorbance at three wavelengths: 470, 646, and 663 nm (spectrophotometer Hitachi U-2001) was measured, which then was used to calculate chlorophyll *a*, *b*, *a+b* and carotenoid concentrations with the following formulas Lichtenthaler and Buschmann (2001):

$$Chl\ a = 12.25 \times A_{663} - 2.79 \times A_{646}$$

$$Chl\ b = 21.50 \times A_{646} - 5.10 \times A_{663}$$

$$Chl\ a + b = 7.15 \times A_{663} + 18.71 \times A_{646}$$

$$Carotenoids = (1000 \times A_{470} - 1.82Chl\ a - 85.02Chl\ b)/198$$

Pigment assays were performed in at least five replicates ( $n = 5$ ).

### Relative Water Content (RWC)

Determination of the RWC was performed according to Barrs (1968). RWC was calculated as follows: RWC [%] = [(FW-DW)/(SW-DW)] × 100%, where FW is the fresh weight, DW is the dry weight determined after 48 h in an oven at 90°C and SW is the saturated weight measured after 4 h of saturation in

deionized water at room temperature in the dark. Experiment was performed in five replicates (five leaf disks per each) ( $n = 5$ ).

## Nitroblue Tetrazolium (NBT) Staining for *Pisum sativum* L. Leaves

Location of  $O_2^{\bullet-}$  in pea leaf disks was performed by NBT staining according to the method of Kawai-Yamada et al. (2004). All leaf disks were investigated at time points:  $T_0$ , 2, 4, 6, 24, and 48 h of incubation in PQ 75  $\mu$ M. The plant material was first infiltrated with 10 mM Na<sub>3</sub>N solution in potassium phosphate buffer (pH 7.8), then with 0.1% (v/v) NBT solution in potassium phosphate buffer (pH 7.8). Subsequently it was incubated for 2 h with NBT solution under light at room temp. After this time, the leaf disks were transferred into wide tubes and boiled in AGE solution (acetic acid: glycerol: ethanol (1: 1: 3 [v/v/v])), up to discolouration of chlorophyll. The stained disks were transferred onto Petri dishes, analyzed using Binocular – Hund-WETZLAR, and then photographed.

## Superoxide Dismutase Extraction and Assay

Protein extraction was performed according to Bałabusta et al. (2016). One gram of leaf disks was ground in a mortar and homogenized with 0.5 g PVP in 5 mL of 0.1 M phosphate buffer (pH 7.5) containing 2.5 mM DTT, 1 mM EDTA, 1.25 mM PEG-4000, and 1 mM PMSF. The homogenate was centrifuged at 20000 g for 30 min at 4°C. The obtained supernatant was filtered through Miracloth, desalted on a PD10 column (Pharmacia, Uppsala, Sweden) and used for the enzyme assays. All steps of the extraction procedure were carried out at 4°C.

Superoxide dismutase (EC1.15.1.1) activity was measured according to Giannopolitis and Ries (1977). The reaction mixture contained 2 mM riboflavin, 13 mM methionine, 0.1 mM EDTA, 70 mM NBT in 0.1 M phosphate buffer (pH7.5), and 100 ml of the enzyme extract in the final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were illuminated with a fluorescent lamp at 25°C (Philips MLL 5000W, Eindhoven, The Netherlands). Identical tubes, which were not illuminated served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. One unit of SOD was defined as the enzyme activity, which inhibited the photoreduction of NBT to blue formazan by 50%. SOD activity was expressed as the enzyme unit per milligram of protein (U mg<sup>-1</sup> prot.).

## Statistical Analyses

The results represent the average values  $\pm$  standard error ( $\pm$ SEM) of the mean. The data were analyzed using STATISTICA v.10.0\_MR1\_PL [StatSoft] software. The two-way analysis of variance (ANOVA) and then the *post hoc* Duncan multiple range tests were carried out to find the significant differences at  $p < 0.001$  in each experiment.

## RESULTS

Tolerance of studied pea leaves to PQ was evaluated on the basis of their reaction to the first 6 h of PQ treatments. In all investigated variants the  $F_v/F_m$  value did not exceed 0.8 at  $T_0$  (Figure 1A). After 6 h of PQ incubation, a sharp decline in this parameter was observed in C and H leaf disks (by about 70%), whereas in H-MEL50 and H-MEL200 leaves,  $F_v/F_m$  ratio remained high, representing 85% and almost 100% of  $T_0$  value, respectively.

**Figure 1B** shows the  $F_v/F_0$  ratio which is more sensitive than the  $F_v/F_m$ . In C, H, and H-MEL50 leaves, the decline of this parameter started after 2 h after PQ treatment and gradually progressed to 6 h; however, in H-MEL50 leaves, this decline was not as rapid (Figure 1B). After 6 h of PQ incubation,  $F_v/F_0$  ratio in H-MEL200 leaves, was even higher than after 2 and 4 h of treatments, and was only 8.5% lower than that at  $T_0$ .

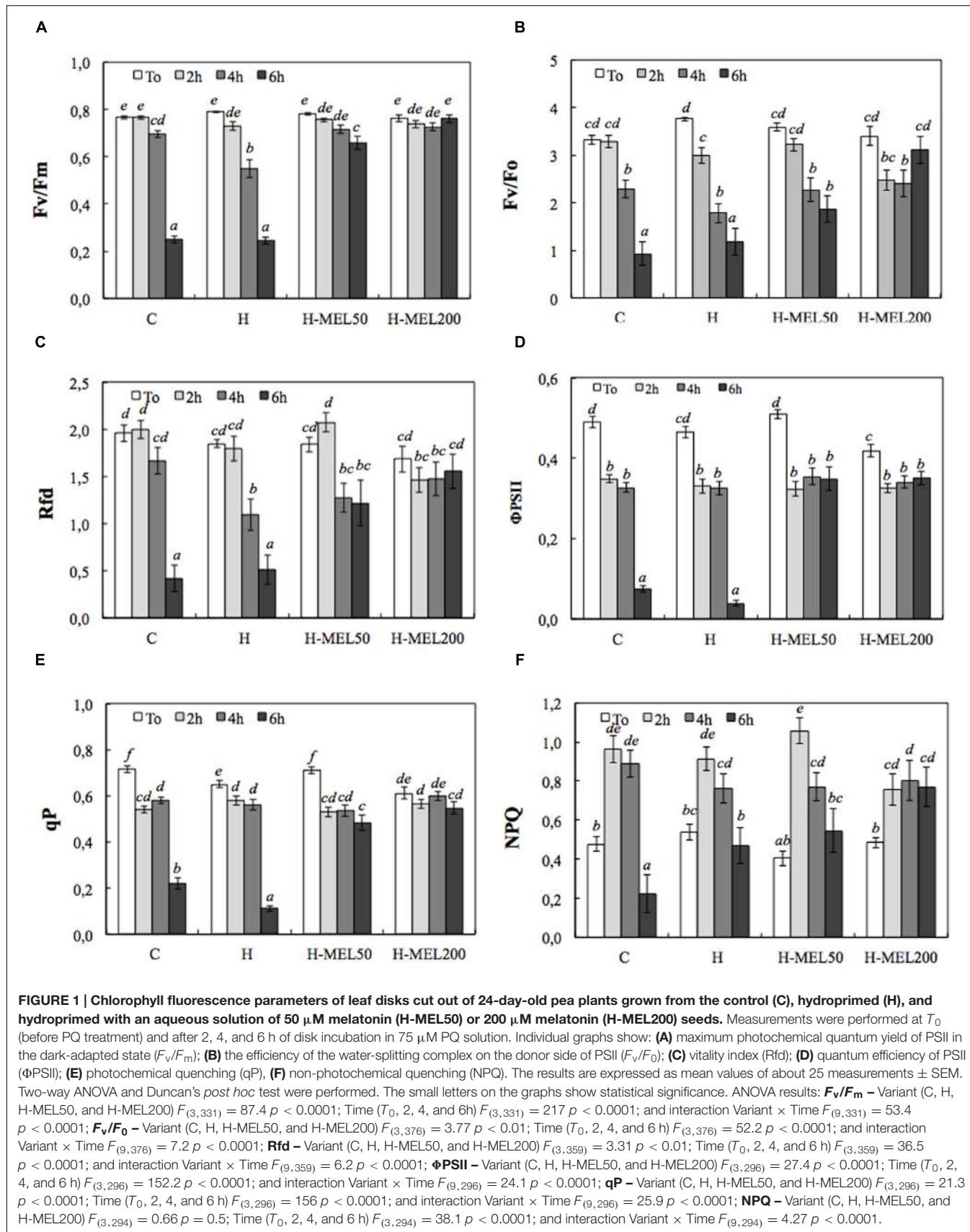
The profiles of the chlorophyll fluorescence decline ratio (Rfd) and the  $F_v/F_m$  changes were similar in C, H, and H-MEL200, while in H-MEL50 after 2 h of PQ treatment Rfd slightly increased (by 13%), after 4 h decreased (by 30%) and after 6 h it remained stable (Figure 1C). The differences between melatonin untreated variants (C and H) and those treated with melatonin (H-MEL50 and H-MEL200) after 6 h of PQ incubation were statistically significant.

The quantum efficiency of PSII ( $\Phi_{PSII}$ ), decreased by about 30% in all studied variants after 2 h of PQ treatment and although the additional hours of incubation triggered drastic an ETR decline in C and H leaf disks (by about 90%), in the variants treated with melatonin it remained at the same level (Figure 1D).

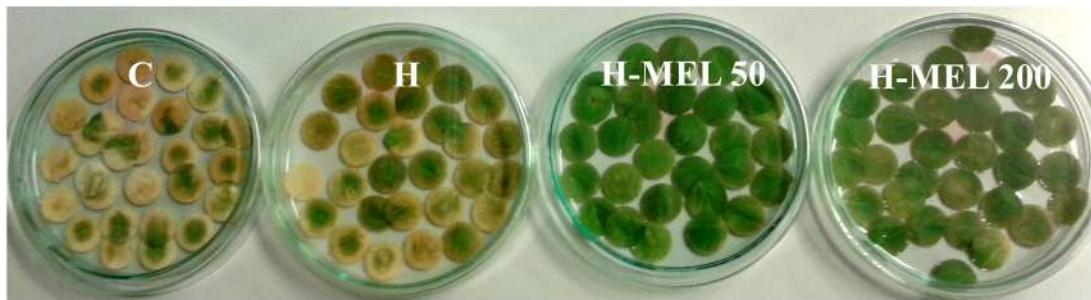
The tendencies of photochemical quenching (qP) changes were similar; in the C and H they sharply decreased after 6 h of PQ incubation (relatively to  $T_0$  by about 70 and 90%, respectively), whereas in H-MEL50 and H-MEL200 it remained at a high level (decreased only by about 30 and 10%, respectively) (Figure 1E). These differences were statistically significant.

The NPQ exhibited a significant increase after 2 h of PQ treatment in all studied variants and in H-MEL50 it reached 260% of  $T_0$  value (Figure 1F). Along with the prolonged time of PQ incubation NPQ levels decreased and after 6 h in C and H leaves were lower than at  $T_0$ , but in H-MEL50 and H-MEL200 leaves this decline was less pronounced.

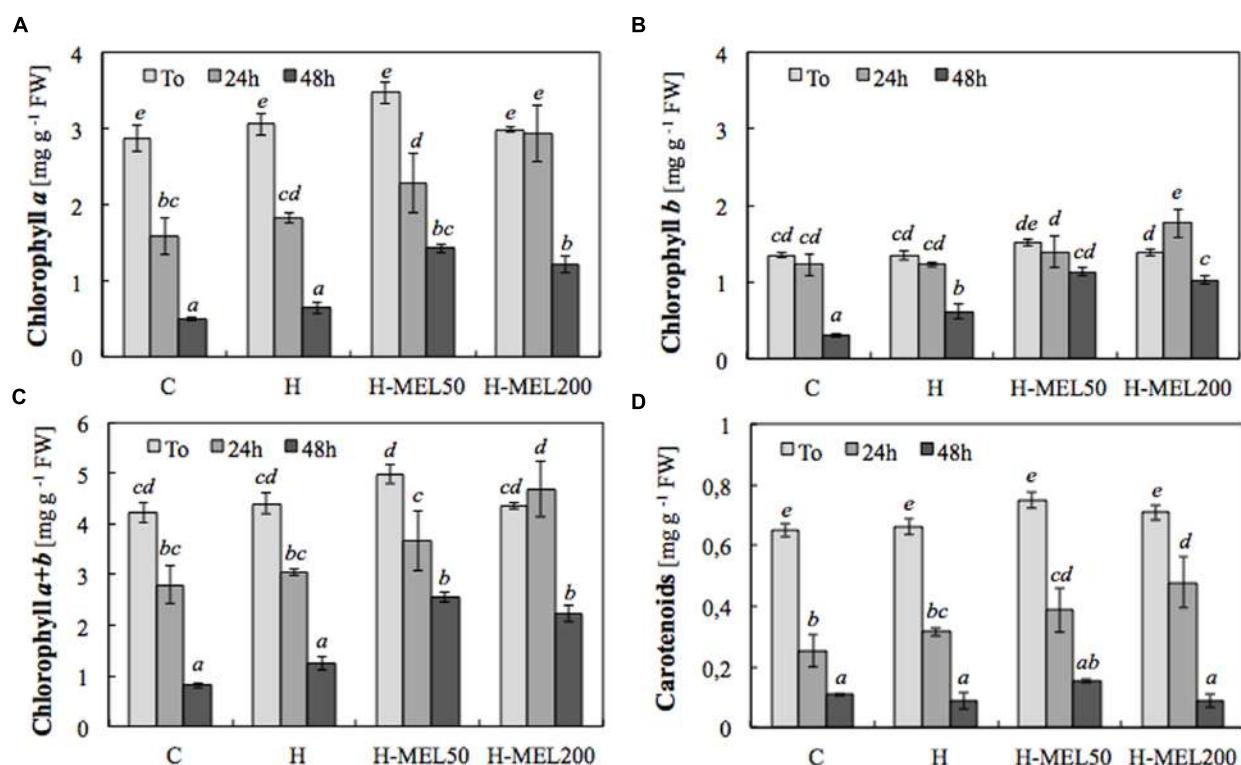
To obtain significant differences in chlorophyll and carotenoid contents, PQ incubation time was extended to 24 and 48 h. Treatment of the leaves with 75  $\mu$ M PQ significantly influenced their color intensity but even if after 24 h the differences between variants were hardly visible (data not shown); 48 h of PQ incubation resulted in nearly 100% discoloration of C leaf disks with slightly less depigmentation of H leaves. In H-MEL50 leaves the green color was almost completely preserved (Figure 2). This visual observation was confirmed by biochemical assays. After 24 h of PQ treatment the chlorophyll *a* level was reduced in C, H, and H-MEL50 leaves by 45, 40, and 34%, respectively; in the H-MEL200 leaves almost no change was observed. In all investigated variants prolonged PQ stress (48 h) caused a significant reduction in the chlorophyll *a* content, but in H-MEL50 it was the greatest and almost 2.5 times greater



**FIGURE 1 | Chlorophyll fluorescence parameters of leaf disks cut out of 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu$ M melatonin (H-MEL50) or 200  $\mu$ M melatonin (H-MEL200) seeds. Measurements were performed at To (before PQ treatment) and after 2, 4, and 6 h of disk incubation in 75  $\mu$ M PQ solution. Individual graphs show: (A) maximum photochemical quantum yield of PSII in the dark-adapted state ( $F_v/F_m$ ); (B) the efficiency of the water-splitting complex on the donor side of PSII ( $F_v/F_0$ ); (C) vitality index ( $Rfd$ ); (D) quantum efficiency of PSII ( $\Phi_{PSII}$ ); (E) photochemical quenching ( $qP$ ); (F) non-photochemical quenching ( $NPQ$ ). The results are expressed as mean values of about 25 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's post hoc test were performed. The small letters on the graphs show statistical significance. ANOVA results:  $F_v/F_m$  – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,331)} = 87.4 p < 0.0001$ ; Time ( $T_0$ , 2, 4, and 6h)  $F_{(3,331)} = 217 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(9,331)} = 53.4 p < 0.0001$ ;  $F_v/F_0$  – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,376)} = 3.77 p < 0.01$ ; Time ( $T_0$ , 2, 4, and 6h)  $F_{(3,376)} = 52.2 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(9,376)} = 7.2 p < 0.0001$ ;  $Rfd$  – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,359)} = 3.31 p < 0.01$ ; Time ( $T_0$ , 2, 4, and 6h)  $F_{(3,359)} = 36.5 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(9,359)} = 6.2 p < 0.0001$ ;  $\Phi_{PSII}$  – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,296)} = 27.4 p < 0.0001$ ; Time ( $T_0$ , 2, 4, and 6h)  $F_{(3,296)} = 152.2 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(9,296)} = 24.1 p < 0.0001$ ;  $qP$  – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,296)} = 21.3 p < 0.0001$ ; Time ( $T_0$ , 2, 4, and 6h)  $F_{(3,296)} = 156 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(9,296)} = 25.9 p < 0.0001$ ;  $NPQ$  – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,294)} = 0.66 p = 0.5$ ; Time ( $T_0$ , 2, 4, and 6h)  $F_{(3,294)} = 38.1 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(9,294)} = 4.27 p < 0.0001$ .**



**FIGURE 2 |** Changes in green color intensity of leaf disks cut out of 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu\text{M}$  melatonin (H-MEL50) or 200  $\mu\text{M}$  melatonin (H-MEL200) seeds. Photographs were taken after 48 h of disks incubation in 75  $\mu\text{M}$  PQ solution. The content of leaf pigments with statistical analysis at this time point (48 h) is presented in **Figure 3** as dark gray bars.



**FIGURE 3 |** Contents of chlorophyll a (A), b (B), a+b (C) and carotenoids (D) in leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu\text{M}$  melatonin (H-MEL50) or 200  $\mu\text{M}$  melatonin (H-MEL200) seeds. Measurements were performed at  $T_0$  (before PQ treatment) and after 24 and 48 h of disk incubation in 75  $\mu\text{M}$  PQ solution. The results are expressed as mean values of about 5 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's post hoc test were performed. The small letters on the graphs show statistical significance. ANOVA results: **Chlorophyll a** – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,24)} = 11.6$   $p < 0.0001$ ; Time ( $T_0$ , 2, 4, and 6 h)  $F_{(2,24)} = 127$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6,24)} = 2.68$   $p < 0.05$ ; **Chlorophyll b** – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,24)} = 12.8$   $p < 0.0001$ ; Time ( $T_0$ , 2, 4, and 6 h)  $F_{(2,24)} = 12$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6,24)} = 3.91$   $p < 0.01$ ; **Chlorophyll a+b** – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,24)} = 12.6$   $p < 0.0001$ ; Time ( $T_0$ , 2, 4, and 6 h)  $F_{(2,24)} = 97.6$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6,24)} = 2.84$   $p < 0.05$ ; **Carotenoids** – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,24)} = 4.31$   $p < 0.01$ ; Time ( $T_0$ , 2, 4, and 6 h)  $F_{(2,24)} = 218$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6,24)} = 1.78$   $p = 0.14$ .

than in C leaves (**Figure 3A**). These differences were statistically significant. The content of chlorophyll b was much lower than that of chlorophyll a and 24 h of PQ treatment triggered only a slight decrease in C, H, and H-MEL50. In H-MEL200 this parameter even increased by 28%. A significant reduction in

chlorophyll b level was observed after 48 h, especially in C and H leaves, while in the variants treated with melatonin it was still relatively high (**Figure 3B**). The schema of carotenoid content changes was similar. Twenty-four hours of PQ incubation caused significant reductions in all variants, and the highest level being

preserved in H-MEL200 leaves. After 48 h of PQ treatment, this decline was dramatic but finally the highest level of carotenoids was noted in H-MEL50 leaves (**Figure 3D**).

The RWC was higher in the leaves derived from melatonin primed seeds even before they were transferred to the PQ solution ( $T_0$ ) (**Table 1**). After 24 h of PQ treatment in C, H, H-MEL50, and H-MEL200 leaves these values rose by about 16, 15, 17, and 20%, respectively. Forty-eight hours of PQ stress caused RWC reduction in all investigated variants, but it remained the lowest in the C.

To visualize  $O_2^{•-}$  in leaf tissues, NBT staining was used. At the starting point ( $T_0$ ) and for the first 2 h of PQ treatment this anion was located only on the rim of the leaf disks as the result of mechanical injury (**Figure 4**). After 6 h of PQ stress the ROS appeared inside the C disks, while in the other variants, they were still not present. Even 24 h of PQ incubation did not change this status and only after 48 h  $O_2^{•-}$  appeared also inside H disks. In H-MEL50 and H-MEL200 leaves throughout the experiment the presence of  $O_2^{•-}$  was not detected (**Figure 4**).

The lack of  $O_2^{•-}$  accumulation in the leaves of plants grown from the seeds pre-treated with melatonin was probably due to the elevated SOD activity in these plants (**Figure 5**). Under oxidative stress induced by PQ, SOD activity increased in all experimental variants, but the highest was in H-MEL50 and H-MEL200 leaf disks (**Figure 5**), where  $O_2^{•-}$  was not revealed (**Figure 4**).

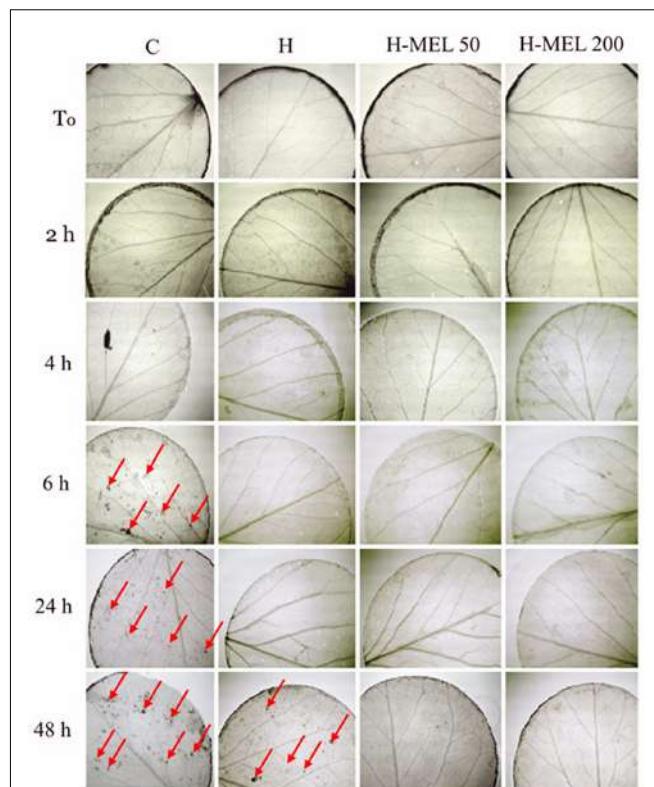
## DISCUSSION

Long-term exposure of plants to melatonin before or during stress is a common popular means to verify the impact of exogenous melatonin application on defense mechanisms activated under harmful environmental conditions. There are several reports on such melatonin application and its influence on protein and photosynthetic pigment degradation during aging (Wang et al., 2012, 2013a,b; Liang et al., 2015). However, there is little information concerning single application of melatonin

**TABLE 1 |** Relative water content (RWC) in leaf disks of 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu\text{M}$  melatonin (H-MEL50) or 200  $\mu\text{M}$  melatonin (H-MEL200) seeds.

Variant	RWC (%)			
	C	H	H-MEL50	H-MEL200
<b>Time</b>				
$T_0$	90.4 $\pm$ 0.36 <sup>a</sup>	90.8 $\pm$ 1.36 <sup>a</sup>	92.3 $\pm$ 1.75 <sup>a</sup>	91.9 $\pm$ 0.88 <sup>a</sup>
24 h	102.9 $\pm$ 4.02 <sup>c</sup>	104.0 $\pm$ 1.74 <sup>c</sup>	108.6 $\pm$ 3.73 <sup>d</sup>	110.0 $\pm$ 3.89 <sup>d</sup>
48 h	98.5 $\pm$ 2.68 <sup>b</sup>	105.8 $\pm$ 2.30 <sup>cd</sup>	102.5 $\pm$ 0.75 <sup>c</sup>	104.8 $\pm$ 2.88 <sup>c</sup>

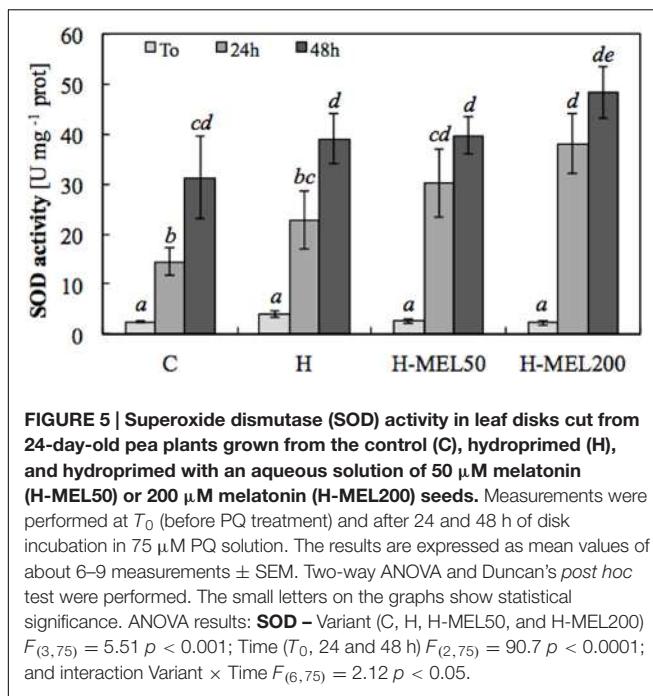
Measurements were performed at  $T_0$  (before PQ treatment) and after 24 and 48 h of disk incubation in 75  $\mu\text{M}$  PQ solution. The results are expressed as mean values of about 5 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's post hoc test were performed. The small letters next to the values show statistical significance. RWC ANOVA results: Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3;32)} = 9.11$   $p < 0.0002$ ; Time ( $T_0$ , 2, 4, and 6 h)  $F_{(2;32)} = 166$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6;32)} = 3$   $p < 0.02$ .



**FIGURE 4 |** Detection of  $O_2^{•-}$  in leaf disks of 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu\text{M}$  melatonin (H-MEL50) or 200  $\mu\text{M}$  melatonin (H-MEL200) seeds. Photographs were taken at  $T_0$  (before PQ treatment) and after 2, 4, 6, 24, and 48 h of disk incubation in 75  $\mu\text{M}$  PQ solution. Red arrows indicate dark spots of formazan derived from NBT oxidized by  $O_2^{•-}$ .

to seeds and its effect on subsequent processes that occur in plants grown from these seeds. Some publications shown that the positive priming effects that occur when beneficial compounds are applied to the seeds are not only visible during germination and sprouting, but also prolonged into later plant developmental stages and observed in more mature plants, especially when subjected to different environmental stresses. There are suggestions that seed conditioning may be a method of improving tolerance through 'priming memory' (Savvides et al., 2016). Generally it is provoked by induction of stress reaction syndrome without negative metabolic disorders and injuries, e.g., through epigenetic modifications and/or synthesis of corresponding anti-stress proteins (Kołodziejczyk et al., 2016a,b).

Considering the well-known antioxidant properties of melatonin (Zhang and Zhang, 2014; Manchester et al., 2015) we tested if this molecule, applied into the seeds, could protect the photosynthetic apparatus of growing seedlings against oxidative stress generated in the 24-day-old pea leaves by 75  $\mu\text{M}$  PQ. Due to potent action of PQ for blocking the photosynthesis process, its influence on the chlorophyll fluorescence parameters has been widely investigated; these parameters are highly sensitive



**FIGURE 5 |** Superoxide dismutase (SOD) activity in leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu$ M melatonin (H-MEL50) or 200  $\mu$ M melatonin (H-MEL200) seeds. Measurements were performed at  $T_0$  (before PQ treatment) and after 24 and 48 h of disk incubation in 75  $\mu$ M PQ solution. The results are expressed as mean values of about 6–9 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's *post hoc* test were performed. The small letters on the graphs show statistical significance. ANOVA results: **SOD** – Variant (C, H, H-MEL50, and H-MEL200)  $F_{(3,75)} = 5.51$   $p < 0.001$ ; Time ( $T_0$ , 24 and 48 h)  $F_{(2,75)} = 90.7$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6,75)} = 2.12$   $p < 0.05$ .

indicators of stress intensity (Guo et al., 2007; Rodriguez et al., 2012; Iriel et al., 2014; Moustaka and Moustakas, 2014; Moustaka et al., 2015). Herein, we performed PAM analyses after leaves were incubated for 2, 4, or 6 h with PQ. Generally, the analysis of photosynthesis parameters allows the estimation of plant susceptibility or tolerance to stress.

The  $F_v/F_m$  ratio reflects the maximum photochemical quantum yield of PSII; values between 0.75 and 0.85 in non-stressed plants are considered normal (Silva et al., 2014). This is an important coefficient that indicates how efficiently the light reactions proceed. A rapid decline in  $F_v/F_m$  ratio in the plants that were not pre-treated with melatonin was observed not only in our leaf disks, as shown here, but also in tomato and maize plants subjected to drought stress (Liu et al., 2015; Ye et al., 2016), in detached apple leaves during dark- and drought-induced senescence (Wang et al., 2012, 2013b) and in cucumber plants under salinity stress (Wang et al., 2016); in tissues treated with exogenous melatonin this ratio always remains high. It may be that melatonin improves the rate of electron transport and the efficiency of photochemical conversion (Liu et al., 2015). If the decline in  $F_v/F_m$  is due to photoinhibition of PSII units or to other causes it is a less reliable index. Since  $F_v/F_m$  is a relatively inert and measured in a dark-adapted state, stress-induced changes are detected rather late. The  $F_v/F_0$  ratio is generally more sensitive, since it expresses the efficiency of the water-splitting complex on the donor side of PSII, which is the most sensitive component in the photosynthetic electron transport chain. This ratio includes the same basic information but exhibits higher values and a higher dynamic range than the  $F_v/F_m$  (Lichtenthaler et al., 2005). This is consistent with the current findings which show that changes in  $F_v/F_0$  ratio are more rapid and greater than the  $F_v/F_m$  ratio, especially in leaves treated

with MEL50. Usually a reduction in  $F_v/F_0$  parameter results from photosynthetic electron transport impairment (Pereira et al., 2000); this is consistent with our results. This more sensitive ratio under stress conditions (PQ treatment) remained at the higher level in the plant variants pre-treated with melatonin (H-MEL50 and H-MEL200).

The vitality index (Rfd), which represents the chlorophyll fluorescence decline ratio is another parameter that complements the information regarding the PSII photochemistry. The omission of this factor often leads to false conclusions about the function of the photosynthetic apparatus. Under various stress conditions, Rfd value markedly decreases even if no changes in the  $F_v/F_m$  ratio is observed. This may indicate a decline in photosynthetic quantum conversion. In the present work, we observed that  $F_v/F_m$  ratio was very high in the melatonin treated variants, but Rfd of these leaves began to decline during PQ-mediated stress. At the end of the experiment, however, the Rfd index was still higher in H-MEL50 and H-MEL200 leaves than in the C and H leaves; this confirms the positive effect of melatonin on the function of the photosynthetic apparatus under PQ-induced oxidative stress. Additionally, the persistence of high  $\Phi_{PSII}$  in the melatonin-treated variants after 6 h of PQ-incubation is consistent with this conclusion. Similarly, a higher  $\Phi_{PSII}$  in melatonin pre-treated apple leaves subjected to drought stress were observed by Wang et al. (2013b) and Ye et al. (2016).

In the current study, the lower quantum efficiency of PSII ( $\Phi_{PSII}$ ), and photochemical quenching (qP) was accompanied by an elevation in NPQ, especially after first 2 and 4 h of PQ-treatment. Changes in qP, that indicates the amount of PSII reactive centers (RCs), that are open, are due to saturation of photosystem with light which results in closure of RCs. A significant decrease of qP in C and H leaves caused by 6 h of PQ treatment indicates that this herbicide reduced the number of open PSII centers; after melatonin pre-treatment, the both leaf variants (H-MEL50 and H-MEL200) had a greater capacity for photochemical quenching under oxidative stress.

Non-photochemical quenching reflects heat dissipation of excitation energy in the antenna system and serves as a photoprotective mechanism. It is related to proton concentrations inside thylakoids and induces the quenching of thermal energy through the xanthophylls cycle (Jahns and Holzwarth, 2012). This cycle transforms the excitation energy into heat and thereby prevents the formation of harmful ROS (Wang et al., 2010). After 2 h of PQ treatment in all studied variants, only a small qP decline occurred, whereas NPQ exhibited a significant increase. This demonstrates that qP, as a measure of the fraction of open PSII RCs, changes little under short term PQ stress; a significantly increased NPQ suggests that the prevailing processes causing the fluorescence quenching are of a photoprotective nature (Serôdio and Lavaud, 2011). During induced apple leaf senescence, a gradual increase in NPQ levels was also observed, but when the plants were treated with melatonin they dropped significantly. This was in accordance with the relatively high rate of photosynthesis and  $\Phi_{PSII}$  (Wang et al., 2013b). Similar effects were observed in cucumber leaves treated with melatonin and subjected to PEG stress (Zhang et al., 2013).

Fluorescence measurements, in tandem with photosynthetic pigment level analysis, leads to a more complete understanding of the energy dissipation pathways at the RCs of PSII and the pigment-light harvesting complexes. It is known that degradation of photosynthetic pigments is closely linked to the aging process. The precise function of melatonin in delaying leaf senescence in plants remains largely undefined, although remarkable advances have been made in understanding its role *in vivo*. The majority of previous studies were focused on positive effect of melatonin in senescence process induced by different stress factors. Studies on salt-stressed rice discovered that melatonin treatment significantly reduced chlorophyll degradation, delayed leaf senescence, and enhanced salt stress tolerance (Liang et al., 2015). Melatonin suppressed expression of four senescence-associated genes involved in chlorophyll degradation [stay-green (SGR), non-yellow coloring 1 (NYC1) and 3 (NYC3) genes, and red chlorophyll catabolite reductase 1 (RCCR1)], as well as four senescence-induced genes (OsNAP, Osh36, Osh69, and OsI57), which are widely used as age-dependent or dark-induced leaf senescence markers in rice (Liang et al., 2015). Soybean seed-coating with melatonin improved their tolerance to salt and drought stress, probably due to enhanced expression of genes related to photosynthesis, carbohydrate/fatty acid metabolism, and ascorbate biosynthesis (Wei et al., 2015). In these studies melatonin upregulated two subunits of photosystem I (PS I) (PsaK and PsaG), two elements (PsbO and PsbP) related to the oxygen-evolving complex of PS II (oxygen-evolving enhancer proteins), the ferredoxin gene PetF, and the VTC4 gene, encoding the L-galactose 1-P-phosphatase involved in ascorbate biosynthesis (Wei et al., 2015). Dark-induced senescence of apple leaves was also inhibited by exogenous melatonin application (Wang et al., 2012). Melatonin delayed the normal chlorophyll degradation and reduced the decline of  $F_v/F_m$  ratio. It also suppressed the transcript levels of a key chlorophyll degradation gene, pheide a oxygenase (PAO), and the senescence-associated gene 12 (SAG12). The slower process of protein degradation during apple leaf senescence was also noticed, probable as a result of melatonin-linked inhibition on the expression of autophagy-related genes (ATGs) (Wang et al., 2013a). Additionally, in melatonin-treated plants the expression of genes encoding the small subunit of Rubisco (RBCS), and proteins binding chlorophyll a/b (CAB), was inhibited much more slowly than in the control non-treated group. Moreover, in these plants the process of photosynthesis was more efficient, and concentrations of sucrose, starch and sorbitol were higher (Wang et al., 2013a). In cucumber plants under salinity stress, the addition of melatonin efficiently alleviated the decrease in the net photosynthetic rate, the maximum quantum efficiency of PSII, and the total chlorophyll content (Wang et al., 2016). Additionally, melatonin enhanced the activity of antioxidant enzymes (including SOD, POD, CAT, and APX) and concentrations of antioxidants (ascorbic acid and glutathione), reducing in this way the oxidative damage and increasing salinity tolerance of plants. Natural senescence of *Arabidopsis thaliana* leaves was also delayed by exogenous melatonin treatment and one positive regulator of natural leaf senescence -AtIAA17 [gene of auxin-resistant 3 (AXR3)/indole-3-acetic acid-inducible 17]

was significantly repressed (Shi et al., 2015). Transcriptome analysis of *A. thaliana* suggests that melatonin may play critical role(s) in plant defense systems (Weeda et al., 2014). Authors discovered that out of nearly 900 genes that were significantly up- or down-regulated by melatonin with at least twofold changes, almost 40% of them were related to plant stress defense, including many stress receptors, kinases and transcription factors, as well as downstream genes encoding end products that were directly used for stress defense. In the presented work melatonin supported chlorophyll preservation during 48 h of PQ-induced stress, and although the mechanism of its action is not fully explored, it could indicate that melatonin pre-treatment enhances synthesis and/or slows down decomposition of chlorophyll under oxidative stress.

Carotenoids also function as accessory pigments and act as photo-protectants and serve as safety valves releasing excess energy before it can damage plant cells (Shumskaya and Wurtzel, 2013). In this study, carotenoids in tandem with chlorophylls seem to aid melatonin pre-treated plants to counteract PQ-induced stress. However, when a very high dose of herbicide was applied, even increasing the carotenoid content was not sufficient to prevent injuries and the consequential death, as was shown in fodder radish plants (Silva et al., 2014).

Paraquat as a fast-acting herbicide directly penetrates leaves and within a few hours can lead to death of plants due to loss of water (Ekmekci and Terzioglu, 2005; Watts, 2011). To determine plant tissue hydration, RWC is usually measured (Zhang et al., 2013). In the present study, melatonin application to pea seeds resulted in rise of RWC in the leaves under PQ-induced oxidative stress. In contrast, C leaf disks revealed a significant loss of water after 48 h of PQ exposure, even when they were incubated in an aqueous medium. These results indirectly testify to the loss of membrane integrity in C plants and to the maintenance membrane integrity in H-MEL50 and MEL200 plants. This finding verifies that melatonin enrichment of seeds significantly inhibited oxidative damage to membrane under PQ-generated stress. A similar effect was observed under drought stress in *Malus* species pre-treated with melatonin (Li et al., 2015). According to these authors, water status can be improved by dual protective mechanisms (reduced contents of both ABA and H<sub>2</sub>O<sub>2</sub>) working synergistically to improve stomata functioning. A mitigating effect of melatonin on RWC was also found in wheat seedlings under cold stress (Turk et al., 2014), although in soybean plants subjected to drought stress, RWC was slightly lower than in the control plants (Wei et al., 2015).

Paraquat is a herbicide which blocks the process of photosynthesis under light conditions due to free radicals excess production, when O<sub>2</sub> is rapidly converted to O<sub>2</sub><sup>•-</sup> and subsequently to other ROS. NBT staining did not reveal O<sub>2</sub><sup>•-</sup> in the tissues of H-MEL50 and H-MEL200 leaves which confirms the radical scavenging activity of melatonin under PQ-stress. These observations are consistent with the results of Hasan et al. (2015), whose histochemical study showed reduced levels of ROS in tomato leaves pre-treated with melatonin under Cd stress. They also demonstrated a significant ameliorative effect of melatonin on  $F_v/F_m$  ratio and net photosynthesis rate during Cd stress.

The analysis of SOD activity, the key enzyme regulating the  $O_2^-$  status in leaves, confirmed the positive indirect effect of melatonin on this radical elimination. Usually SOD is activated in the face of oxidative stress, which can be clearly seen here in all experimental variants at 24 and 48 h of PQ treatment. However, the highest, statistically important increase in SOD activity was noted in H-MEL50 and H-MEL200 variants. Thus, once again our group confirmed that melatonin has positive influence on SOD activity, as it was shown previously in cucumber seeds (Bałabusta et al., 2016). On the other hand, in our experiment no spectacular changes in the activity of the other AOX enzymes, such as: CAT, POX, APX, etc., which regulate the  $H_2O_2$  status, were observed under the influence of melatonin.

The current findings suggest that seed priming fluid supplemented with melatonin enhances oxidative stress tolerance in growing plants. This beneficial effect was reflected by reduced accumulation of  $O_2^-$  in leaf tissues, probably due to increased

SOD activity; preservation of photosynthetic pigments; improved functioning of the photosynthetic apparatus and higher water content in the tissues during PQ-mediated stress. Although detailed molecular mechanisms of melatonin action still need elucidation, these findings provide evidence for the physiological role of this molecule applied during seed priming and serve as a platform for its possible applications in agricultural or related areas of research.

## AUTHOR CONTRIBUTIONS

KS: Work conception, all experiments concerning pea seeds and seedlings realization, data acquisition and analysis, drafting of the manuscript. RR: Research consultation/discussion, manuscript revision: language and editorial corrections. MP: Methodological consultant, statistical calculations, data analysis, and interpretation, manuscript revision.

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# Melatonin Improves the Photosynthetic Apparatus in Pea Leaves Stressed by Paraquat via Chlorophyll Breakdown Regulation and Its Accelerated *de novo* Synthesis

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The positive effect of melatonin on the function of the photosynthetic apparatus is known, but little is known about the specific mechanisms of melatonin's action in plants. The influence of melatonin on chlorophyll metabolism of 24-day-old *Pisum sativum* L. seedlings during paraquat (PQ)-induced oxidative stress was investigated in this study. Seeds were hydro-primed with water (H), 50 and 200 µM melatonin/water solutions (H-MEL50, H-MEL200), while non-primed seeds were used as controls (C). Increases in chlorophyllase activity (key enzyme in chlorophyll degradation) and 5-aminolevulinic acid contents (the first compound in the porphyrin synthesis pathway) were observed in H-MEL50 and H-MEL200 leaf disks. This suggests that melatonin may accelerate damaged chlorophyll breakdown and its *de novo* synthesis during the first hours of PQ treatment. Elevated level of pheophytin in control leaf disks following 24 h of PQ incubation probably was associated with an enhanced rate of chlorophyll degradation through formation of pheophytin as a chlorophyll derivative. This validates the hypothesis that chlorophyllide, considered for many years, as a first intermediate of chlorophyll breakdown is not. This is indicated by the almost unchanged chlorophyll to chlorophyllide ratio after 24 h of PQ treatment. However, prolonged effects of PQ-induced stress (48 h) revealed extensive discolouration of control and water-treated leaf disks, while melatonin treatment alleviated PQ-induced photobleaching. Also the ratio of chlorophyll to chlorophyllide and porphyrin contents were significantly higher in plants treated with melatonin, which may indicate that this indoleamine both retards chlorophyll breakdown and stimulates its *de novo* synthesis during extended stress. We concluded that melatonin added into the seeds enhances the ability of pea seedlings to accelerate chlorophyll breakdown and its *de novo* synthesis before stress appeared and for several hours after, while during prolonged PQ incubation melatonin delays chlorophyll degradation.

**Keywords:** ALA, chlorophyll, chlorophyllase, chlorophyllide, hydropriming, melatonin, oxidative stress, pheophytin

## INTRODUCTION

Melatonin (MEL) as a small amphiphilic particle can easily cross cell membranes and penetrate all cellular compartments. Due to the ability to scavenge reactive oxygen and nitrogen species (ROS and RNS), and its high diffusibility, it is considered as a powerful antioxidant with a very large range of actions (Manchester et al., 2015; Reiter et al., 2016). ROS and RNS generated under harmful environmental conditions inflict damage to critical macromolecules, such as lipids, proteins, DNA, etc.; thus MEL plays an important role in the defense system of plants (Reiter et al., 2015). A potent antioxidant function of MEL is associated not only with its ability to directly scavenge ROS and RNS or to stimulate antioxidant enzymes activities, but also due to its ability to generate a highly effective free radical scavenging cascade of its metabolites, including cyclic 3-hydroxymelatonin (C-3HOM), N<sup>1</sup>-acetyl-N<sup>2</sup>-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) (Galano et al., 2013). Because of this cascade, MEL is considered a more effective universal antioxidant than many other substances with known and proven antioxidant properties, such as vitamin C, vitamin E, glutathione, and NADH (Tan et al., 2015). The antioxidant cascade provided by MEL and its metabolites makes this indoleamine, even at low concentrations, highly effective in protecting organisms against oxidative stress (Galano et al., 2013).

Although a multitude of physiological, biochemical, and molecular processes determine plant growth and development, photosynthesis is a key factor. It is the most fundamental and intricate physiological process in all green plants which is severely affected by environmental stresses (Ashraf and Harris, 2013). Degradation and loss of chlorophyll (Chl) is one of the biochemical markers of aging but a positive effect of MEL on this process has been reported. According to Wang et al. (2012, 2013a,b), MEL delays drought- and dark-induced leaf senescence in apple due to maintaining the photosystem II (PSII) function under stress and reducing the typical decline in Chl content. Moreover, in leaves treated with MEL the expression of a key genes for the Chl degradation - pheide *a* oxygenase (PAO), senescence-associated gene 12 (SAG12) and sugar-sensing and senescence associated hexokinase-1 gene (HXK1)- are also inhibited. In *Arabidopsis thaliana* leaves treated with paraquat (PQ), MEL significantly decreased the expression of chlorophyllase (CLH1) gene that is a light-regulated enzyme participating in Chl degradation (Weeda et al., 2014). In these

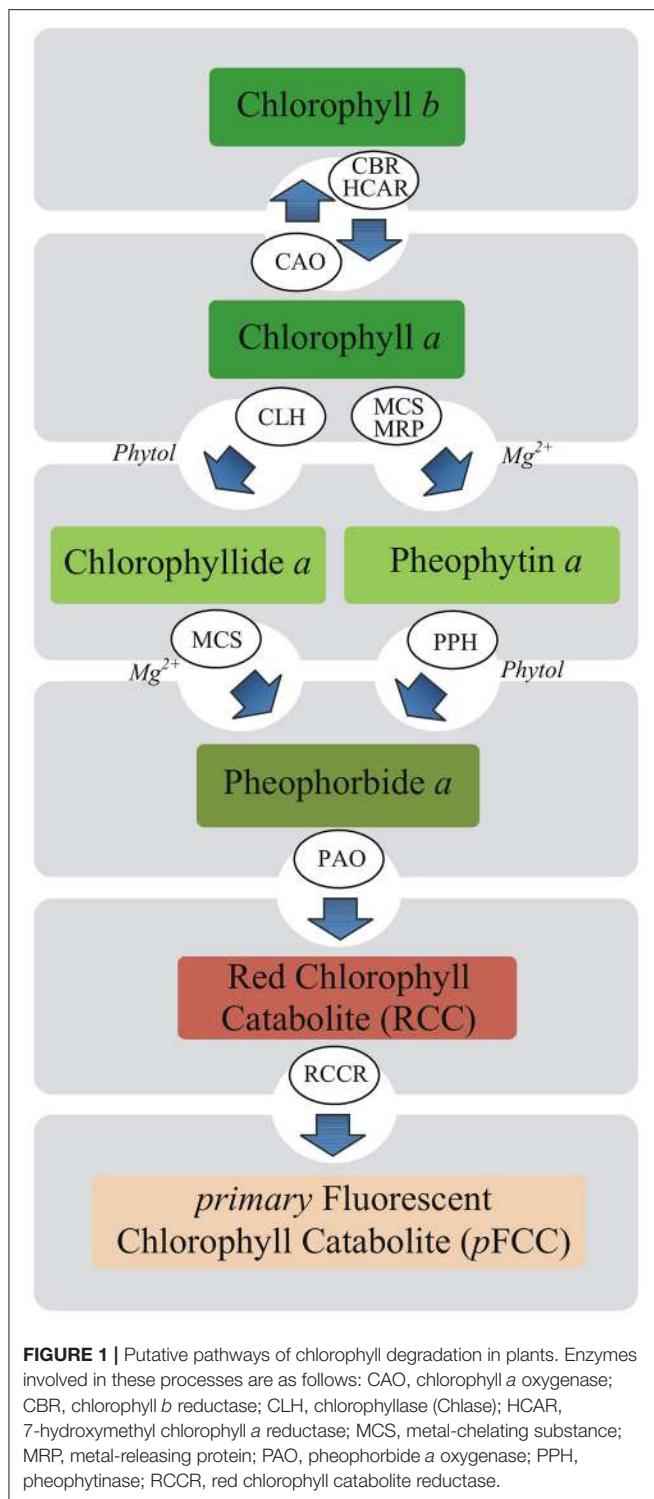
leaves exogenous MEL clearly slowed Chl loss, as also seen in barley leaves during senescence (Arnao and Hernandez-Ruiz, 2009) and in rice leaves under salt stress (Liang et al., 2015). Hence, due to its ability to support photosynthesis, MEL plays an important role in senescence delay.

The mechanism whereby MEL influences photosynthesis remains unknown. In plants under stressful conditions, maintaining a balance between the breakdown of damaged Chl and its *de novo* synthesis is important. Rapid degradation of free Chl or its colored derivatives is necessary to avoid cell damage due to their photodynamic action (Takamiya et al., 2000). Chl breakdown is also a direct precondition for the remobilization of proteins, chloroplast lipids, and metals (Christ and Hörtensteiner, 2014). Therefore, this process takes place not only under stressful conditions but also during various phases of the life cycle of plants. Chl breakdown is a multistep enzymatic process and Chl degradation into phytol, and the first decomposition product of the porphyrin ring, takes place in four successive steps catalyzed by chlorophyllase (Chlase), Mg-dechelatase, PAO and red chlorophyll catabolite reductase (Harpaz-Saad et al., 2007; **Figure 1**). Chlase catalyzes the reaction of Chl hydrolysis to chlorophyllide (Chlide) and phytol and it is the first enzyme of Chl catabolism during fruit ripening and leaf senescence (Takamiya et al., 2000; Hörtensteiner, 2006). However, in some plants Chlase was found not to be essential for dephytylation. Recent studies have shown that the chlorophyll degradation may progress through the formation of pheophytin *a* (Pheo *a*) as a chlorophyll derivative (Schelbert et al., 2009; Dissanayake et al., 2012). Based on these findings, it is assumed that Chl degradation pathway is not necessarily *via* Chlide *a* but also involves Pheo *a*.

A marked decline in content of important photosynthetic pigments also occurs due to stress-induced impairment in pigment biosynthetic pathways (Ashraf and Harris, 2013; **Figure 2**). Down-regulation of Chl biosynthesis may be attributed to reduced accumulation of 5-aminolevulinic acid (ALA) being the precursor of all tetrapyrroles and protochlorophyllide (Santos, 2004), as well as to decreased activities of Chl biosynthetic pathway enzymes including: ALA dehydratase (4), hydroxymethylbilane synthase (5), coproporphyrinogen III oxidase (8), protoporphyrinogen IX oxidase (9), Mg-protoporphyrin IX methyltransferase (12) and protochlorophyllide oxidoreductase (15) (Turan and Tripathy, 2015; **Figure 2**). Since the regulation of Chl biosynthesis and degradation under stress conditions is important, it is essential to check what role in these processes may relate to MEL.

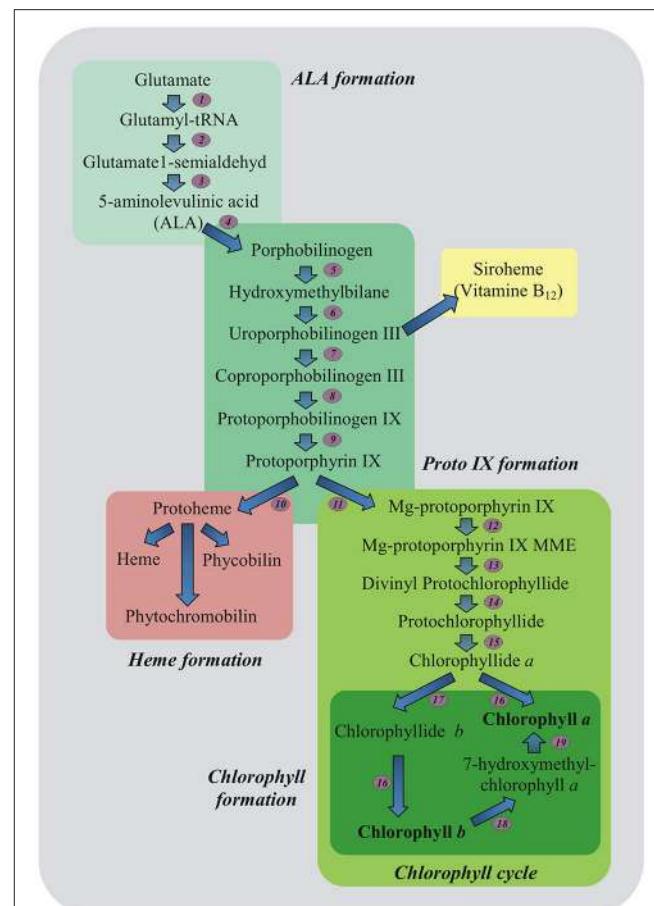
Much of the research on the role of MEL in plants is based on watering or spraying them with MEL solution. In present work, the method of MEL application was seed priming, which is a well-known technique for seed quality improvement (Jisha et al., 2013). To determine the role of MEL in Chl protection, pea leaves were subjected to oxidative stress. PQ was used as a stress agent; this is a fast-acting, non-selective herbicide that works in the chloroplast by diverting electrons from photosystem I (PSI). By accepting a single electron, PQ generates a stable reduced cation radical rapidly reacting with dioxygen to generate superoxide ( $O_2^{\bullet-}$ ) (Hawkes, 2014). This in turn produces a variety

**Abbreviations:** AFMK, N<sup>1</sup>-acetyl-N<sup>2</sup>-formyl-5-methoxykynuramine; ALA, 5-aminolevulinic acid; ALA-S, D-aminolevulinate synthase; AMK, N-acetyl-5-methoxykynuramine; C-3HOM, cyclic 3-hydroxymelatonin; Cars, carotenoids; Chl, chlorophyll; Chlase, chlorophyllase; Chlide, chlorophyllide; CLH1, chlorophyllase gene; H, plants grown from hydro-primed seeds; MEL200, plants grown from seeds hydroprimed with 200  $\mu$ M melatonin; MEL50, plants grown from seeds hydroprimed with 50  $\mu$ M melatonin; HXK1, sugar-sensing and senescence associated hexokinase-1 gene; MEL, melatonin; MgProto, Mg-protoporphyrin; PAO, pheide *a* oxygenase; Pchlde, protochlorophyllide; Pheo, pheophytin; PPH, pheophytin-specific phytol hydrolase (pheophytinase); PQ, paraquat; Proto, protoporphyrin; PS I, photosystem I; PS II, photosystem II; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAG12, senescence-associated gene 12.



**FIGURE 1 |** Putative pathways of chlorophyll degradation in plants. Enzymes involved in these processes are as follows: CAO, chlorophyll a oxygenase; CBR, chlorophyll b reductase; CLH, chlorophyllase (Chlase); HCAR, 7-hydroxymethyl chlorophyll a reductase; MCS, metal-chelating substance; MRP, metal-releasing protein; PAO, pheophorbide a oxygenase; PPH, pheophytinase; RCCR, red chlorophyll catabolite reductase.

of ROS, including hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $\bullet OH$ ); the latter is an especially highly reactive agent that induces protein and pigment degradation, lipid peroxidation and nucleic acid damage. By affecting key components of plant cell metabolism these changes eventually cause cell death (Lascano et al., 2012).



**FIGURE 2 |** Plant tetrapyrrole biosynthesis pathway. 5-aminolevulinic acid (ALA, the universal precursor of all tetrapyrroles) is synthesized from glutamate. ALA is then converted into protoporphyrin IX before the pathway branches into haem and chlorophyll biosynthesis. The ratio of chlorophyll a and b is balanced in the chlorophyll cycle. Biosynthetic enzymes: 1-GluRS, glutamyl-tRNA synthetase; 2-GluTR, glutamyl-tRNA reductase; 3-GSAT, glutamate-1-semialdehyde aminotransferase; 4-ALAD, ALA dehydratase; 5-HB synthase, hydroxymethylbilane synthase; 6-Uro III synthase, uroporphyrinogen III synthase; 7-Uro III decarboxylase, uroporphyrinogen III decarboxylase; 8-Copro III oxidase, coproporphyrinogen III oxidase; 9-PPOX, protoporphyrinogen IX oxidase; 10-FeCh, Fe chelatase; 11-Mg chelatase; 12-MTF, Mg protoporphyrin IX methyltransferase; 13-cyclase; 14-DVR, divinyl protopchlorophyllide reductase; 15-POR, light-dependent NADPH-protopchlorophyllide oxidoreductase; 16-Chl synthase, chlorophyll synthase; 17-CAO, chlorophyll a oxygenase; 18-CBR, chlorophyll b reductase; 19-HCAR, 7-hydroxymethyl chlorophyll a reductase; MME, monomethylester (Czarnecki and Grimm, 2012, modified).

The aim of this study was to verify whether the positive effect of MEL in PQ-treated pea leaves is associated only with limited degradation of Chl, or perhaps with increased *de novo* synthesis as well.

## MATERIALS AND METHODS

### Plant Material

*Pisum sativum* L. seeds obtained from TONSEED (Torun, Poland) were hydro-primed with water (H) or with 50 and

200  $\mu\text{M}$  melatonin/water solutions (H-MEL50, H-MEL200), while non-primed seeds were used as a controls (C) (Posmyk et al., 2008; Szafranska et al., 2016). Melatonin concentrations were chosen as optimal, on the basis of previous experiments (Szafranska et al., 2014). A fungicide, Thiuram (Organica-Sarzyna, Poland), was used for seed sterilization, and thereafter they were placed in plastic boxes with cottonwool moistened with distilled water and germinated at 25°C for 3 days. The young seedlings were transplanted into plastic pots filled with sterilized universal soil and perlite (3:1); they were grown for 21 days in a breeding room at constant temperature of 25°C and a fixed photoperiod (16 h light/8 h dark) with intensity of light: 770–840  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

## Stress Conditions

Paraquat (PQ, methyl viologen, 1,1'-dimethyl 4,4'-bipyridinium dichloride), purchased in Sigma-Aldrich (Germany), was used to generate oxidative stress in tissues. Leaf disks 18 mm in diameter were cut from 24-day-old pea plants. One part of the leaf disks was immediately used for analysis ( $T_0$ ), and the other was transferred into Petri dishes filled with 15 mL of 75  $\mu\text{M}$  PQ. They were incubated in a growth chamber (Orbis DATA LOG) with constant light (350 – 370  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), at 25°C for specified time for different analysis: 1, 2, 4, and 6 h for Chlase activity and ALA content, and 24, 48 h for Chl, carotenoid (Car), Pheo, Chlide, and porphyrin contents. After the times indicated, leaf disks were removed from the Petri dishes, dried on a paper towels, frozen in liquid nitrogen and stored at –80°C for further analysis. Only Chlase activity and ALA content were analyzed in the fresh tissues.

## Chlorophyllase (Chlase) Activity

Enzyme extraction and assay were performed according to Gupta et al. (2012). The leaf disks (0.8 g) were homogenized in 5 mL of cold extraction buffer (100 mM potassium phosphate (pH 7.0), 50 mM potassium chloride, 5 mM sodium diethyldithiocarbamate (DECA), 1 mM diethylenetriaminepentaacetic acid (DPTA), 0.24% (v/v) Triton X-100) and 0.2 g pre-swollen insoluble polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15,000  $\times g$  for 15 min and the supernatant was used as a crude enzyme. All these steps were performed on ice.

Chlase activity was determined in 2 mL reaction mixture containing 80 mM phosphate buffer (pH 7.0), 0.24% (v/v) Triton X-100, 0.22  $\mu\text{mol}$  Chl *a* dissolved in acetone and 1 mL of crude enzyme. The reaction was run for 15 min in dark at 40°C. Aliquots of 0.5 ml were added to 5 mL of phase separation mixture containing acetone/hexane/10 mM KOH (2:3:0.2, v/v) and mixed by vortexing for 30–40 s. The resulting emulsion was centrifuged at 12,000  $\times g$  for 5 min for quick phase separation. Chlide *a* was estimated in acetone phase at 667 nm (spectrophotometer Hitachi U-2001) and its amount was calculated using extinction coefficient 74.9  $\text{mM}^{-1} \text{cm}^{-1}$ . One unit of activity was defined as the amount of enzyme hydrolyzing 1 nmol Chl *a* per s at 40°C. Protein in the enzyme extract was assayed according to Bradford (1976) using bovine serum albumin as a standard. Chlase activity was measured in 9 replicates ( $n = 9$ ).

## 5-Aminolevulinic Acid (ALA) Content

5-Aminolevulinic acid (ALA) contents were estimated according to modified method of Turan and Tripathy (2015). The leaf disks (0.2 g) were collected at different time points (0, 1, 2, 4, and 6 h of PQ treatment). They were homogenized in a cooled mortar and pestle in 2 mL of 1 M sodium acetate buffer (pH 4.6). Next homogenate was centrifuged at 10,000  $\times g$  for 10 min and obtained supernatant was used for analysis. The reaction mixture consisting of: 4 mL of distilled water, 1 mL of supernatant, and 250  $\mu\text{L}$  of acetyl-acetone was mixed and boiled for 10 min. Next it was cooled and an equal volume of Ehrlrich reagent (2 g DMBA, 30 mL of glacial acetic acid, 16 mL of 70% perchloric acid filled up to 50 mL with glacial acetic acid) was added and mixed. After 20 min of incubation absorbance at 555 nm was measured (spectrophotometer Hitachi U-2001). ALA content was evaluated from the calibration curve based on known concentrations of ALA. All assays were performed in 9 replicates ( $n = 9$ ).

## Chlorophyll *a+b*: Carotenoids Ratio

Chls *a+b* and Cars were quantified spectrophotometrically according to Lichtenthaler and Buschmann (2001). The leaf disks (0.025 g) were homogenized in a cooled mortar and pestle in 5 mL of 80% acetone with MgCO<sub>3</sub> and filtered. In the obtained supernatant absorbance at 470, 646, and 663 nm (spectrophotometer Hitachi U-2001) was measured. Chls *a*, *b*, *a+b* and Car concentrations were calculated with the following formulas:

$$\text{Chl } a [\mu\text{g mL}^{-1}] = 12.25 \times A_{663} - 2.79 \times A_{646},$$

$$\text{Chl } b [\mu\text{g mL}^{-1}] = 21.50 \times A_{646} - 5.10 \times A_{663},$$

$$\text{Chls } a + b [\mu\text{g mL}^{-1}] = 7.15 \times A_{663} + 18.71 \times A_{646},$$

$$\text{Cars } [\mu\text{g mL}^{-1}] = (1,000 \times A_{470} - 1.82 \text{ Chl } a - 85.02 \text{ Chl } b)/198.$$

Pigment assays were performed in at least 5 replicates ( $n = 5$ ).

## Chlorophyll: Pheophytin Ratio

Chl and Pheo contents were assayed by the modified method of Radojević and Bashkin (2006). The leaf disks (0.025 g) were homogenized in a cooled mortar and pestle in 5 mL of 90% acetone with 1% of magnesium carbonate and filtered through filter paper. Aliquots of 3 mL of final extracts were placed into 1 cm cuvettes and absorbance at 664 and 750 nm was measured ( $A_{664a}$ ,  $A_{750a}$ ) (spectrophotometer Hitachi U-2001). Then 0.1 mL of 0.1 M HCl was added, mixed and absorbance at 665 and 750 nm was measured ( $A_{665b}$ ,  $A_{750b}$ ). Chl *a* and Pheo *a* concentrations were calculated from formulas:

$$\text{Chl } a [\text{mg g}^{-1} \text{ FW}] = [(A_{664a} - A_{750a}) - (A_{665b} - A_{750b})] \times V_1 / 26.7 \times d \times E \times V_2,$$

$$\text{Pheo } a [\text{mg g}^{-1} \text{ FW}] = [(1.7 \times (A_{665b} - A_{750b}) - (A_{664a} - A_{750a})) \times V_1] / 26.7 \times d \times E \times V_2.$$

The value 26.7 above in the equations is the absorbance correction and equal  $A \times K$  where  $A$  is the absorbance coefficient for chlorophyll *a* at 664 nm (11.0) and  $K$  is a ratio expressing the correction for acidification (2.43 = 1.7  $\times$  0.7) (APHA et al., 2005). If the ratio of  $A_{664}$  before acidification and  $A_{665}$  after

acidification ( $A_{664}/A_{665}$ ) is 1.7, the sample is considered to be free of Pheo *a*. Explanation of abbreviations: d-optical distance [cm]; E-equivalent of fresh weight [g FW mL<sup>-1</sup>]; V<sub>1</sub>-sample volume [L], V<sub>2</sub>-extract volume in a sample [mL]. These assays were performed in 5 replications ( $n = 5$ ).

## Chlorophyll: Chlorophyllide Ratio

Chl and Chlide contents were determined according to modified method of Harpaz-Saad et al. (2007). The leaf disks (0.2 g) were homogenized in a prechilled mortar and pestle in 6 mL of 100% acetone and filtered through filter paper. Aliquots of 2 mL from each sample were added to a centrifuge tubes containing 3 mL of hexane and 0.5 mL of 10 mM KOH, shortly vortexed and centrifuged for phase separation at 12,000  $\times g$  for 2 min. Chl levels were measured in the hexane phase, and Chlide levels were measured in the acetone phase spectrophotometrically (Hitachi U-2001) using formulas:

$$\text{Chl } a (\text{Chlide } a) [\mu\text{g mL}^{-1}] = 12.7 \times A_{663} - 2.69 \times A_{645} \text{ and,}$$

$$\text{Chl } b (\text{Chlide } b) [\mu\text{g mL}^{-1}] = 22.9 \times A_{645} - 4.68 \times A_{663}, \text{ as reported Arnon (1949).}$$

Experiment was performed in 3 replicates ( $n = 3$ ).

## Porphyrin Content

Porphyrin content was estimated by method described by Sarropoulou et al. (2012). The leaf disks (0.1 g) were placed in glass test tubes and 15 mL of 96% (v/v) ethanol was added. The samples were incubated in a water bath at temperature of 65°C until total discolouration of samples (3 h). Protoporphyrin (Proto), Mg-protoporphyrin (MgProto) and protochlorophyllide (Pchlde) concentrations were calculated using the following three equations:

$$\text{Proto } [\mu\text{g g}^{-1} \text{ FW}] = [(12.25 \times A_{665} - 2.55 \times A_{649}) \times \text{volume of supernatant (mL)/sample weight (g)}]/892,$$

$$\text{MgProto } [\mu\text{g g}^{-1} \text{ FW}] = [(20.31 \times A_{649} - 4.91 \times A_{665}) \times \text{volume of supernatant (mL)/sample weight (g)}]/906,$$

$$\text{Pchlde } [\mu\text{g g}^{-1} \text{ FW}] = [(196.25 \times A_{575} - 46.6 \times A_{590} - 58.68 \times A_{628}) + (61.81 \times A_{590} - 23.77 \times A_{575} - 3.55 \times A_{628}) + (42.59 \times A_{628} - 34.32 \times A_{575} - 7.25 \times A_{590})] \times \text{volume of supernatant (mL)/sample weight (g)} \times 1,000.$$

Porphyrin determinations were performed in at least 8 replicates ( $n = 8$ ).

## Statistical Analyses

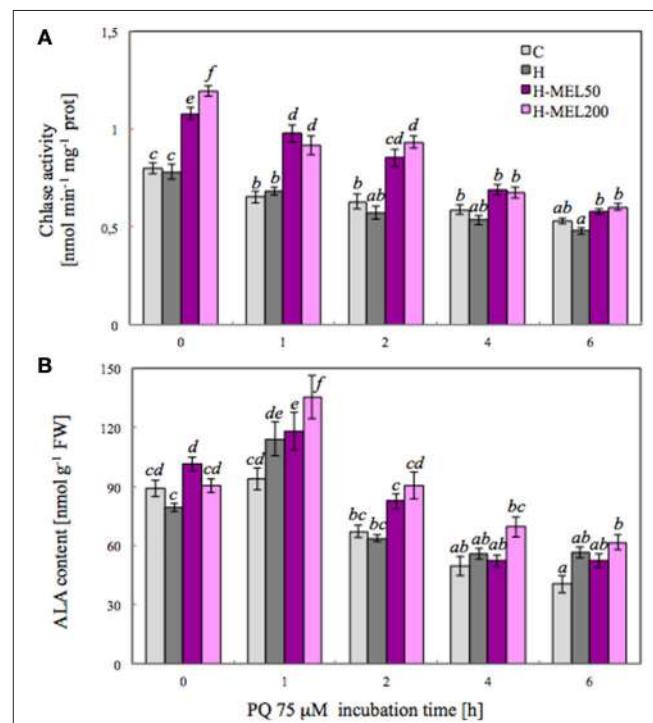
The results represent the average values  $\pm$  standard error ( $\pm$ SEM) of the mean. The data were analyzed using STATISTICA v.10.0\_MRI1\_PL [StatSoft] software. The two-way or one-way (the latter applies only to Figures 8A–C data) analysis of variance (ANOVA) and then the *post-hoc* Duncan multiple range test were performed to find the significant differences at least  $p < 0.001$  in each experiment.

## RESULTS

To investigate the influence of exogenous MEL on Chl metabolism under PQ-induced oxidative stress, the activity of Chlase, a key enzyme involved in the chlorophyll breakdown, was

analyzed. Activity of this enzyme measured after PQ treatment gradually decreased in all examined variants (Figure 3A). There were no differences between control and H leaf disks, but in variants with MEL Chlase activity was significantly higher. Before PQ treatment ( $T_0$ ), Chlase activity in MEL50 and MEL200 leaf disks was accounted for 135 and 150% of activity in control disks, respectively. This marked difference between non-treated (control and H) disks and those treated with MEL (H-MEL50, H-MEL200) variants remained at a similar level after 1 and 2 h of PQ treatment, but after 4 and 6 h the differences were reduced (Figure 3A).

5-Aminolevulinic acid (ALA) is a key precursor in the biosynthesis of porphyrin, such as Chl, so we analyzed its content. At  $T_0$  time point the highest ALA content was detected in H-MEL50 leaf disks (Figure 3B). PQ triggered a rapid, significant increase after 1 h of treatment and the maximum was noticed in H-MEL200 leaf disks. In H and H-MEL50 leaf disks, this level was slightly reduced, but in control variants it was lower by about 30%, compared with H-MEL200. The next 2, 4, and 6 h of PQ



**FIGURE 3 |** Chlorophyllase (Chlase) activity (A) and 5-aminolevulinic acid (ALA) content (B) in leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu\text{M}$  melatonin (H-MEL50) or 200  $\mu\text{M}$  melatonin (H-MEL200) seeds. Measurements were performed at  $T_0$  (before PQ treatment) and after 1, 2, 4, and 6 h of disks incubation in 75  $\mu\text{M}$  PQ solution. The results are expressed as mean values of about 9 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's *post-hoc* test were performed. The small letters on the graphs show statistical significance. ANOVA results: Chlase activity-Variant  $F_{(3; 157)} = 89.6$ ,  $p < 0.0001$ ; Time ( $T_0$ , 1, 2, 4, and 6 h)  $F_{(4; 157)} = 108$ ,  $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(12; 157)} = 5.7$ ,  $p < 0.0001$ ; ALA-Variant  $F_{(3; 181)} = 16.1$ ,  $p < 0.0001$ ; Time ( $T_0$ , 1, 2, 4, and 6 h)  $F_{(4; 181)} = 87.6$ ,  $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(12; 181)} = 2.4$ ,  $p < 0.001$ .

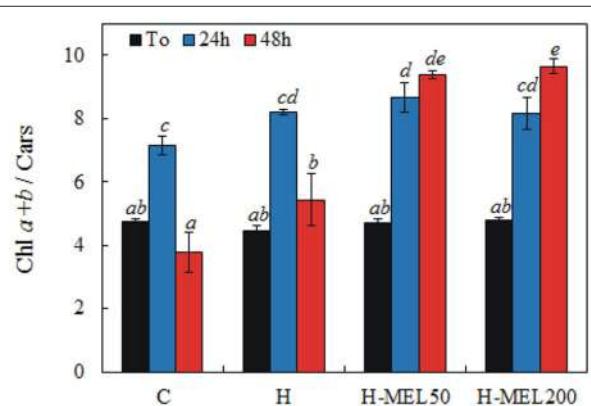
incubation resulted in gradual reduction of ALA content, but still the lowest content was detected in control disks and the highest in H-MEL200 leaf disks (**Figure 3B**).

Next, we performed analyses on leaf disks incubated in 75  $\mu\text{M}$  PQ for 24 or 48 h (**Figure 4**). After 24 h of PQ incubation there were no significant visual changes between the variants or in Chls *a+b* contents. 48 h PQ incubation triggered almost a 100% depigmentation of control and H leaf discs, while H-MEL50 and H-MEL200 preserved the green color and these disks contained only 50% less Chls *a+b* than those treated with PQ for 24 h (**Figure 4**).

The ratio of Chl *a+b* and Cars before PQ treatment was almost the same in all investigated variants. 24 h of incubation in a PQ solution caused a significant increase in this ratio, which reached the highest value in H-MEL50 leaf disks. After 48 h of PQ treatment, Chl *a+b* and Cars ratio markedly decreased in control and H leaf disks, while in the variants with MEL the ratio continued to grow (**Figure 5**).

Pheo *a*, a degradation product of Chl *a*, may interfere if it is present in the sample. The scans of Chl and Pheo and their quantitative analysis are presented in **Figure 6**. The level of Chl *a* slightly decreased after 24 h of PQ treatment in all investigated variants. Prolonged incubation time (48 h) caused a marked decline in Chl *a* content in control and H leaf disks, reaching only 3 and 4% of  $T_0$  values, respectively. In H-MEL50 and H-MEL200 leaf disks, this decline was not so drastic and Chl *a* levels achieved 33 and 23% of  $T_0$  values, respectively (**Figure 6A**). Before PQ treatment ( $T_0$ ) the lowest Pheo content

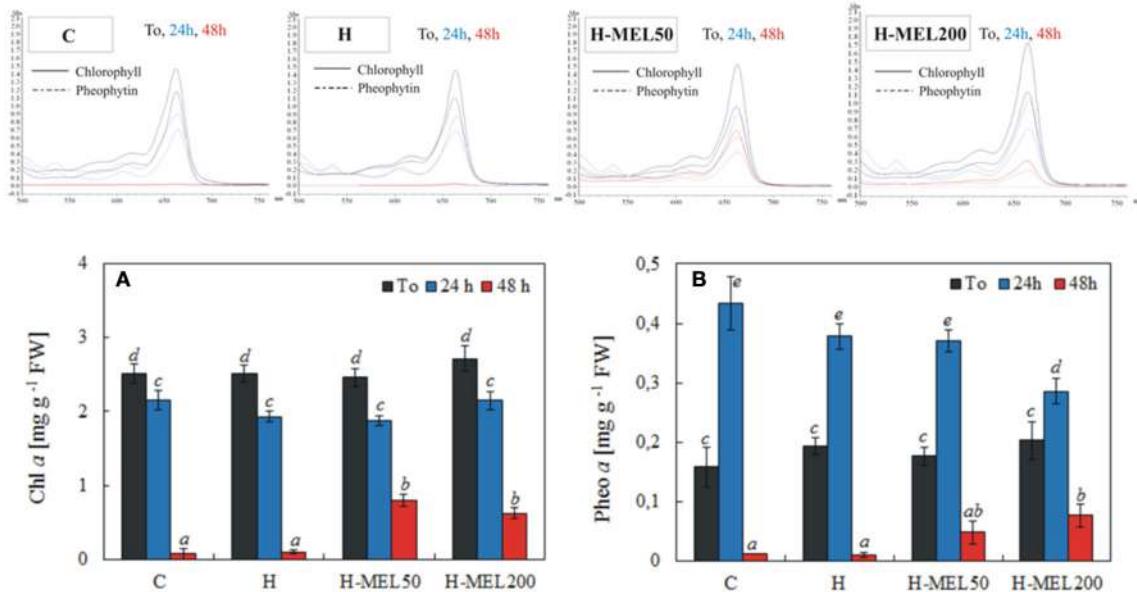
was determined in control leaf disks, but after 24 h it increased almost 3 fold (**Figure 6B**). Although 24 h PQ-induced stress caused a significant rise in Pheo levels in all studied variants, the lowest accumulation was noticed in H-MEL200 leaf disks.



**FIGURE 5 |** Ratio of chlorophyll *a+b* to carotenoids (Chl *a+b*/Cars) in leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu\text{M}$  (H-MEL50) or 200  $\mu\text{M}$  melatonin (H-MEL200) seeds. Measurements were performed at  $T_0$  (before PQ treatment) and after 24 and 48 h of disk incubation in 75  $\mu\text{M}$  PQ solution. The results are expressed as mean values of about 5 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's *post-hoc* test were performed. The small letters on the graphs show statistical significance. ANOVA results: Chl *a+b*/Cars-Variant  $F_{(3; 44)} = 26.6 p < 0.0001$ ; Time ( $T_0$ , 24, 48 h)  $F_{(2; 44)} = 85.9 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6; 44)} = 15.4 p < 0.0001$ .

Variant Time	C	H	H-MEL50	H-MEL200
24h				
Chl <i>a+b</i>	$2.817 \pm 0.21$ c	$2.645 \pm 0.40$ c	$2.665 \pm 0.25$ cd	$3.287 \pm 0.59$ d
48h				
Chl <i>a+b</i>	$0.100 \pm 0.06$ a	$0.095 \pm 0.04$ a	$1.347 \pm 0.42$ b	$1.465 \pm 0.67$ b

**FIGURE 4 |** Changes in green color intensity and Chl *a+b* contents in leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu\text{M}$  (H-MEL50) or 200  $\mu\text{M}$  melatonin (H-MEL200) seeds. Photographs were taken after 24 and 48 h of disks incubation in 75  $\mu\text{M}$  PQ solution. As concerns Chl *a+b* contents [ $\text{mg g}^{-1}_{\text{FW}}$ ] two-way ANOVA and Duncan's *post-hoc* test were performed. The small letters in the table show statistical significance. ANOVA results: Chl *a+b*-Variant  $F_{(3; 32)} = 14.4 p < 0.0001$ ; Time (24, 48 h)  $F_{(1; 32)} = 281.9 p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(3; 32)} = 6.8 p < 0.001$ .



**FIGURE 6 |** Chl a (**A**) and pheophytin a (Pheo a) (**B**) contents and scans of extracts from leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu$ M (H-MEL50) or 200  $\mu$ M melatonin (H-MEL200) seeds. Measurements were performed at T<sub>0</sub> (before PQ treatment) and after 24 and 48 h of disk incubation in 75  $\mu$ M PQ solution. The results are expressed as mean values of about 5 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's post-hoc test were performed. The small letters on the graphs show statistical significance. ANOVA results: Chl a-Variant  $F_{(3; 140)} = 5.3$   $p < 0.001$ ; Time (T<sub>0</sub>, 24, 48 h)  $F_{(2; 140)} = 443.8$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6; 140)} = 4.8$   $p < 0.0001$ ; Pheo a-Variant  $F_{(3; 41)} = 2.9$   $p < 0.1$ ; Time (T<sub>0</sub>, 24, 48 h)  $F_{(2; 41)} = 154.9$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6; 41)} = 3.8$   $p < 0.005$ .

With the rapid decline of Chl content after 48 h of PQ treatment (**Figure 6A**), in control and H leaf disks a trace amount of Pheo was also noticed, while in H-MEL50 and H-MEL200 leaf disks its content accounted for 27 and 47% of T<sub>0</sub> values, respectively (**Figure 6B**).

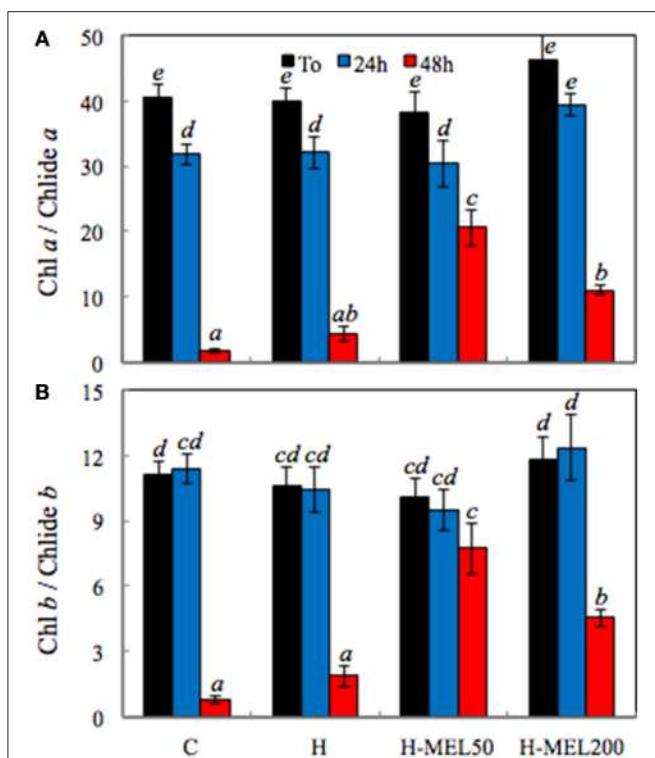
Chl decomposition is initiated with the separation of the phytol residue and the porphyrin ring of the Chl molecule, what results in Chlide formation (Kariola et al., 2005). The highest ratio of both Chl a/Chlide a and Chl b/Chlide b before PQ treatment was found in H-MEL200 leaf disks (**Figures 7A,B**). After 24 h of PQ-treatment Chl a / Chlide a ratio decreased about 20% in all investigated variants (**Figure 7A**), while Chl b/Chlide b remained at almost the same level (**Figure 7B**). Substantial changes appeared only after 48 h of PQ incubation, where both indicators sharply declined in control and H leaf disk, but in variants with MEL they remained high, especially after MEL50 treatment.

To assess the relationship between porphyrin biosynthesis and exogenous MEL applied to the pea seeds, the effects of PQ-induced oxidative stress on the regulation of porphyrin biosynthetic intermediates in leaves were also examined. 48 h of PQ treatment caused a drastic decline in protoporphyrin (Proto), Mg-protoporphyrin (MgProto), and protochlorophyllide (Pchlide) in control and H leaf disks, while in H-MEL50 and H-MEL200 their levels were about twice as high. The content of all investigated porphyrins was slightly higher in the leaves of H-MEL50 variant (**Figure 8**).

## DISCUSSION

Photosynthesis is a basic physiological process to maintain plant survival and stress factors disrupt this process. Research on the role of MEL in plants under different stress conditions has repeatedly demonstrated its positive impact on the functioning of the photosynthetic apparatus (Arnao and Hernandez-Ruiz, 2009; Sarropoulou et al., 2012; Wang et al., 2012, 2013a; Weeda et al., 2014; Liang et al., 2015; Szafranska et al., 2016), but precise mechanism of MEL action still needs elucidation.

The first enzyme in Chl degradation is Chlase, so checking its activity in all examined leaf disks was necessary. In *A. thaliana*, MEL treatment inhibited Chlase (CLH1) gene expression (Weeda et al., 2014), but in our experiment activity of this enzyme was significantly higher in variants treated with MEL (**Figure 3A**). Chlase gene expression and *in vitro* activity in plant tissues sometimes do not correlate well with de-greening during physiological fruit ripening and senescence (Minguez-Mosquera and Gallardo-Guerrero, 1996; Fang et al., 1998), implying that either Chlase is not the rate-limiting enzyme in Chl breakdown or that Chlase action is regulated post-translationally—what clarifies its latent function (Harpaz-Saad et al., 2007). This would explain some of the apparent discrepancies regarding the inhibition of CLH1 gene expression in *A. thaliana* treated with MEL, and the elevated activity of this enzyme in the analyzed pea leaves (H-MEL50 and H-MEL200). Because Chl levels are constantly modulated throughout the plant life and the catabolic pathway must be strictly controlled to avoid the

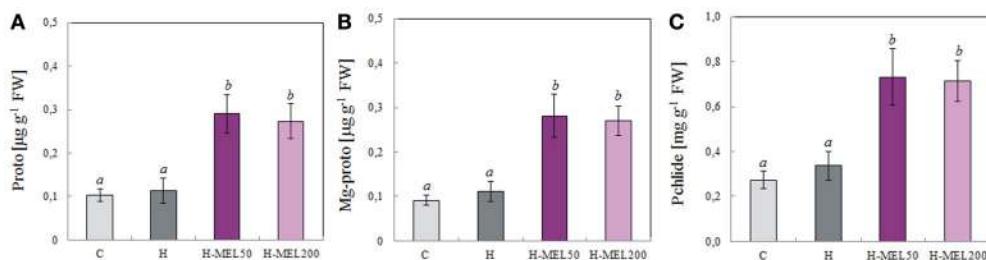


**FIGURE 7 |** Ratio of chlorophyll a/chlorophyllide a (Chl a/Chlide a) (**A**) and chlorophyll b/chlorophyllide b (Chl b/Chlide b) (**B**) in leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50  $\mu$ M (H-MEL50) or 200  $\mu$ M melatonin (H-MEL200) seeds. Measurements were performed at T<sub>0</sub> (before PQ treatment) and after 24 and 48 h of disk incubation in 75  $\mu$ M PQ solution. The results are expressed as mean values of about 3 measurements  $\pm$  SEM. Two-way ANOVA and Duncan's post-hoc test were performed. The small letters on the graphs show statistical significance. ANOVA results: Chl a/Chlide a-Variant  $F_{(3; 24)} = 7.0$   $p < 0.0001$ ; Time (T<sub>0</sub>, 24, 48 h)  $F_{(2; 24)} = 169.8$   $p < 0.001$ ; and interaction Variant  $\times$  Time  $F_{(6; 24)} = 4.6$   $p < 0.001$ ; Chl b/Chlide b-Variant  $F_{(3; 23)} = 3.9$   $p < 0.01$ ; Time (T<sub>0</sub>, 24, 48 h)  $F_{(2; 23)} = 84.6$   $p < 0.0001$ ; and interaction Variant  $\times$  Time  $F_{(6; 23)} = 5.3$   $p < 0.001$ .

excess of photodynamically active pigments, a post-translational regulatory mechanism is ergonomic and makes physiological sense; this allows the plant more flexibility (quick adaptation) in the response to environmental factors (Harpaz-Saad et al., 2007). It is also worth mentioning that transcriptome analysis of melatonin-treated *Arabidopsis* (Weeda et al., 2014) revealed upregulation of numerous genes implicated in the signaling of senescence-promotion but also stress-induced phytohormones, such as ethylene, abscisic, jasmonic, and salicylic acids (Jibran et al., 2013; Khan et al., 2014). This also might indicate that MEL provokes a stress response syndrome in plants as suggested by Kołodziejczyk et al. (2016a,b), although this issue still requires clarification. Since the breakdown of the damaged Chl and *de novo* synthesis are a natural processes occurring in plant cells, the next step was to examine if under oxidative stress MEL affects the contents of its precursor-ALA. Biosynthesis of ALA is the first step of Chl biosynthesis, and is therefore supposed to be a crucial control point in the regulation of Chl supply (Figure 2; Tanaka and Tanaka, 2006).

A wide variety of stresses cause a reduction in Chl biosynthesis due to downregulation of gene expression, decrease in protein abundance or post-translational modification of several enzymes participating in tetrapyrrole metabolism (Turan and Tripathy, 2015). In this work the question arose whether the positive effect of MEL on the function of the photosynthetic apparatus is associated rather with the accelerated synthesis of Chl and not with its delayed degradation. MEL applied into the pea seeds significantly enhanced ALA level immediately after PQ treatment of the leaves (Figure 3B), suggesting that MEL plays an important role in inducing *de novo* synthesis. In non-MEL treated plants (control and H) Chl biosynthesis pathway was downregulated by reduced ALA synthesis, as in sunflower leaves under salinity stress (Santos, 2004). Prolonged incubation (48 h) in 75  $\mu$ M PQ medium triggered extensive discolouration of control and H leaf disks, while MEL at 50 or 200  $\mu$ M alleviated PQ-induced photobleaching (Figure 4). This is consistent with the observations of Weeda et al. (2014), who noted that *A. thaliana* leaves treated with PQ in the absence of MEL became completely photobleached, while leaves treated with 1 mM MEL remained green.

Photosynthetic pigments including Chl *a*, Chl *b* and Cars are necessary for the photosynthetic process. The content of foliar pigments varies depending on species. Cars are thought to protect Chl from the absorption of excess energy which might otherwise photobleach the Chl. Variation in leaf pigments (Chls and Cars) and their proportion may be due to internal factors and environmental conditions (Sumanta et al., 2014). Their concentrations are closely related and the ratio of Chls *a+b* to total Cars is the greenness index of plants. This ratio generally decreases in senescing, unhealthy plants and rises in vigorously growing plants. In our experiment, before PQ-treatment (T<sub>0</sub>) there were no differences in Chls *a+b* and Car ratios between leaf variants, but after 24 h this ratio increased, reaching the maximum in H-MEL50 leaf disks (Figure 5). A significant decrease of this ratio in control and H leaf disks was observed after 48 h, while in variants with MEL (H-MEL50 or H-MEL200) it was still growing. This was associated with drastic decline of Chl and Car levels in control and H leaf disks and their much higher levels in variants due to MEL treatment (data not shown); this may confirm a positive influence of MEL on Chl and Car biosynthesis. Although in the present study a MEL effect on Car levels was shown, MEL did not appear to influence its concentration in shoot tip explants of the cherry rootstock PHL-C treated with different MEL solutions (Sarropoulou et al., 2012). These authors stated that there were no links between MEL concentrations and the Car biosynthesis pathway. However, a relationship between MEL concentrations and porphyrin contents was found, as in the present work. In H-MEL50 or H-MEL200 leaf disks, contents of all three investigated porphyrins (Proto, MgProto and Pchlde) were significantly higher than in control and H variants (Figure 8); this confirms that Chl biosynthesis is stimulated by MEL. This effect may depend on the concentration of MEL since as shown Rodriguez et al. (1994) and Kalka et al. (1997) in mammals, a high MEL concentration (1 mM) reduced the porphyrin content and decreased D-aminolevulinate synthase



**FIGURE 8 |** Contents of porphyrins: protoporphyrin (Proto) (A), Mg-protoporphyrin (Mg-proto) (B) and protochlorophyllide (Pchlde) (C) in leaf disks cut from 24-day-old pea plants grown from the control (C), hydroprimed (H), and hydroprimed with an aqueous solution of 50 μM (H-MEL50) or 200 μM melatonin (H-MEL200) seeds. Measurements were performed after 48 h of disk incubation in 75 μM PQ solution. The results are expressed as mean values of about 8 measurements ± SEM. One-way ANOVA and Duncan's post-hoc test were performed. The small letters on the graphs show statistical significance. ANOVA results: Proto-Variant  $F_{(3; 22)} = 10.2$   $p < 0.001$ ; Mg-proto-Variant  $F_{(3; 22)} = 9.7$   $p < 0.001$ ; Philide-Variant  $F_{(3; 23)} = 9.8$   $p < 0.001$ .

(ALA-S) activity. Because still little or no information for this aspect of the plant kingdom exist, further investigation are required to ascertain the precise role of MEL in Chl biosynthesis pathway.

Chlide has been long considered to be an intermediate of both Chl biosynthesis and breakdown (Takamiya et al., 2000; Hörtensteiner, 2006). However, recent studies have revealed that Chlide seems not to be a true intermediate of Chl catabolism and Chl degradation may process through formation of Pheo *a* as a Chl derivative (Schenk et al., 2007; Schelbert et al., 2009; Hu et al., 2013; **Figure 1**). According to Guyer et al. (2014) genes encoding highly conserved pheophytin-specific phytol hydrolase (PPH) are prevalent in higher plants, allowing the suggestion that Pheo-specific dephytylation by PPHs may be a common attribute of Chl decomposition during leaf senescence. Therefore, we checked the level of Pheo in all studied pea leaf disks following PQ treatment. After 24 h of PQ incubation, the Pheo level significantly increased in control leaf disks, while in H-MEL200 only a slight rise was observed (**Figure 6B**). These results may confirm a positive role of MEL in delaying Chl breakdown by formation of Pheo during PQ-induced oxidative stress. Supposing that in the first hours of PQ treatment Chlase activity increased in H-MEL50 or H-MEL200 leaf disks (**Figure 3A**), while after 48 h Chl/Chlide

ratio was much higher in those variants (**Figure 7**), this may indicate that the long-term Chl breakdown involving Chlase was also limited by MEL.

The current findings suggest that under PQ-induced oxidative stress, MEL preserves the Chl content in pea leaves by delaying Chl breakdown and simultaneously accelerating its *de novo* synthesis. The current results are consistent with some other published findings. It is known that the activation of genes, or transcript measurements, do not always correlate with physiological effects because of post-transcriptional and post-translational regulation. In this work we documented such effects on the biochemical and physiological levels of several constituents.

## AUTHOR CONTRIBUTIONS

KS-work conception, all experiments concerning pea seeds and seedlings realization, data acquisition and analysis, drafting of the manuscript. RR-research consultation/discussion, manuscript revision: language and editorial corrections. MP-methodological consultant, statistical calculations, data analysis and interpretation, manuscript revision.

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# Melatonin Regulates Root Meristem by Repressing Auxin Synthesis and Polar Auxin Transport in *Arabidopsis*

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Melatonin (*N*-acetyl-5-methoxytryptamine) plays important roles in regulating both biotic and abiotic stress tolerance, biological rhythms, plant growth and development. Sharing the same substrate (tryptophan) for the biosynthesis, melatonin and auxin also have similar effects in plant development. However, the specific function of melatonin in modulating plant root growth and the relationship between melatonin and auxin as well as underlying mechanisms are still unclear. In this study, we found high concentration of melatonin remarkably inhibited root growth in *Arabidopsis* by reducing root meristem size. Further studies showed that melatonin negatively regulated auxin biosynthesis, the expression of PINFORMED (PIN) proteins as well as auxin response in *Arabidopsis*. Moreover, the root growth of the triple mutant *pin1pin3pin7* was more tolerant than that of wild-type in response to melatonin treatment, suggesting the essential role of PIN1/3/7 in melatonin-mediated root growth. Combination treatment of melatonin and 5-Triiodobenzoic acid (TIBA) did not enhance melatonin-mediated reduction of root meristem size, indicating that polar auxin transport (PAT) may be necessary for the regulation of root meristem size by melatonin treatment. Taken together, this study indicates that melatonin regulates root growth in *Arabidopsis*, through auxin synthesis and polar auxin transport, at least partially.

**Keywords:** melatonin, auxin, root meristem, auxin synthesis, polar auxin transport, *Arabidopsis*

## INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine), a widely distributed endogenous bio-molecule in mammals, was first discovered in the bovine pineal gland in 1958 (Lerner et al., 1958). Melatonin regulates many important physiological processes in mammals, including sleep, body temperature regulation, circadian rhythms, mood, immune processes, etc. (Jan et al., 2009; Hardeland et al., 2012; Carrillo-Vico et al., 2013; Reiter et al., 2014).

The existence and discovery of melatonin in other species, especially in higher plants, indicates its extensive functions (Poeggeler et al., 1991; Dubbels et al., 1995; Hattori et al., 1995). Numerous studies have shown that melatonin is widely involved in regulating both the biotic and abiotic stress tolerance, biological rhythms, plant growth and development (seed germination, root architecture, shoot development, plant flowering, fruit ripening, and yield;

**Abbreviations:** IAA, indolyl-3-acetic acid; TIBA, 5-Triiodobenzoic acid.

Kang et al., 2010; Okazaki et al., 2010; Wang et al., 2012; Byeon et al., 2013, 2014b; Yin et al., 2013; Zhang et al., 2013, 2014; Zhao et al., 2013; Byeon and Back, 2014a; Shi and Chan, 2014; Weeda et al., 2014; Shi et al., 2015a,b,c,d,e, 2016).

In higher plants, melatonin is synthesized from tryptophan as substrate by four key enzymes [tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), serotonin N-acetyltransferase (SNAT), and N-acetylserotonin O-methyltransferase (ASMT)] (Kang et al., 2007a,b, 2011, 2013; Okazaki et al., 2009; Fujiwara et al., 2010; Byeon and Back, 2014b, 2015; Zuo et al., 2014; Arnao and Hernández-Ruiz, 2015). Recent studies found that N-acetylserotonin can also be synthesized via caffeic acid O-methyltransferase (COMT) in *Arabidopsis thaliana*, except ASMT (Byeon et al., 2014a; Lee et al., 2014). Interestingly, there are two different pathways for the synthesis of auxin (IAA) in plants, one is tryptophan-independent, and the other is tryptophan-dependent, sharing the same substrate with melatonin (Wang et al., 2015). Tryptophan-dependent pathway is dependent on precursor tryptophan, through tryptamine (TAM), indole-3-pyruvic acid (IPyA) and indole-3-acetaldoxime (IAOx) pathways (Benjamins and Scheres, 2008; Strader and Bartel, 2008; Chandler, 2009).

Melatonin also showed some similar functions as auxin in the higher plants, in addition to sharing the same substrate for their biosynthesis. Previous studies suggested that melatonin acts as a growth-stimulating molecule in *lupin* tissues and some monocots, including *canary grass*, *wheat*, *barely*, and *oat*; its IAA-like activity is 10–55% of that of auxin (Hernández-Ruiz et al., 2004, 2005). However, there are also reports indicating that melatonin regulates *Arabidopsis* root growth independent of auxin signaling (Pelagio-Flores et al., 2012; Koyama et al., 2013).

To date, the function of melatonin in regulating root growth and the underlying mechanisms are still unclear in higher plants. Moreover, the relationship between melatonin and auxin remains unknown. In the present work, different concentrations of melatonin were used to treat the wild-type (WT, Col-0) *Arabidopsis*. The results showed that melatonin significantly suppressed root growth by reducing the size of root meristem. Additionally, comprehensive analyses of auxin synthesis, PIN (PINFORMED) proteins and auxin response marker line of *Arabidopsis* (DR5 promoter marker line) suggested that melatonin might regulate the root growth through auxin signaling, at least partially.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

The ecotype Columbia-0 (Col-0) of *Arabidopsis thaliana* was used as the WT plant in this study. Other plant materials are listed as follows: *pin1* (Salk\_047613), *pin3* (CS9364), and *pin7* (CS9367) from the Arabidopsis Biological Resource Centre (ABRC), *pin3pin7* (Benková et al., 2003), *pin1pin3pin7* (Blilou et al., 2005), *PIN1::PIN1-GFP* (Benková et al., 2003), *PIN3::PIN3-GFP* (Blilou et al., 2005), *PIN7::PIN7-GFP* (Blilou et al., 2005), *DR5::GUS* (Sabatini et al., 1999), and *DR5::GFP*

(Frml et al., 2003). *Arabidopsis* seeds were sterilized with 70% (v/v) ethanol for 1 min and 1% sodium hypochlorite for 16 min. After washing with distilled water for 3–5 times, seeds were sown on 1/2 Murashige and Skoog medium with 1% sucrose and 0.8% agar. The plates with seeds were placed at 4°C for 2 days to break dormancy prior to transfer to a culture room under dark/light cycles of 8 h/16 h at the temperature of 22°C. Plates were maintained in a vertical position for 3 days in the culture room before various treatments.

### Drug Treatments and Root Assay

As described above, 3-day-old *Arabidopsis* seedlings were transferred to 1/2 MS medium containing different concentrations of chemical components [melatonin, 2,3,5-Triiodobenzoic acid (TIBA) and IAA] for treatments. To limit the effect of solvent, the same volume of solvent including ethanol was used as a control. Thereafter, photos were taken by a digital camera, and the length of primary roots was determined by software Image J<sup>1</sup> (version 1.47 g). For the root meristem size measurement, every five roots were cut and transferred onto a glass slide, and were treated with clearing solution (30 mL ddH<sub>2</sub>O, 53.3 g chloral hydrate and 10 mL glycerol) for 5 min before microscope analyses. Images were captured by Leica DM6000 differential interference contrast microscope. The zone between two white arrows in images include both the apical meristem and the transition zone (Verbelen et al., 2006; Baluska et al., 2010). Root meristem size was quantified as previously described (Liu et al., 2015; Yuan and Huang, 2016). Results presented are average values of more than 30 seedlings per treatment from three independent experiments. Statistical analysis was conducted in KaleidaGraph 4.03.

### GUS Staining

GUS staining was performed as described previously (Jefferson et al., 1987). Samples were cleared as mentioned above before observation. For DR5::GUS marker line, 2 h was enough for staining. The images of GUS staining were taken with a Leica DM6000 microscope equipped with Leica Application Suite software.

### Confocal Microscopy

*Arabidopsis* seedlings expressing *PIN1::PIN1-GFP*, *PIN3::PIN3-GFP*, *PIN7::PIN7-GFP*, and *DR5::GFP* were observed under Leica TCS SP8 laser scanning confocal microscope, with excitation of 488 nm argon laser, and emission wavelength range of 505–525 nm. The intensity of argon laser in laser configuration and intensity of laser line 488 in acquire section was set to 20 and 15%, respectively. Pinhole was set to 1.8 Airy units for all materials. To compare the fluorescent intensity of GFP in roots between control and samples treated, all optical sections were acquired under identical conditions. Quantification of the fluorescent intensity was performed by measuring the mean gray value using Image J software. Since PIN1 is mainly localized in the provascularure in roots, and that both PIN3 and PIN7 are expressed in provascularure and root cap. For the

<sup>1</sup><http://rsbweb.nih.gov/ij/>

*PIN1::PIN1-GFP* roots, only the signals in the provascularure were quantified, while for *PIN3::PIN3-GFP* and *PIN7::PIN7-GFP* roots, signals both in the provascularure and root cap were quantified separately. And we did not distinguish signals at the plasma membrane from signals in the cytoplasm.

### Quantitative Real-Time PCR Analysis

Three-day-old *Arabidopsis* seedlings were transferred to new 1/2 MS medium and medium containing 600  $\mu$ M melatonin. After another 7 days' treatment, root tips (sections from root meristem to the tip) of control and samples were dissected under a dissecting microscope, and total RNA was isolated from root tips treated with TRIzol reagent (Invitrogen). For cDNA synthesis, 2  $\mu$ g of total RNA from different samples was used for reverse transcription with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's recommendations. To analyze the transcript levels of auxin-related genes in control and treated roots, quantitative real-time PCR was performed with Applied Biosystems 7500 (Foster City, CA, USA) in a 20- $\mu$ L reaction volume containing SYBR Green dye (SYBR Premix Ex Taq, TAKARA). *PDF2* (protein phosphatase 2, AT1G13320) was chosen as an internal control (Czechowski et al., 2005). Relative expression levels were estimated using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). All the primers used in the study are listed in Supplementary Table S1.

### Determination of Endogenous Melatonin and IAA Levels in *Arabidopsis* Roots

For the endogenous melatonin and IAA measurements, 3-days-old seedlings were transferred to new control 1/2 MS medium and medium containing IAA or melatonin for another 8 days. Endogenous melatonin in *Arabidopsis* root tips was extracted as previously described (Pape and Lüning, 2006). The levels of melatonin and IAA in root extracts were quantified using melatonin enzyme linked immunosorbent assay kit (EK-DSM; Buhlmann Laboratories AG, Schonenbuch, Switzerland)

and Plant IAA enzyme-linked immunosorbent assay (EIASA) Kit (Jianglai Biotechnology, Shanghai, China), respectively, according to the instructions.

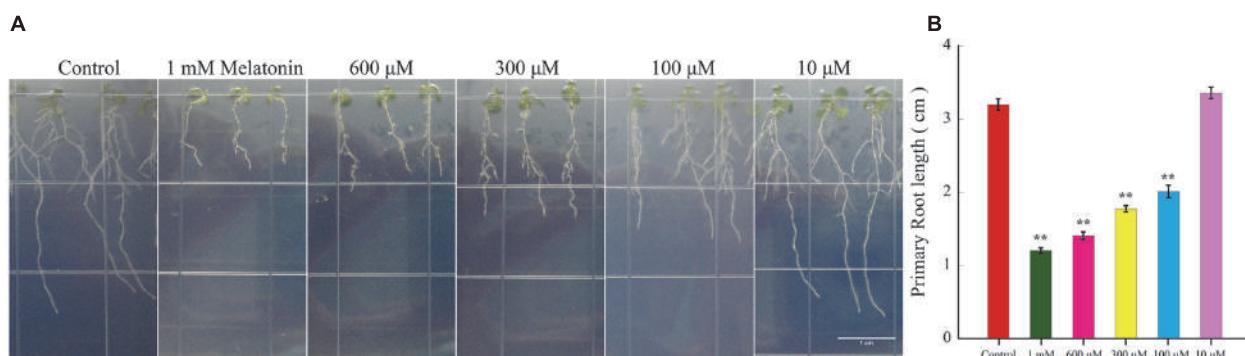
## RESULTS

### Melatonin Suppressed the Primary Root Growth in *Arabidopsis* by Reduced Root Meristem

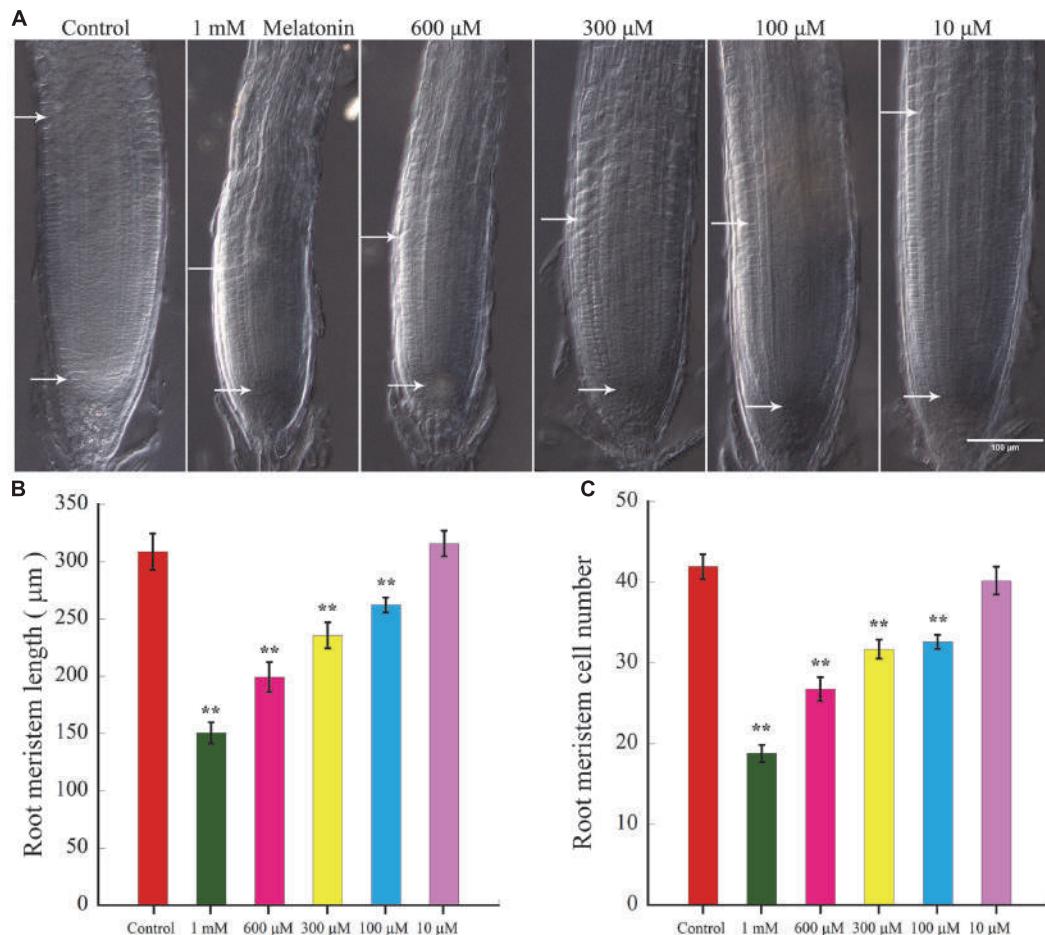
To investigate the effects of melatonin on primary root growth in *Arabidopsis*, 3-day-old WT (Col-0) seedlings were transferred to new 1/2 MS media with different concentrations of melatonin for another 6 days (Figure 1). By measuring and statistical analysis, we found that the primary root length was decreased after melatonin treatment, and the inhibition effect of melatonin exhibited dose-dependent (Figure 1). The result suggested that high concentration of melatonin could suppress the primary root growth in *Arabidopsis*.

In plants, postembryonic root growth is sustained by the root apical meristem (RAM), which consists of stem cell-like cells that are the precursors of all differentiated cell types (Laux and Mayer, 1998; Dinneny and Benfey, 2008). So we wonder if melatonin to reduce the primary root length by affecting root meristem. To test our hypothesis, 3-day-old seedlings were kept growing under different concentrations of melatonin for another 6 days, and we found that both the number of meristem cells and the length of meristem are significantly reduced with increased concentration of melatonin (Figures 2B,C), indicating that melatonin-mediated repression of primary root growth might be due to reduced root meristem.

Our data showed that 10  $\mu$ M melatonin had no effect on primary root growth (Figures 1 and 2), and our previous work suggested that 10–50  $\mu$ M melatonin had litter effect on endogenous melatonin content (Shi et al., 2015c). Therefore, we chose high concentration of melatonin for further analyses in this study.



**FIGURE 1 | Effect of high concentration of Melatonin on the length of *Arabidopsis* primary root.** After 3 days' culture, seedlings were transferred to 1/2 MS medium with indicated concentrations of melatonin for other 6 days, and the primary root length were measured with software Image J. **(A)** Digital images of wild-type *Arabidopsis* seedlings treated with different concentrations of melatonin. Scale bar = 1 cm. **(B)** Primary root length of *Arabidopsis* growing on medium with control and increasing concentration of melatonin. More than 25 seedlings per experiment from three independent experiments were measured for statistic analysis. Values represent mean  $\pm$  SD, \*\* $P$  < 0.01 by a Student's *t*-test.



**FIGURE 2 | Effects of Melatonin on the size of the *Arabidopsis* root meristem.** Three-day-old seedlings were kept growing under different concentrations of melatonin for other 6 days. More than 25 seedlings per experiment from three independent experiments were cleared for imaging. Values represent mean  $\pm$  SD, \*\* $P < 0.01$  by a Student's *t*-test. **(A)** Images of *Arabidopsis* root tips treated with melatonin for 6 days were present. Scale bar = 100  $\mu\text{m}$ . **(B)** Comparison of root meristem length of the *Arabidopsis* seedlings treated with different levels of melatonin. **(C)** Quantification of root meristem cell number of seedlings treated with different levels of melatonin. More than 25 seedlings per experiment from three independent experiments were measured for statistic analysis. Values represent mean  $\pm$  SD, \*\* $P < 0.01$  by a Student's *t*-test.

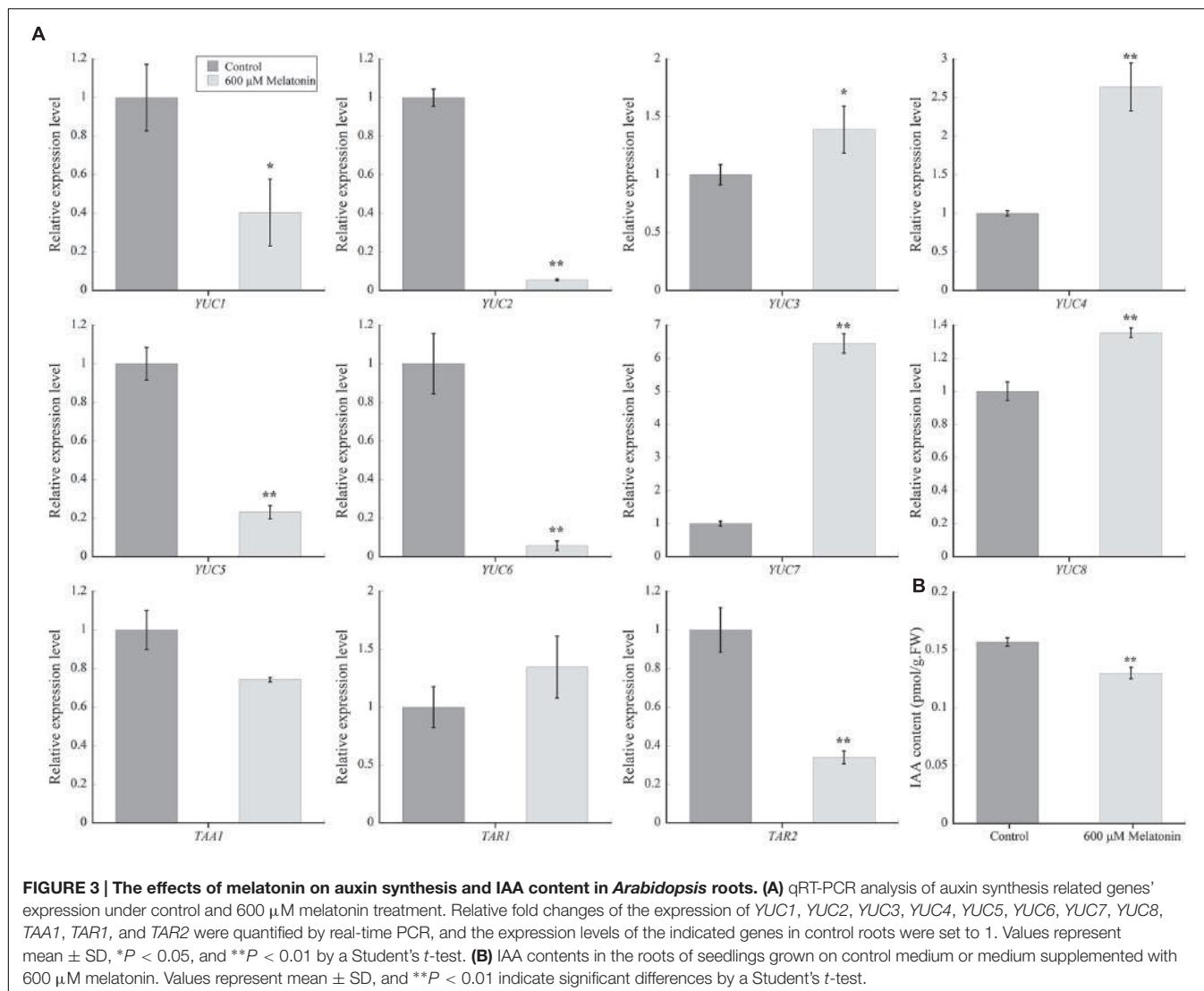
## Melatonin Negatively Regulated Auxin Biosynthesis

Since defective auxin response can cause reduced meristem phenotype, the first question we wanted to known was whether melatonin actually affects auxin biosynthesis. YUCCA (YUC) proteins, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family, TAA RELATED 1 and 2 play important roles in auxin (IAA) biosynthesis during plant development (Cheng et al., 2006; Yamamoto et al., 2007; Stepanova et al., 2008; Tao et al., 2008), so we investigated the effects of melatonin on the transcript levels of these genes (Figure 3A). Quantitative real-time PCR showed that the transcript levels of *YUC1*, *YUC2*, *YUC5*, *YUC6*, and *TAR2* significantly decreased after 600  $\mu\text{M}$  melatonin treatment. The transcript levels of *YUC3*, *YUC4*, *YUC7*, and *YUC8* increased after treatment, while the relative expression levels of *YUC3* and *YUC8* in roots with treatment were less than 1.5-fold in control.

Indeed, the endogenous IAA content in melatonin-treated roots was significantly lower than that of control (Figure 3B).

## Melatonin Repressed Polar Auxin Transport in *Arabidopsis*

PINFORMED proteins, especially PIN1, PIN3, and PIN7, directly participate in auxin transport in plant roots (Friml et al., 2003; Blilou et al., 2005), and play important roles in controlling the size of root meristem. In order to examine whether melatonin treatment affected the levels of these proteins in root, we measured the relative fluorescence intensity of GFP using the marker lines *PIN1::PIN1-GFP*, *PIN3::PIN3-GFP*, and *PIN7::PIN7-GFP*. As shown in Figure 4, signals of PIN1 in provascular, and signals of PIN3 and PIN7 deriving from both root cap and provascular region were decreased significantly after 600  $\mu\text{M}$  treatment. The quantitative real-time PCR demonstrated that the relative transcript levels of *PIN1*, *PIN3*, and *PIN7* were also



**FIGURE 3 |** The effects of melatonin on auxin synthesis and IAA content in *Arabidopsis* roots. **(A)** qRT-PCR analysis of auxin synthesis related genes' expression under control and 600  $\mu$ M melatonin treatment. Relative fold changes of the expression of *YUC1*, *YUC2*, *YUC3*, *YUC4*, *YUC5*, *YUC6*, *YUC7*, *YUC8*, *TAA1*, *TAR1*, and *TAR2* were quantified by real-time PCR, and the expression levels of the indicated genes in control roots were set to 1. Values represent mean  $\pm$  SD, \* $P$  < 0.05, and \*\* $P$  < 0.01 by a Student's *t*-test. **(B)** IAA contents in the roots of seedlings grown on control medium or medium supplemented with 600  $\mu$ M melatonin. Values represent mean  $\pm$  SD, and \*\* $P$  < 0.01 indicate significant differences by a Student's *t*-test.

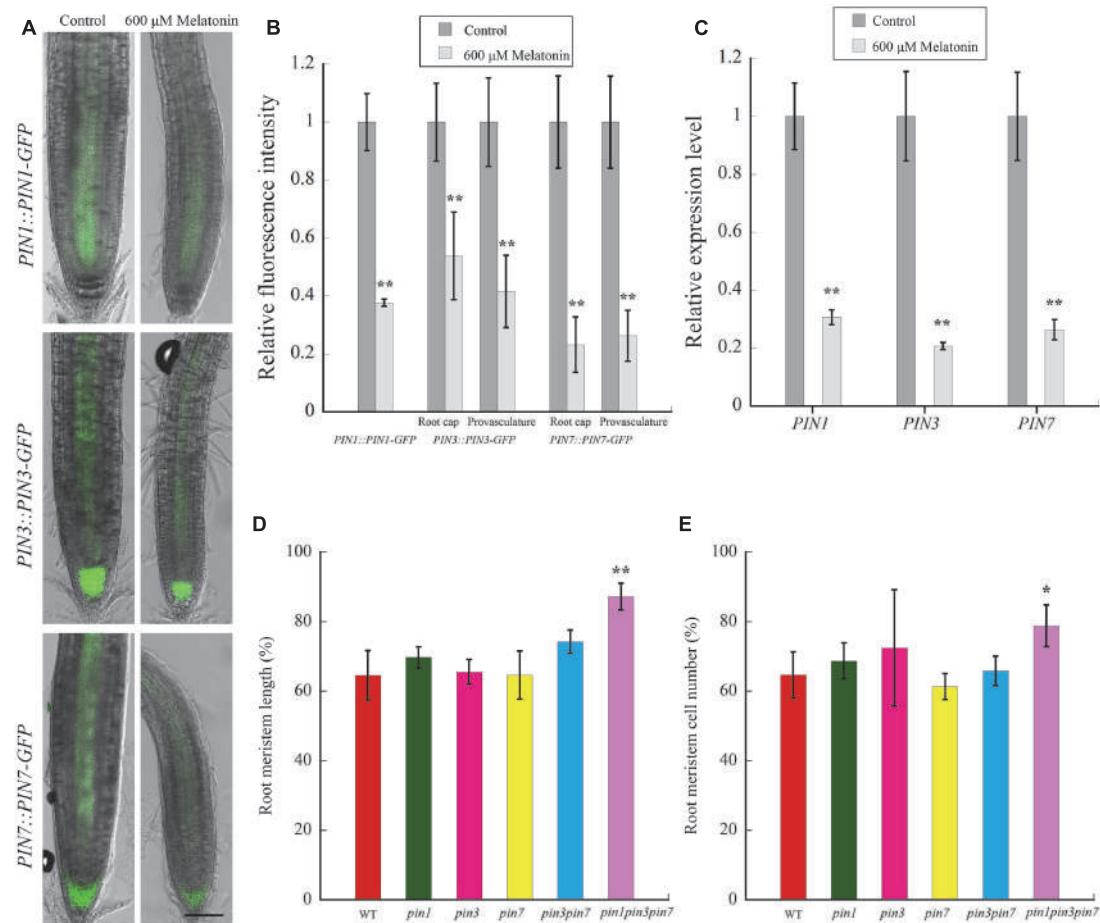
significantly reduced in melatonin-treated roots, suggesting that melatonin treatment repressed the expression of *PIN1*, *PIN3* and *PIN7*. To further confirm the involvement of PINs in melatonin-mediated root development, the meristem length and cell number of the roots of PIN mutants (including *pin1*, *pin3*, *pin7*, *pin3pin7*, and *pin1pin3pin7*) were also determined. Notably, we found that the root growth of triple mutant *pin1pin3pin7* was more tolerant to melatonin treatment than WT and other mutants (Figures 4D,E), indicating the essential role of PIN1/3/7 in melatonin-mediated repression of root meristem.

## Melatonin Repressed Auxin Response in *Arabidopsis* in an IAA Similar Manner

Endogenous auxin level is directly related to development of plant roots. To further dissect the underlying mechanism of melatonin during *Arabidopsis* root growth and the relationship between melatonin and auxin, exogenous IAA and auxin transport inhibitor (TIBA) were used to treat the seedlings.

Firstly, 3-day-old seedlings were treated with melatonin containing medium in the presence or absence of 2  $\mu$ M TIBA for 8 days. The root meristem length and cell number were measured. The results showed that both melatonin and TIBA treated roots reduced root meristem length and cell number, but the inhibition caused by melatonin was not intensified by the presence of TIBA (Figures 5A,B), indicating that polar auxin transport (PAT) might be necessary for the regulation of root meristem size by melatonin treatment.

Since melatonin-treated roots had lower IAA levels (Figure 3B), we wonder whether the root meristem size was reduced by melatonin through decreasing IAA content. If so, the reduction of root meristem size could be complemented, or partially complemented by exogenous IAA at a certain concentration. Two concentrations of IAA (0.5 and 100 nM) were used to treat the seedlings. We found that treatment of 0.5 nM IAA for 8 days alone did not affect the root meristem size, but 0.5 nM IAA and 600  $\mu$ M melatonin co-treatment significantly reduced the root meristem size, similar

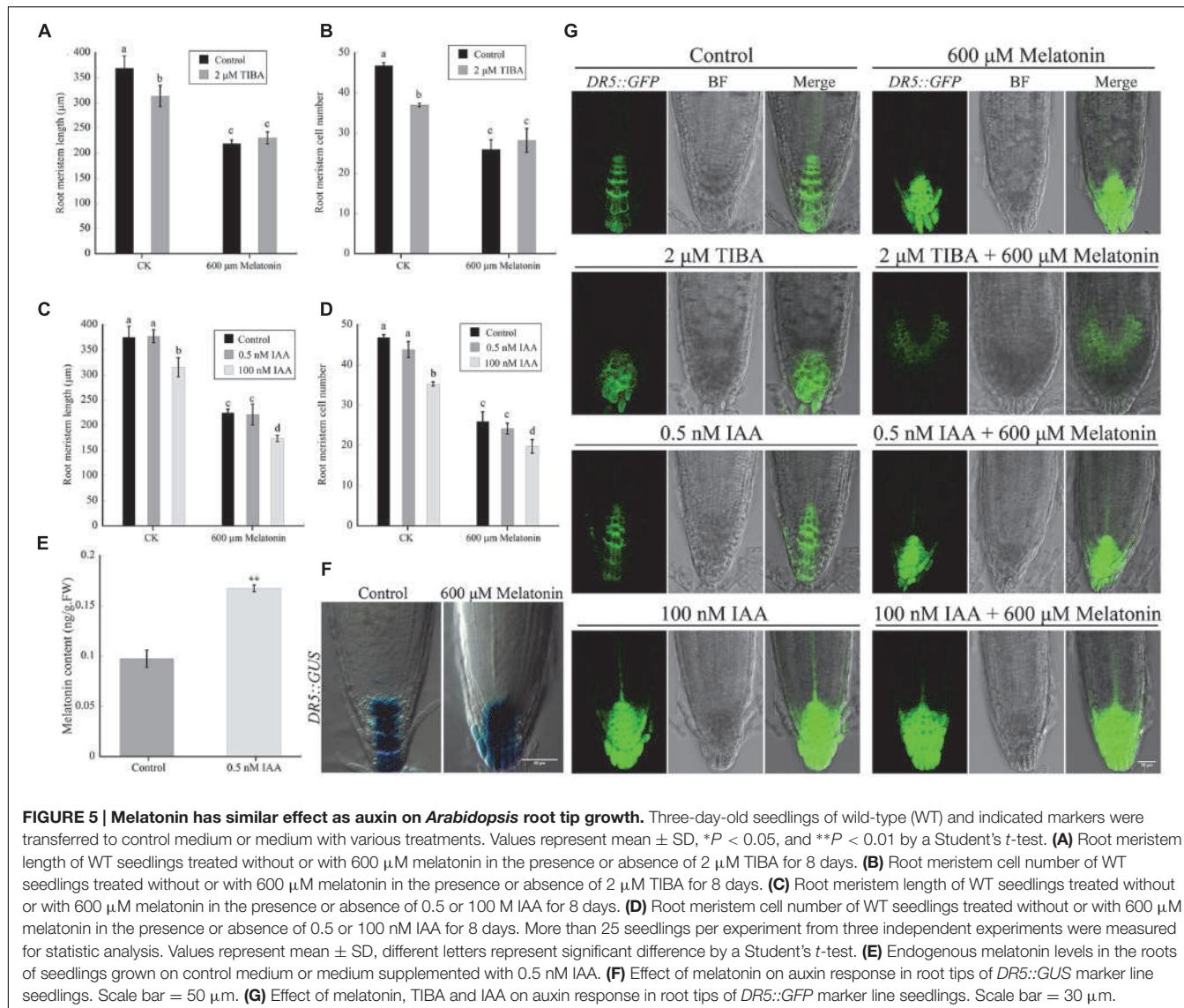


**FIGURE 4 |** The expression of auxin efflux components PINFORMEDS (PINs) were down-regulated after treatment of 600  $\mu$ M melatonin. **(A)** Effects of Melatonin on the abundance of PIN proteins in *Arabidopsis*. Three-day-old seedlings harboring indicated markers were transferred to control medium or medium with 600  $\mu$ M melatonin for 6 days. Scale bar = 100  $\mu$ m. **(B)** Comparison of GFP fluorescence intensity in plants treated without or with melatonin as in **(A)** by Image J. The fluorescence intensity levels of the control roots were set to 1. Values represent mean  $\pm$  SD, \*\* $P$  < 0.01 by a Student's *t*-test. **(C)** qRT-PCR analysis of *PIN1*, *PIN3*, and *PIN7* in *Arabidopsis* roots under 600  $\mu$ M melatonin treatment. The expression levels of the indicated genes in control roots were set to 1. Values represent mean  $\pm$  SD, \*\* $P$  < 0.01 by a Student's *t*-test. **(D)** Quantification of relative root meristem length of various mutants treated without or with 600  $\mu$ M melatonin for 6 days. Values represent mean  $\pm$  SD, \*\* $P$  < 0.01 by a Student's *t*-test. **(E)** Quantification of relative root meristem cell number of various mutants treated without or with 600  $\mu$ M melatonin for 6 days. Values represent mean  $\pm$  SD, \* $P$  < 0.05, and \*\* $P$  < 0.01 by a Student's *t*-test.

to melatonin-treated roots alone (**Figures 5C,D**). Application of 100 nM IAA caused reduced root meristem size, as previously reported (Rahman et al., 2007; Strader et al., 2011), but the inhibition of 100 nM was less severe than that of 600  $\mu$ M melatonin. To our surprise, 100 nM IAA and 600  $\mu$ M melatonin co-treatment led to a more serious decrease in root meristem size than that of 600  $\mu$ M melatonin (**Figures 5C,D**). In the meanwhile, we examined the content of endogenous melatonin in the roots of control and 0.5 nM IAA treated seedlings, and found that 0.5 nM IAA treatment resulted in increased level of melatonin (**Figure 5E**).

*DR5* promoter contains seven tandem repeat sequences of an auxin-responsive element, and it is widely used as a reporter for auxin signaling responses and auxin distribution in *Arabidopsis* (Ulmasov et al., 1997; Friml et al., 2003). After GUS staining, we found that the distribution of auxin was dramatically changed

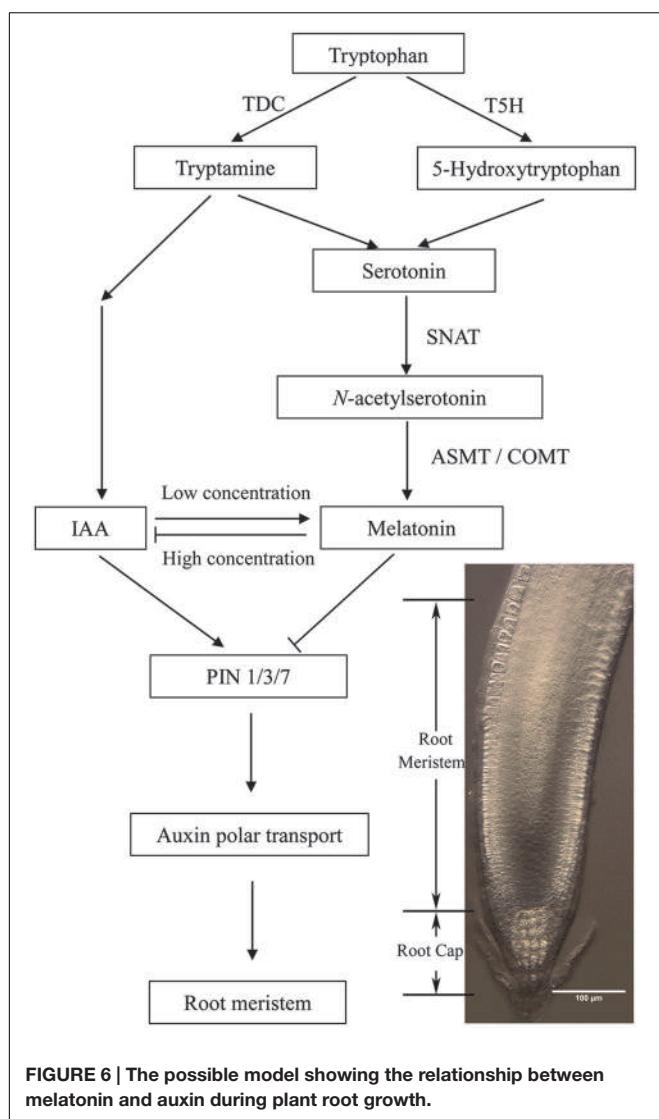
upon melatonin treatment, as it diffused into lateral root cap (LRC) cells from columella cells (**Figure 5F**). Similar results could be obtained by the observation of *DR5::GFP* line seedlings treated with melatonin for 5 days (**Figure 5G**) while 2  $\mu$ M TIBA changed the expression pattern of *DR5::GFP* in root tips. In combination with melatonin, TIBA-induced auxin signals spread to LRC cells and adjacent meristem cells further (**Figure 5G**). Exogenous application of 100 nM IAA, but not that of 0.5 nM IAA, caused expansion of auxin signals to the lower part of LRC cells, similar to that of melatonin treatment. Interestingly, in association with melatonin, IAA-induced fluorescence signals spread to the whole distal tips of the roots below QC (**Figure 5G**), just like seedlings treated with higher concentrations of IAA as reported before (Ottenschläger et al., 2003), suggesting that melatonin aggravated the accumulation of auxin signals in the whole distal tips of roots, in an IAA similar manner.



## DISCUSSION

As an important plant hormone, auxin plays vital roles in root cell division, differentiation, elongation, and the overall growth of roots (Benjamins and Scheres, 2008). In recent decades, more attention has been paid to the role of melatonin as a growth regulator of plants (Arnao and Hernández-Ruiz, 2015). Both of auxin and melatonin have been suggested to regulate similar growth processes. Our data showed 10  $\mu$ M melatonin had no effect on primary root growth (Figures 1 and 2), and our previously work suggested that 10–50  $\mu$ M melatonin had litter effect on endogenous melatonin content (Shi et al., 2015c). Based on previous studies and our preliminary experiments, 10–50  $\mu$ M melatonin had litter effect on endogenous melatonin content (Shi et al., 2015c), and lower melatonin concentration also had litter effect on plant root development (Bajwa et al., 2014). Moreover, 100–600  $\mu$ M

melatonin were also widely used in other studies (Pelagio-Flores et al., 2012; Bajwa et al., 2014). In our study, we first tested the effect of melatonin on root growth of *Arabidopsis* and found that 100  $\mu$ M melatonin had already shown an inhibitory effect on root growth, in accordance with previous reports (Chen et al., 2009). However, Bajwa et al. (2014) showed that 100, 200, and 400  $\mu$ M melatonin treatment had no significant effects on plant root growth. The difference might be attributed to the big values of SD of their results and solvent effect. To limit the effect of solvent, the same volume of solvent including ethanol was used as a control in this study. Moreover, the average values of more than 30 seedlings per treatment from three independent experiments. The higher the concentration of melatonin, the more suppression of the root length (Figure 1B), indicating that melatonin inhibit root length in a dose-dependent manner. Although moderate auxin promotes root growth of plants, overproduction of auxin



levels can cause a decay of root growth (Teale et al., 2005; Strader et al., 2011). After digging deeper into the effects of melatonin, we found high concentrations of melatonin reduced root meristem size, consistent with its effects on primary root length (Figures 2B,C).

The effects of auxin on root growth, is largely dependent on its biosynthesis and polar transport, which cause optimal auxin accumulation and distribution in the root apex during the whole developmental process (Blilou et al., 2005; Kim et al., 2007; Li et al., 2011). Quantitative real-time PCR showed that the expression levels of *YUC1*, *YUC2*, *YUC5*, *YUC6*, and *TAR2*, key genes of auxin biosynthesis, were significantly down-regulated after 600  $\mu$ M melatonin treatment consistent with lower IAA content in 600  $\mu$ M melatonin-treated roots (Figure 3). If we set the transcript level of *YUC1* in control material as 1, the relative expression level of *YUC2*, *YUC3*, *YUC4*, *YUC5*, *YUC6*, *YUC7*, *YUC8*, *TAI1*, *TAR1*, and *TAR2* was 28.7, 406.7, 4.6, 0.37, 151.9, 20.6, 170.3,

43.5, 41, and 693 separately. In melatonin-treated material, the relative transcript level of *YUC1* and other genes were 0.4, 1.6, 564.1, 11.9, 0.1, 1, 132.8, 230.8, 23.1, 29.6, and 234.3, respectively. After melatonin treatment, the total relative expression abundance was decreased significantly. The decrease in expression of *YUC1*, *YUC2*, *YUC5*, *YUC6*, and *TAR2*, together with the effects of melatonin on auxin transport may cause the decrease in IAA levels in roots, at least partially. As reported recently, application of 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) results in a decay in the transcript levels of *YUC1*, *YUC2*, *YUC4*, *YUC6*, and *TAR2* in *Arabidopsis* seedlings (Suzuki et al., 2015). We noticed there is a difference in the endogenous IAA level between this study and previous results (Zhao et al., 2001; Lee et al., 2012), which could be resulted from two possible reasons. One reason could be the sample differences. In our work, only the root tips were harvested and used for analysis. Another possibility is that different methods were used. In this study, we used ELISA method. Based on the consistence between the transcript levels of auxin biosynthesis genes and IAA level, we concluded that melatonin negatively regulated auxin biosynthesis.

Polar auxin transport is essential for the distribution of auxin in *Arabidopsis* root tips, and the auxin efflux machinery PIN proteins play important roles in controlling the size of root meristem (Friml et al., 2003; Blilou et al., 2005). Expression levels of PINs were always found down-regulated in the shortened root meristem after stresses (Liu et al., 2015; Yuan and Huang, 2016). Even in *PIN1::GFP* line roots exogenously treated with IAA, a significant decrease in GFP fluorescence was detected under high concentrations (5 and 10  $\mu$ M; Omelyanchuk et al., 2016). Coincidentally, our results demonstrated a decrease in both of the transcript levels and the protein expression levels of *PIN1*, *PIN3*, and *PIN7* in melatonin-treated roots (Figures 4A,C, revised Figure 4B). Auxin transport inhibitor TIBA could also decrease the root meristem size and root growth (Blilou et al., 2005). Our study showed auxin transport inhibitor TIBA did not enhance melatonin-mediated reduction of root meristem size, indicating that PAT might be necessary for the regulation of root meristem size by melatonin treatment (Figures 5A,B). Moreover, the triple mutant *pin1pin3pin7* was more tolerant than WT in response to melatonin treatment, suggesting that *PIN1/3/7*-mediated PAT might contribute to melatonin-regulated root meristem.

Previous study showed application of low concentration of melatonin (0.1  $\mu$ M), increased the endogenous levels of IAA in *Arabidopsis* roots (Chen et al., 2009), and our findings demonstrated that exogenous application of low concentration of IAA (0.5 nM), also raised the endogenous melatonin content in roots (Figure 5E). On the contrary, 100 nM IAA caused reduced root meristem size, as previously reported (Rahman et al., 2007; Strader et al., 2011). In this study, simultaneous presence of 100 nM IAA and 600  $\mu$ M melatonin led to more serious decrease in root meristem size than that of 600  $\mu$ M melatonin alone (Figures 5C,D).

The expression of *DR5* promoter marker line in root tips represents the responses and distribution pattern of auxin.

Unlike the results showed by Pelagio and Koyama (Pelagio-Flores et al., 2012; Koyama et al., 2013), here we found that melatonin was able to change the expression pattern in both of *DR5::GUS* and *DR5::GFP* line roots (**Figure 5G**), just like the effects of IAA (Ottenschläger et al., 2003). In combination with 600  $\mu\text{M}$  melatonin, 100 nM IAA caused a more expansion pattern of fluorescence signals in the whole root caps, including columella cells and lateral root cells, indicating that exogenous application of melatonin intensified the effect of IAA on the auxin responses in root tips. Considering altered auxin synthesis in root tip of treated seedlings, decreased signals of PIN1 in provascular, reduced PIN3 and PIN7 signals in both root cap and provascular, the DR5::GFP signal should not expand that strongly toward the lateral cap and tip cells after melatonin treatment. Then we also observed the expression and localization of PIN2 in the root cells, however, no significant difference was shown about the expression of PIN2 in cell membrane without and with 600  $\mu\text{M}$  melatonin treatment (revised **Supplementary Figures S1A–C**). What's surprised is that PIN2 signals in cytoplasm in melatonin-treated roots were obviously increased, although the signals in cytoplasm were much lower than that in cell membrane. This may, at least partially explain that why DR5 signals spread into the whole root cap after melatonin treatment. These results indicated that melatonin may have dual and complex effects on auxin transport. Besides PIN1/2/3/7, there may be other issues contribute to melatonin-mediated auxin signaling, which need to be further investigated.

Based on our results, we proposed a working model for the mechanisms by which melatonin regulates root meristem (**Figure 6**). Melatonin and auxin share the same substrate tryptophan during biosynthetic pathways, and exogenous application of IAA at low concentration increases melatonin production, while high concentration of melatonin decreases the level of IAA and PIN1, 3, 7 in *Arabidopsis* roots. Thus, melatonin regulates root meristem by repressing auxin synthesis and polar auxin transport in *Arabidopsis*. In summary, this study provides a direct link between melatonin and root growth, and indicates the novel involvement of auxin responses in melatonin-mediated root growth in *Arabidopsis*. We highlight the relationship between of melatonin and auxin in *Arabidopsis* root growth.

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## AUTHOR CONTRIBUTIONS

HS conceived and directed this study, revised the manuscript; QW designed and performed the experiments, analyzed the data, wrote and revised the manuscript; BA performed the experiments, analyzed the data and revised the manuscript; YW provided help in the melatonin and IAA content analysis; RR provided suggestions and revised the manuscript; HL designed the experiments and revised the manuscript; CH designed the experiments and revised the manuscript. All authors approved the manuscript and the version to be published.

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## SUPPLEMENTARY MATERIAL

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**FIGURE S1 | The expression of PIN2 in cytoplasm of root cells was altered after 600  $\mu\text{M}$  melatonin treatment.** Three-day-old seedlings harboring indicated markers were transferred to control medium or medium with 600  $\mu\text{M}$  melatonin for 6 days. **(A)** Effects of Melatonin on the expression pattern of PIN2 in *Arabidopsis*. Scale bar = 50  $\mu\text{m}$ . **(B)** Localization of PIN2 in root cells in control seedling and seedling treated with 600  $\mu\text{M}$  melatonin. Scale bar = 5  $\mu\text{m}$ . **(C)** Comparison of GFP fluorescence intensity in plants treated without or with melatonin as in **(A)** by Image J. The fluorescence intensity levels of the control roots were set to 1. Values represent mean  $\pm$  SD, \* $P$  < 0.05, and \*\* $P$  < 0.01 by a Student's *t*-test.

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# Isolation and Functional Characterization of Bidirectional Promoters in Rice

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Bidirectional promoters, which show great application potential in genetic improvement of plants, have aroused great research interest recently. However, most bidirectional promoters were cloned individually in the studies of single genes. Here, we initiatively combined RNA-seq data and cDNA microarray data to discover the potential bidirectional promoters in rice genome. Based on the expression level and correlation of each adjacent and oppositely transcribed gene pair, we selected four candidate gene pairs. Then, the intergenic region between each pair was isolated and cloned into a dual reporter vector pDX2181 for functional identification. GUS and GFP assays of the transgenic plants indicated that all the intergenic regions showed bidirectional expression activity in various tissues. Through 5' and 3' deletion analysis on one of the above bidirectional promoters, we identified the enhancing region which sharply increased its bidirectional expression efficiency and the essential regions respectively responsible for its 5' and 3' basic expression activity. The bidirectional arrangement of the four gene pairs in six gramineous plants was also analyzed, showing the conserved characteristics of the four bidirectional promoters identified in our study. In addition, two novel *cis*-sequences conserved in the four bidirectional promoters were discovered by bioinformatic identification. Our study proposes a feasible method for selecting, cloning, and functionally identifying bidirectional promoters as well as for discovering their bidirectional regulatory regions and conserved sequences in rice.

**Keywords:** rice, bidirectional promoter, stable transformation, GUS assay, GFP assay, deletion analysis, conservation analysis

## INTRODUCTION

Plant architecture, development, and interaction with environment are controlled by the expression of a series of genes (Chen F. et al., 2010; Li C. et al., 2011; Zhu et al., 2011). As a critical regulator of gene expression, promoters are important in plant biotechnology and functional genomics research for their great application potential in genetic engineering and theoretical significance in the exploration of transcriptional regulation mechanism (Cai et al., 2007; Yi et al., 2011; Walcher and Nemhauser, 2012; Ye et al., 2012; Balasubramani et al., 2014). Many researches have been focused on the cloning and analysis of unidirectional promoters, such as constitutive promoters, spatiotemporal promoters, and inducible promoters (McElroy et al., 1990; Pan et al., 2015; Vijayan et al., 2015). Bidirectional promoters, which generally refer to the intergenic region between two

adjacent genes transcribed in opposite directions, show better applicability than unidirectional promoters in genetic improvement (Trinklein et al., 2004; Mitra et al., 2009; Yang et al., 2013). That is because a bidirectional promoter can drive the expression of two genes simultaneously, and thus can be time-saving in constructing expression vectors and pyramiding of multiple genes (Kumar et al., 2015). Besides, it is very critical for transgenic breeding to enable the functionally related genes to express in the same pattern in the receptors (Ha et al., 2010; Ogo et al., 2013). However, the unidirectional promoters with the same specific expression patterns are only available in limited quantities, and repetitious use of the promoters may have a negative impact on the stability and expression of transgenes (Peremarti et al., 2010). According to previous reports, the expression patterns of bidirectional promoters in opposite directions are similar in many cases due to the co-expression of the adjacent genes (Huang et al., 2007; Wang et al., 2009; Chen W. et al., 2010; Didych et al., 2013). Therefore, bidirectional promoters could also compensate for the lack of unidirectional promoters with the same expression pattern.

Much work has been done to analyze the bidirectional promoters in mammalian genome with experimental and bioinformatic methods (Trinklein et al., 2004; Yang and Elnitski, 2008; Uwanogho et al., 2010). The results suggest that the divergent gene pairs regulated by bidirectional promoters exhibit the characteristics of conserved arrangement, coexpression, and functional association (Xu et al., 2012; Didych et al., 2013; Meersseman et al., 2014; Yang and Elnitski, 2014). Since the sequences of promoters are known to be variable (Müller et al., 2007), for discovering bidirectional promoters, it is particularly helpful to investigate the characteristics of the divergent gene pairs regulated by them.

Bidirectional promoters have become a research focus in plants in recent years. With the development of plant genome sequencing, bioinformatic analyses in plants like rice, *Arabidopsis* and *Populus* have revealed that the divergent gene pairs regulated by bidirectional promoters have similar characteristics, such as coexpression, functional association, and conserved arrangement (Krom and Ramakrishna, 2008; Dhadi et al., 2009; Wang et al., 2009; Chen W. et al., 2010). The structural characteristics of bidirectional promoters in plants are similar to those in mammals, such as higher GC content and less TATA boxes (Dhadi et al., 2009). Besides, bidirectional promoters have been cloned in many species. In *Arabidopsis*, the tissue-specific and light-inducible bidirectional promoter between *cab1* and *cab2*, the tissue-specific bidirectional promoter between *At5g06290* and *At5g06280*, and the tissue-specific and stress-inducible bidirectional promoter between *At4g35985* and *At4g35987* have been cloned successively (Bondino and Valle, 2009; Mitra et al., 2009; Banerjee et al., 2013). All of these promoters can be widely used in gene functional analysis in *Arabidopsis*. Several bidirectional promoters from other species such as melon and *Capsicum annuum*, have been also cloned gradually (Shin et al., 2003; Wang et al., 2008). In rice, a few promoters have been found to show bidirectional expression activities (Huang et al., 2007; Singh et al., 2009; Dhadi et al., 2013). So far, there has been no report about the cloning and identification of bidirectional

promoters using two reporter genes simultaneously with stable transformation in rice.

Rice is one of the most important food crops in the world and a model plant for functional genomic researches in cereals (Zhang, 2007). Therefore, it is highly necessary to introduce multiple genes into rice for genetic improvement (Ha et al., 2010; Yang et al., 2011; Ogo et al., 2013). Accordingly, discovery of bidirectional promoters in rice is very critical. Besides, more complete genomic information (Goff et al., 2002; Yu et al., 2002; Pan et al., 2014) and more explicit gene expression information (Wang et al., 2010; Kawahara et al., 2013; Sakai et al., 2013) will greatly facilitate the development of a high-throughput method for discovering bidirectional promoters.

Most of the known bidirectional promoters were found during the researches of single genes. In this study, we reported a method of selection, cloning, functional identification, and deletion analysis of bidirectional promoters for their *de novo* discovery in rice. We first selected four adjacent and oppositely transcribed gene pairs based on their expression levels and correlations, which were derived from the RNA-seq data of the Michigan State University Rice Genome Annotation Project Database (MSU), the Rice Annotation Project Database (RAP), and the microarray data of the rice cDNA microarray database (CREP). Subsequently, the intergenic regions between the four gene pairs were cloned for functional identification. GUS and GFP assays of the transgenic plants indicated that all the intergenic regions showed bidirectional expression activity in various tissues. With 5' and 3' deletion analysis of one bidirectional promoter above, we found the regulatory region responsible for its bidirectional expression activity. Meanwhile, the bidirectional arrangement of the four gene pairs in six gramineous plants showed the conserved characteristics of the four bidirectional promoters identified in our study. Then, we discovered two *cis*-sequences conserved in the four bidirectional promoters with MEME. The two *cis*-sequences showed overrepresentation in the intergenic regions between divergent gene pairs in rice genome under the reference of random promoters. Our study proposes a feasible method for selecting, cloning, and functionally identifying bidirectional promoters as well as for the discovery of their bidirectional expression regulatory regions and conserved sequences in rice.

## METHODS

### Selection of Candidate Bidirectional Promoters

RNA-seq data were obtained from the Michigan State University Rice Genome Annotation Project Database (MSU) and the Rice Annotation Project Database (RAP; Kawahara et al., 2013; Sakai et al., 2013), and the microarray data were downloaded from the rice cDNA microarray database (CREP; Wang et al., 2010). Based on the expression characteristics, the criteria for candidate divergent gene pairs regulated by bidirectional promoters in our study were set as: the maximum expression value of the gene pair was simultaneously higher than 10 in RNA-seq data and higher than 5000 in microarray data, and the expression

correlation coefficient between the gene pair from RNA-seq data of 95 samples was higher than 0.4. Because only 36 samples were available in microarray data, which were not sufficient for reliable correlation analysis, we did not consider the correlation coefficient of the data from microarray data. According to the criteria, we chose four divergent gene pairs (**Table 1**) and isolated their intergenic regions (here designated as *BIP1*, *BIP2*, *BIP3*, and *BIP4*, respectively) for functional identification.

## Isolation and Vector Construction of *BIP1*, *BIP2*, *BIP3*, and *BIP4*

The genomic DNA of Minghui 63 (*Oryza sativa* L ssp. *indica*) was used as template to amplify *BIP1*, *BIP2*, *BIP3*, and *BIP4* with specific primers (**Table 2**). The PCR-generated fragments were respectively inserted into T-vector (Promega) and confirmed by sequencing with primers SP6 and T7. The sequence-confirmed clone containing *BIP1/BIP2/BIP3/BIP4* was digested by *Pst* I/*Bam*H I/*Bam*H I/*Bam*H I and was respectively cloned into a dual reporter vector pDX2181 (Ye et al., 2012).

## Agrobacterium-Mediated Rice Transformation

The sequence-confirmed clones were transformed into the *Agrobacterium tumefaciens* strain *EHA105* by electroporation. Subsequently, all the constructs were introduced into Zhonghua11 (*O. sativa* L ssp. *japonica*) by *Agrobacterium*-mediated transformation. The callus culture and transformation procedures were carried out as previously described (Hiei et al., 1994).

## Histochemical and Fluorometric Analysis of GUS Activity

Histochemical staining of GUS activity in rice tissues was conducted essentially as previously described (Jefferson et al., 1987). Various tissues of *T<sub>0</sub>* transgenic-positive transformants (root, leaf, sheath, panicle, stem, and mature seed) were

incubated in GUS staining solution (50 mM sodium phosphate at pH 7.0, 10 mM Na<sub>2</sub>-EDTA, 0.1% Triton X-100, 1 mg/mL X-Gluc, 100 µg/ml chloramphenicol, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide and 20% methanol) at 37°C for 2–10 h after 15-min vacuum filtration. After GUS staining, the samples were incubated in 70% ethanol to remove chlorophyll and photographs were taken under a dissecting microscope (Leica MZFLIII).

Quantitative analysis of GUS activity was conducted as previously described (Xu et al., 2010). The total protein concentration in the supernatant was quantified using the Bradford assay (Bradford, 1976). GUS protein in the supernatant was determined fluorometrically with an INFINITE 200 photometer (Tecan Austria GmbH, Ltd, Grodig, Austria). GUS activity was determined fluorometrically by measuring the amount of 4-methylumbellifera (Mu) produced under the catalysis of GUS in 1 mg of total protein per minute. Five biological replicates were assayed for each construct. Ten transgenic lines were randomly divided into five groups and two transgenic lines were considered as one biological replicate.

## Histological and Quantitative Analysis of GFP

Histological analysis of GFP in rice tissues was detected and photographed under fluorescence microscope. Various tissues of *T<sub>0</sub>* transgenic-positive transformants (root, leaf, sheath, panicle, stem, and mature seed) were sampled and observed under a fluorescence microscope (Leica MZ16F) using GFP filter sets and Leica Application Suite software.

The relative expression levels of GFP in rice tissues were detected by quantitative real-time PCR (qRT-PCR). Total RNAs of different rice tissues were extracted and reverse-transcribed as described previously (Wang et al., 2015a), and qRT-PCR was performed according to the same reference. The primers of GFP were GFP-F: 5'-ATCCGCCACAACATCGAGGA-3' and GFP-R: 5'-TCGTCCATGCCGAGAGTGAT-3', and the primers

**TABLE 1 |** Four divergent gene pairs chosen for functional identification.

Intergenic regions	5' gene	Minimum value	Maximum value	Mean value	3' gene	Minimum value	Maximum value	Mean value	Pearson correlation coefficient	Spearman correlation coefficient	Database
<i>BIP1</i>	LOC_Os02 g42314	0.6	21.57	7.88	LOC_Os02 g42320	0	31.16	11	0.67	0.62	MSU
		1.65	35.33	10.35		0	10.25	3.8	0.58	0.51	RAP
		2143.75	9118.85	5662.15		6009.55	15236.5	9940.64			CREP
<i>BIP2</i>	LOC_Os05 g27940	1.58	88.59	17.19	LOC_Os05 g27950	0.38	40.61	8.91	0.45	0.5	MSU
		2472.2	22698.9	13559.4		1381.8	7987.6	4900.6			CREP
		0	30.11	13.56	LOC_Os02 g47000	0.37	16.17	4.96	0.51	0.49	MSU
<i>BIP3</i>	LOC_Os02 g47000	0.05	639.27	19.56	LOC_Os02 g47010	0.23	720.79	45.81	0.84	0.93	RAP
		568.4	4000.9	1680.54		709.45	7154.35	3080.98			CREP
		0	69.12	9.42	LOC_Os03 g22880	0	184.71	15.17	0.65	0.78	MSU
<i>BIP4</i>	LOC_Os03 g22880	0.16	21.51	3.8	LOC_Os03 g22890	1.31	142.75	12.44	0.74	0.81	RAP
		274.55	13458.05	4949.52		1065.65	14857.15	7410.79			CREP

**TABLE 2 |** Polymerase chain reaction (PCR) primers used in this study.

Primer name	Primer sequence (5'-3') <sup>a</sup>	Purpose
BIP1-F	AA <u>CTGCAG</u> CTGGTCTCCTCTACTGTTG	Promoter clone
BIP1-R	AA <u>CTGCAG</u> AGCTGCAAACATAACAAATACCC	Promoter clone
BIP2-F	<u>CGGGAT</u> CCCTTGATAACCCGTAGTG	Promoter clone
BIP2-R	<u>CGGGAT</u> CCCTCTTCTGAAGAACCATC	Promoter clone
BIP3-F	<u>CGGGAT</u> CCCTCGCTGAGCTACCAATAACC	Promoter clone
BIP3-R	<u>CGGGAT</u> CCCTACACCCACACCCCCATT	Promoter clone
BIP4-F	CGGGATCCCTCGCCGGCGCGTCGGC	Promoter clone
BIP4-R	<u>CGGGAT</u> CCCGCAGAGGATTCTTCTTC	Promoter clone
BIP1-2F	AA <u>CTGCAG</u> CAGCTCGCAGCTCCCCT	5' deletion analysis
BIP1-2R	AA <u>CTGCAG</u> GCTGCAAACGAAATGCCAC	3' deletion analysis
BIP1-3R	AA <u>CTGCAG</u> GGGCCGCCGACGCGCAGGCC	3' deletion analysis

<sup>a</sup>The underlined letters indicate the restriction enzyme sites.

of GAPDH (the endogenous control) were GAPDH-F: 5'-CTGCAACTCAGAAGACCGTTG-3' and GAPDH-R: 5'-CCT GTTGTCAACCCTGGAAGTC-3'. Relative expression levels were determined using  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen, 2001).

### Melatonin Treatment

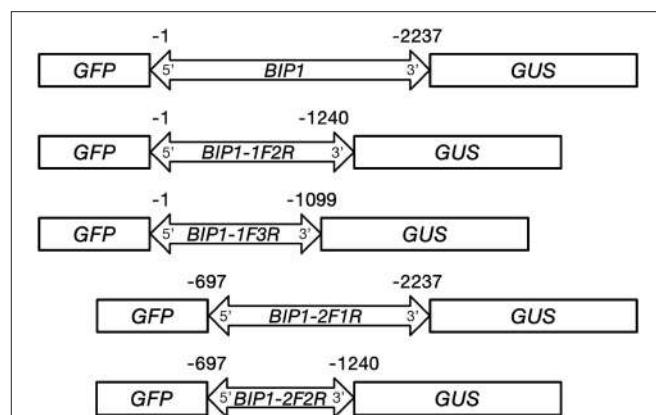
Melatonin (*N*-acetyl-5-methoxytryptamine), which is known as an indispensable hormone related to many physiological activities in animals, has also been identified as an important signaling molecule in response to many stresses in plants. In order to test the response of the four bidirectional promoters to melatonin, melatonin treatment was performed on the transgenic plants according to the procedure described by Shi and Chan (2014).

### 5' and 3' Deletion Analysis of *BIP1*

Among the four bidirectional promoters above, *BIP1* showed the highest expression efficiency in both 5' and 3' orientations. Therefore, it was selected for 5' and 3' deletion analysis in order to find the regulatory regions responsible for bidirectional expression activity (Figure 1). The specific primers used for PCR amplification to generate different 5' and 3' truncated fragments are shown in Table 2. Vector construction, callus culture and transformation, histochemical and fluorometric analysis of GUS activity, histological and quantitative analysis of GFP were performed as described above.

### Conservation Analysis of the Four Bidirectional Promoters and Bioinformatic Identification of Their Conserved Sequences

The conserved arrangements of the four gene pairs in six gramineous plants (*O. sativa*, *Sorghum bicolor*, *Setaria italica*, *Brachypodium distachyon*, *Zea mays*, and *Triticum aestivum*) were identified with the information from the Ensembl Plants database (<http://plants.ensembl.org/index.html>). The bidirectional genes whose homologous genes in other



**FIGURE 1 |** Schemes of constructs carrying *BIP1* and different deleted versions fused with *GFP* and *GUS* reporter genes.

species were still arranged in a bidirectional architecture were considered to be regulated by conserved bidirectional promoters (c-BIP); otherwise, they were considered to be regulated by non-conserved bidirectional promoters (n-BIP).

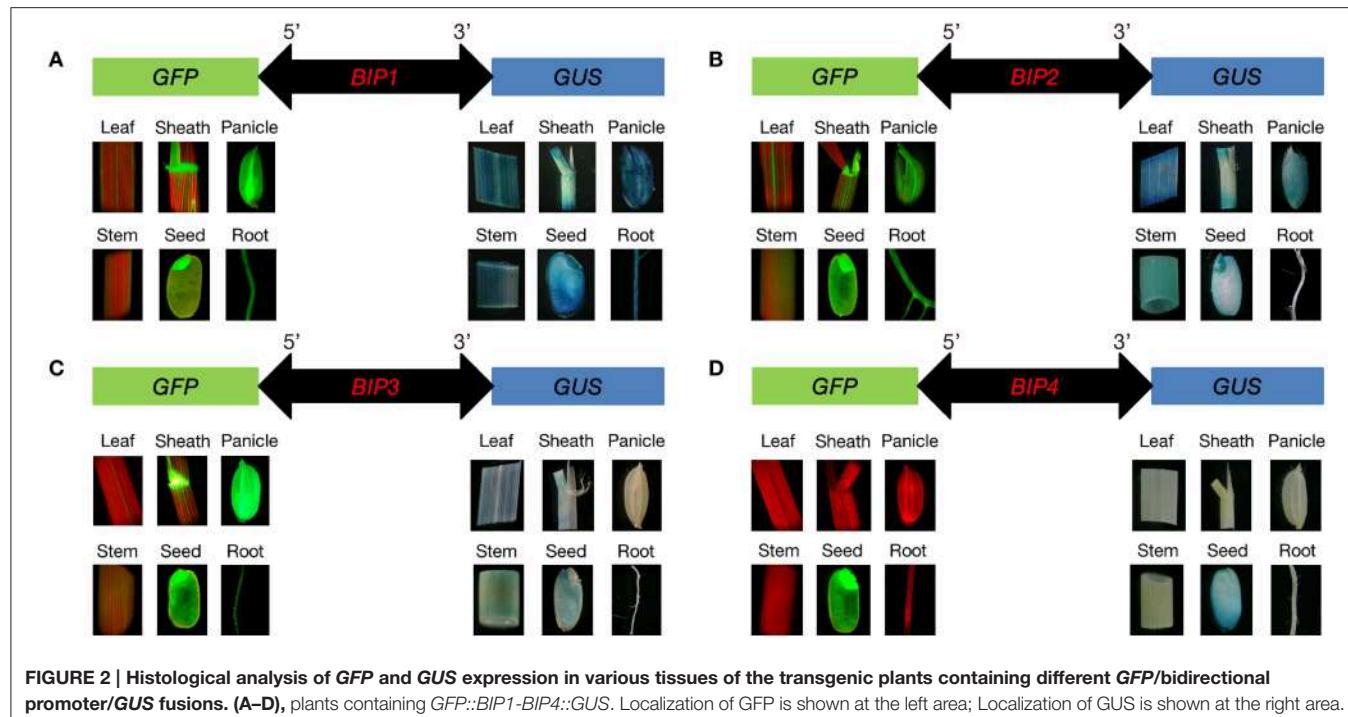
The conserved sequences in the four bidirectional promoters were discovered by MEME (<http://meme-suite.org//tools/meme>) and their frequencies in the intergenic regions between divergent gene pairs in rice genome were identified by FIMO ( $p < 1E-8$ ) using the reference of random promoters (<http://meme-suite.org//tools/fimo>).

## RESULTS

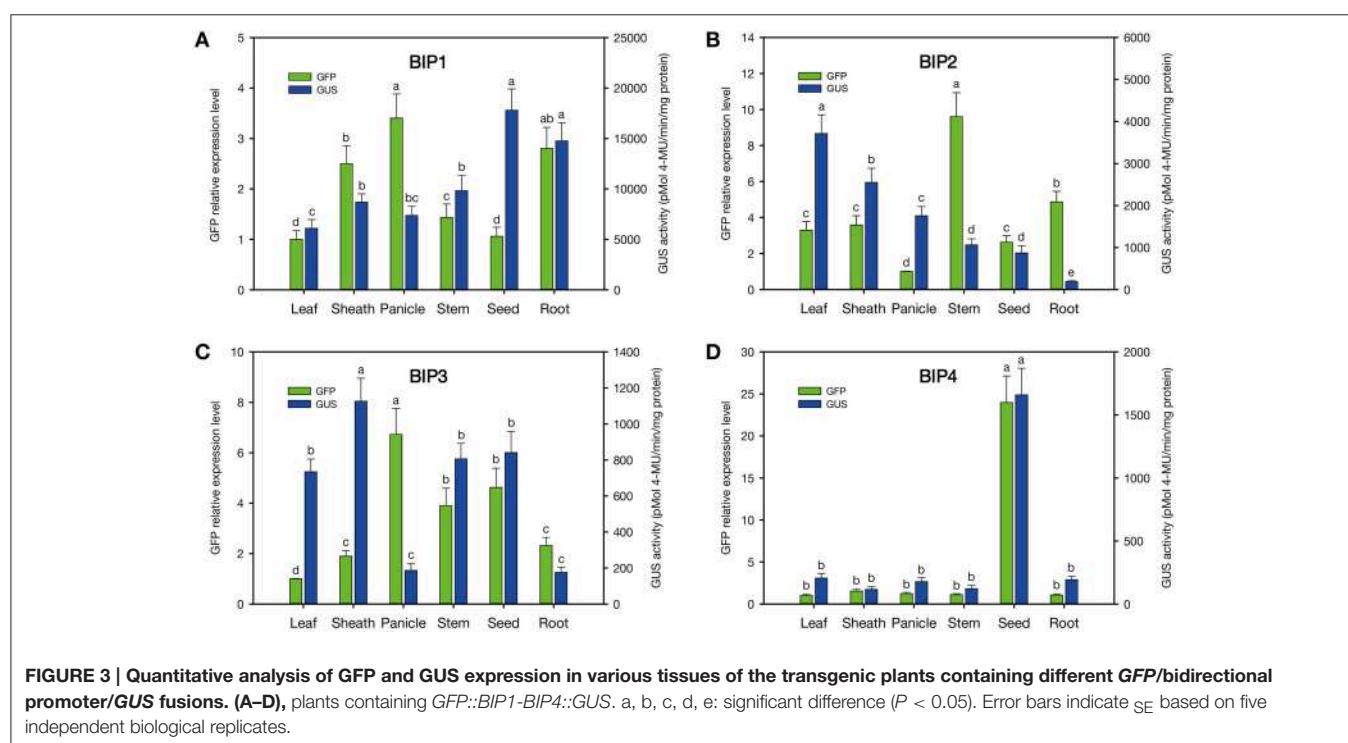
### Selection of Four Novel Bidirectional Promoters in Rice Genome and Their Functional Characterization in Transgenic Plants

Based on RNA-seq and microarray data, we chose four divergent gene pairs (Table 1) according to the criteria in Section Methods and isolated their intergenic regions for functional identification. The four fragments were respectively cloned to a dual reporter vector pDX2181 and transformed into rice variety Zhonghua 11.

According to the results of GUS and GFP assays of the transgenic plants, all the intergenic regions showed bidirectional expression activity in various tissues. Histological GUS and GFP analysis of the transgenic plants showed that four novel bidirectional promoters (*BIP1*, *BIP2*, *BIP3*, and *BIP4*) were successfully identified in our work. Among them, *BIP1*, *BIP2*, and *BIP3* showed bidirectional constitutive expression patterns and *BIP4* showed bidirectional seed-specific expression pattern (Figure 2). Analysis of GUS fluorometric activities in various tissues of *BIP1* transgenic plants (Figure 3) showed that the expression efficiency of *BIP1* toward 3' was the highest in the seed, which showed a GUS enzymatic activity of  $17806 \pm 2108$  pmol 4-MU/min/mg protein, followed by in the root, which exhibited a GUS enzymatic activity of  $14769 \pm 1782$  pmol 4-MU/min/mg protein, while the GUS enzymatic activities in the stem, sheath, panicle, and leaf were  $9825 \pm 1510$ ,  $8681 \pm 834$ ,



**FIGURE 2 |** Histological analysis of GFP and GUS expression in various tissues of the transgenic plants containing different GFP/bidirectional promoter/GUS fusions. **(A–D)**, plants containing GFP::BIP1-BIP4::GUS. Localization of GFP is shown at the left area; Localization of GUS is shown at the right area.



**FIGURE 3 |** Quantitative analysis of GFP and GUS expression in various tissues of the transgenic plants containing different GFP/bidirectional promoter/GUS fusions. **(A–D)**, plants containing GFP::BIP1-BIP4::GUS. a, b, c, d, e: significant difference ( $P < 0.05$ ). Error bars indicate SE based on five independent biological replicates.

$7380 \pm 895$ , and  $6092 \pm 875$  pmol 4-MU/min/mg protein, respectively. Analysis of GFP expression in *BIP1* transgenic plants (Figure 3) revealed that the expression efficiency of *BIP1* toward 5' was the highest in the panicle, which was 2.4-fold higher than that in the leaf. While the expression levels of

GFP in the root, sheath, stem, and seed were 1.8-, 1.5-, 0.4-, and 0.1-fold higher than that in the leaf, respectively. GUS assays of *BIP2* transgenic plants (Figure 3) indicated that the expression efficiency of *BIP2* toward 3' was the highest in the leaf, which showed a GUS enzymatic activity of  $3713 \pm 445$  pmol

4-MU/min/mg protein, and the GUS activities in the sheath, panicle, stem, and seed were  $2548 \pm 339$ ,  $1755 \pm 220$ ,  $1063 \pm 145$ , and  $872 \pm 173$  pmol 4-MU/min/mg protein, respectively, while GUS activity was hardly detected in the root. Analysis of GFP expression in *BIP2* transgenic plants (Figure 3) showed that the expression efficiency of *BIP2* toward 5' was the highest in the stem, which was 8.6-fold higher than that in the panicle, while the expression levels of GFP in the root, sheath, leaf, and seed were 3.9-, 2.6-, 2.3-, and 1.6-fold higher than that in the panicle, respectively. *BIP3* transgenic plants showed GUS enzymatic activities of  $1126 \pm 128$ ,  $841 \pm 117$ ,  $806 \pm 85$ , and  $734 \pm 71$  pmol 4-MU/min/mg protein in the sheath, seed, stem, and leaf, respectively, while GUS activity was hardly detected in the panicle and root. The expression efficiency of *BIP3* toward 5' was the highest in the panicle, which was 5.7-fold higher than that in the leaf, while the expression levels of GFP in the seed, stem, root, and sheath were 3.6-, 2.9-, 1.3-, and 0.9-fold higher than that in the leaf, respectively. *BIP4* showed a bidirectional seed-specific expression pattern, as a GUS enzymatic activity of  $1659 \pm 211$  pmol 4-MU/min/mg protein was detected in the seed while almost no GUS activity was detected in other tissues, and a 23-fold higher expression level of GFP was observed in the seed compared with in the leaf. In addition, these results indicate that all the bidirectional promoters identified here direct gene expression in an orientation-independent manner; namely, the expression patterns in opposite directions of these bidirectional promoters are similar, which is consistent with the co-expression characteristics of the adjacent genes.

Melatonin is one of the most important hormones in plant and animal. In order to test the response of the four bidirectional promoters to melatonin, melatonin treatment was performed on the *BIPs* transgenic plants. The results of GUS and GFP assays indicated that the four bidirectional promoters were not induced by melatonin (Figure 4). Hence, it can be inferred that no melatonin-responsive *cis*-element was harbored in these promoters.

## Identification of the Expression Regulatory Regions in *BIP1*

In order to identify the regulatory regions in *BIP1*, 5' and 3' deletion analysis of this promoter was performed. A series of truncated *BIP1* were respectively cloned to pDX2181 and transformed into Zhonghua 11. Transgenic plants carrying *BIP1-1F2R* showed much lower GUS activity than *BIP1* transgenic plants in various tissues, especially in the root, stem, seed and panicle, whose GUS activities were lower than 10% of that in the corresponding tissues of *BIP1* transgenic plants (Figures 5, 6). It thus could be inferred that region 1 could greatly increase the transcriptional activity of *BIP1* toward 3'. Meanwhile, *BIP1-1F2R* transgenic plants also showed an obvious decrease of GFP expression in various tissues compared with *BIP1* transgenic plants (Figure 6). These results could be integrated to reveal that region 1 is a bidirectional transcription-enhancing region of *BIP1*. Further truncating in 3' of *BIP1* led to complete abolishment of GUS activity in *BIP1-1F3R* transgenic plants, while the GFP expression level was not obviously reduced

in *BIP1-1F3R* transgenic plants compared with in *BIP1-1F2R* transgenic plants. These results suggest that region 2 is the essential region responsible for the basic expression activity of 3' but not for that of 5'. Transgenic plants carrying *BIP1-2F1R* or *BIP1-2F2R* showed no expression of GFP, indicating that truncating 5' of *BIP1* will completely abolish 5' expression activity of the promoter. GUS assays in *BIP1-2F1R* transgenic plants revealed that truncating 5' of *BIP1* caused slight changes of 3' expression activity in most tissues except for the root, which showed obviously decreased GUS activity compared with that of *BIP1* transgenic plants (Figure 6). These results suggest that region 3 is the essential region responsible for the basic expression activity of 5' but not for that of 3'; however, it can positively regulate the expression activity of 3' in the root.

## Conserved Arrangement of the Four Gene Pairs Regulated by Bidirectional Promoters

The sequences of promoters are known to be variable (Müller et al., 2007). Therefore, in order to analyze the conservation of the four bidirectional promoters in different species, we investigated the conservation of the four gene pairs regulated by these promoters.

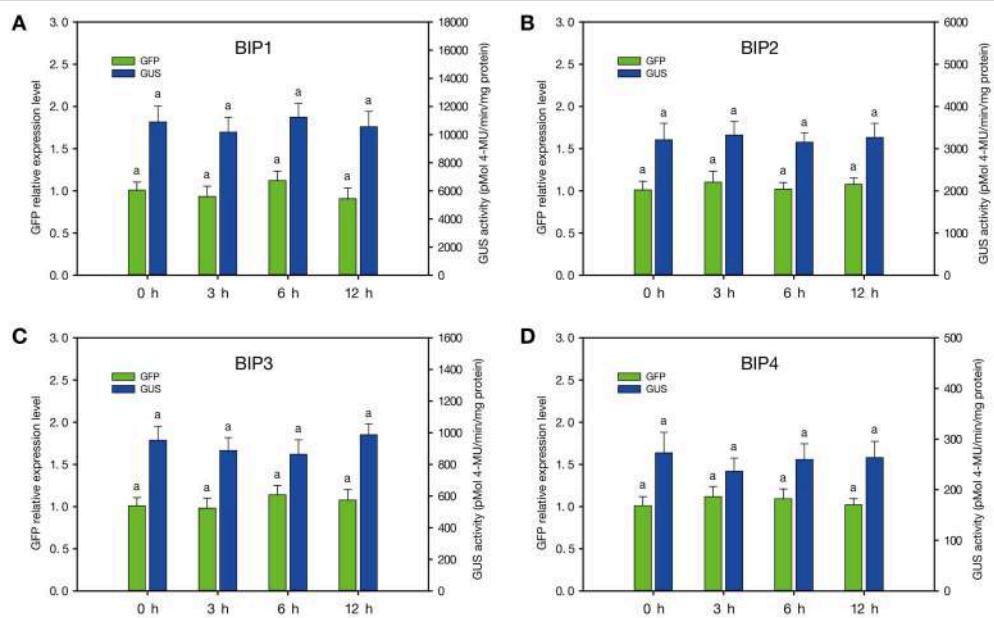
The conserved arrangement of the four gene pairs in six gramineous plants was identified with information from the Ensembl Plants database. The bidirectional genes whose homologous genes in other species were still arranged in a bidirectional architecture were considered to be regulated by c-BIP; otherwise, they were considered to be regulated by n-BIP (Table 3). It was found that *BIP1* and *BIP3* were the most c-BIP in the six gramineous plants. *BIP1* was conserved in *O. sativa*, *S. bicolor*, *B. distachyon*, and *Z. mays*; *BIP3* was conserved in *O. sativa*, *S. bicolor*, *S. italic*, and *B. distachyon*; *BIP4* was conserved in *O. sativa*, *S. bicolor*, and *S. italic*; while *BIP2* was only conserved in *O. sativa* and *T. aestivum*.

## Potential *cis*-Sequences Involved in Bidirectional Expression

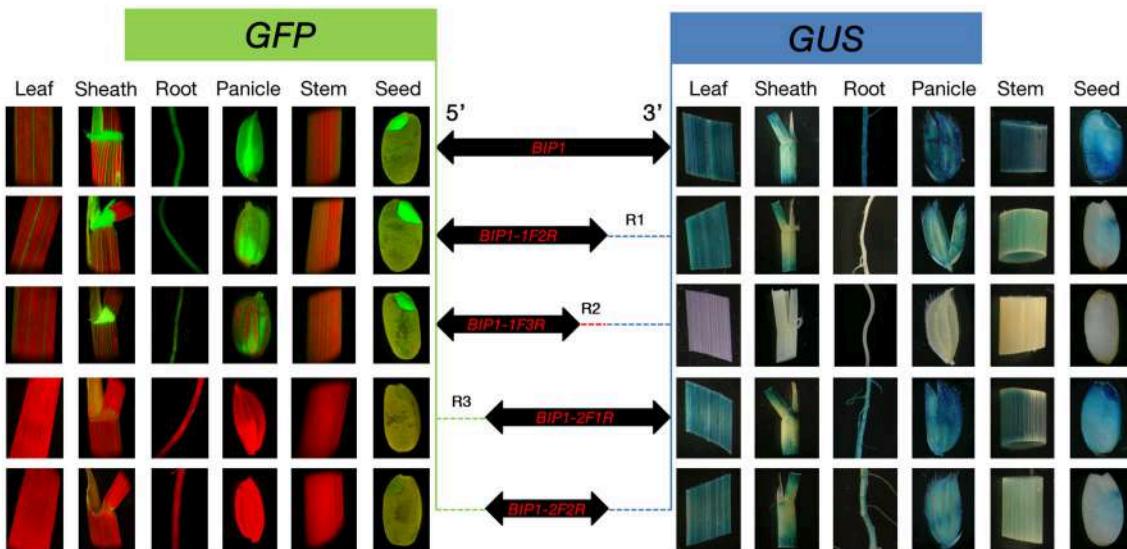
By MEME, two conserved *cis*-sequences in the four bidirectional promoters were identified (Figure 7). *Cis*-sequence 1 was a G/C-rich sequence, while *cis*-sequence 2 was an A/T-rich sequence. Subsequently, the frequencies of these *cis*-sequences in the intergenic regions between divergent gene pairs in rice genome were analyzed by FIMO using the reference of random promoters. Consistent with the expectation, the two *cis*-sequences conserved in the four bidirectional promoters both showed overrepresentation in potential bidirectional promoters in rice genome compared with random promoters. This result further reveals that the two novel *cis*-sequences are probably involved in bidirectional expression.

## DISCUSSION

In this study, we initiatively combined RNA-seq data and cDNA microarray data to discover potential bidirectional promoters in rice. Four adjacent and oppositely transcribed gene pairs were selected based on their expression levels and correlations.



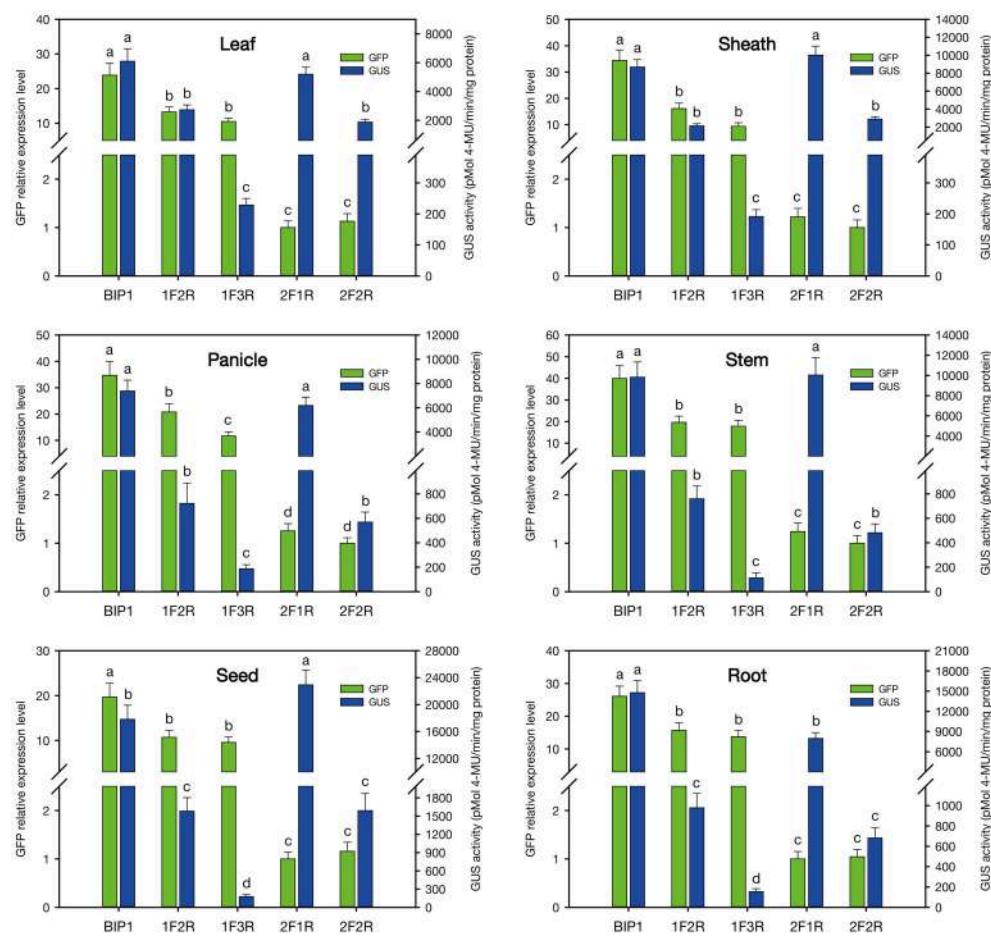
**FIGURE 4 | Quantitative analysis of GFP and GUS expression of the BIPs transgenic plants in response to melatonin. (A–D)**, plants containing GFP::BIP1-BIP4::GUS. a: no significant difference. Error bars indicate  $\text{SE}$  based on five independent biological replicates.



**FIGURE 5 | Histological analysis of GFP and GUS expression in various tissues of the transgenic plants containing different GFP/BIP1 deleted version/GUS fusions.** Localization of GFP is shown at the left area; Localization of GUS is shown at the right area. R1 (blue dashed line), region 1; R2 (red dashed line), region 2; R3 (green dashed line), region 3.

The intergenic regions between the four gene pairs were successfully cloned, and all of them were identified to be bidirectional promoters, confirming the feasibility of our method for discovering bidirectional promoters. This is the first study to clone and identify bidirectional promoters using two reporter genes simultaneously with stable transformation in rice. Among the four identified bidirectional promoters, *BIP1* shows high

expression efficiency in various tissues, and thus has a high application potential in genetic engineering, such as driving two resistant genes simultaneously in transgenic breeding against pest/disease stress, which can confer more strong, broad, and durable resistance in rice (Du et al., 2009; Shah et al., 2009; Yang et al., 2011; Wang et al., 2015a). Rice is one of the most important food crops in the world, and its seed is the edible part



**FIGURE 6 | Quantitative analysis of GFP and GUS expression in various tissues of the transgenic plants containing different GFP/BIP1 deleted version/GUS fusions.** 1F2R, plants containing GFP::BIP1-1F2R::GUS; 1F3R, 2F1R and 2F2R follow the same pattern. a, b, c, d: significant difference ( $P < 0.05$ ). Error bars indicate SE based on five independent biological replicates.

**TABLE 3 | Conservation analysis of the four bidirectional promoters in gramineous plants.**

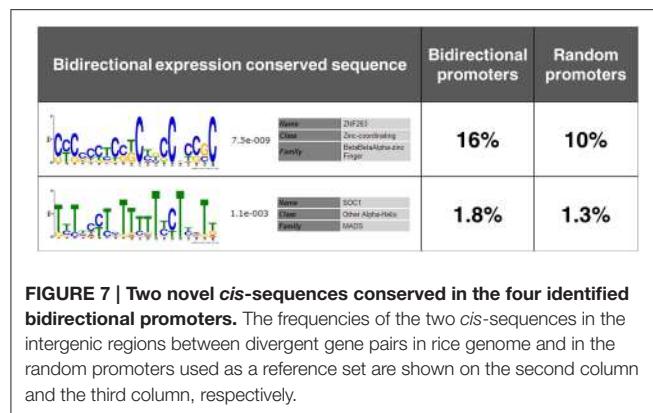
	Oryza sativa	Sorghum bicolor	Setaria italica	Brachypodium distachyon	Zea mays	Triticum aestivum
BIP1	C/C, c-BIP	C/C, c-BIP	C/C, n-BIP	C/C, c-BIP	C/C, c-BIP	C/C, n-BIP
BIP2	C/C, c-BIP	C/C, n-BIP	C/C, n-BIP	C/C, n-BIP	C/C, n-BIP	C/C, c-BIP
BIP3	C/C, c-BIP	C/C, c-BIP	C/C, c-BIP	C/C, c-BIP	C/C, n-BIP	C/C, n-BIP
BIP4	C/C, c-BIP	C/C, c-BIP	C/C, c-BIP	C/C, n-BIP	C/C, n-BIP	C/C, n-BIP

If 5' gene/3' gene of the bidirectional promoters had homologous gene in another species (C/C) and the homologous genes were still arranged in bidirectional architecture, they were considered to be regulated by conserved bidirectional promoters (c-BIP). Otherwise, they were considered to be regulated by non-conserved bidirectional promoters (n-BIP).

consumed by human. Therefore, it is highly necessary to improve the nutrient quality of the seed (Ha et al., 2010; Li Y. et al., 2011, 2014; Ogo et al., 2013). Efficient and specific expression of multiple target genes for seed improvement in rice could hardly be realized without seed-specific promoter. In this work, *BIP4* shows a bidirectional seed-specific expression pattern, indicating its high application potential in the improvement of seed quality by specifically driving multiple genes. The results of 5' and 3' deletion analysis reveal that region 1 is the bidirectional

transcription-enhancing region of *BIP1*; region 2 is the essential region specifically responsible for the basic expression activity of 3'; region 3 is the essential region responsible for the basic expression activity of 5' but not for that of 3', while it can positively regulate the 3' expression activity in the root.

Conservation analysis of the four bidirectional promoters in gramineous plants reveals the possible co-evolution of adjacent genes regulated by these promoters. The bidirectional arrangement of LOC\_Os02g42314 and LOC\_Os02g42320,



which are regulated by *BIP1*, is conserved in four species of six gramineous plants, suggesting that they are relatively conserved during co-evolution. The functional annotations of these two genes, which are “ubiquitin-conjugating enzyme” and “peptidase,” show that both of them are structural genes conserved during evolution. Moreover, the functional relationship between the two genes further supports their co-evolutionary conservation. LOC\_Os02g47000 and LOC\_Os02g47010, which are regulated by *BIP3*, also show conserved arrangement in four species of six gramineous plants. Although the functional annotation of LOC\_Os02g47000 is unclear, considering that LOC\_Os02g47010 is annotated to encode “secretory carrier-associated membrane protein,” we speculate that LOC\_Os02g47000 may encode a structural protein related to secretory pathway.

So far, many *cis*-regulatory sequences have been identified, which are involved in inducible expression (Liu et al., 2010, 2014; Yuan et al., 2011; Koschmann et al., 2012; Walcher and Nemhauser, 2012) and tissue-specific expression (Hartmann et al., 2005; Cai et al., 2007; Ye et al., 2012; Wang et al., 2015b). A previous report also has suggested that several

known *cis*-sequences might be related to bidirectional expression (Dhadi et al., 2009). Here, we used the experimentally verified bidirectional promoters to predict two *cis*-sequences related to bidirectional expression which had not been identified in rice genome. Subsequently, overrepresentation of the two novel *cis*-sequences in the intergenic regions between divergent gene pairs further reveals their involvement in bidirectional expression. Interestingly, *cis*-sequence 1 is a G/C-rich sequence, which is consistent with the characteristics of higher GC content in bidirectional promoters; however, *cis*-sequence 2 is an A/T-rich sequence, which might be a new finding in the sequence characteristics of bidirectional promoters. Overall, the novel bidirectional promoters identified using two reporter genes simultaneously with stable transformation in rice are expected to have high applicability in genetic engineering. Our study proposes a feasible method for selecting, cloning, and functionally identifying bidirectional promoters as well as for discovering their bidirectional regulatory regions and conserved sequences in rice.

## AUTHOR CONTRIBUTIONS

YL and RW conceived and designed the experiments. RW, YY, MZ, and MY performed the experiments. RW and YY performed the data analysis. RW and YL wrote the paper. FZ and HC revised the paper. YL secured the funds to support this research.

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# Comparative Transcriptional Profiling of Melatonin Synthesis and Catabolic Genes Indicates the Possible Role of Melatonin in Developmental and Stress Responses in Rice

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As a well-known animal hormone, melatonin (*N*-acetyl-5-methoxytryptamine) is also involved in multiple plant biological processes, especially in various stress responses. Rice is one of the most important crops, and melatonin is taken in by many people everyday from rice. However, the transcriptional profiling of melatonin-related genes in rice is largely unknown. In this study, the expression patterns of 11 melatonin related genes in rice in different periods, tissues, in response to different treatments were synthetically analyzed using published microarray data. These results suggest that the melatonin-related genes may play important and dual roles in rice developmental stages. We highlight the commonly regulation of rice melatonin-related genes by abscisic acid (ABA), jasmonic acid (JA), various abiotic stresses and pathogen infection, indicating the possible role of these genes in multiple stress responses and underlying crosstalks of plant hormones, especially ABA and JA. Taken together, this study may provide insight into the association among melatonin biosynthesis and catabolic pathway, plant development and stress responses in rice. The profile analysis identified candidate genes for further functional characterization in circadian rhythm and specific stress responses.

**Keywords:** melatonin, rice, gene expression, circadian rhythm, development, immunity, stress response

## INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) was first discovered in the cow's pineal gland (Lerner et al., 1958). Dubbels et al. (1995) and Hattori et al. (1995), melatonin was identified in plants by two research groups. Until now, melatonin has been found in multiple plant species, including alfalfa, almond, anise, apples, *Arabidopsis*, banana, beetroot, bermudagrass, black mustard, cabbage, celery,

**Abbreviations:** 2-ODD, 2-oxoglutarate-dependent dioxygenase; AANAT, arylalkylamine *N*-acetyltransferase; ABA, abscisic acid; ASMT, *N*-acetylserotonin methyltransferase; AXR3, Auxin Resistant 3; BL, indole-3-acetic acid; DAT, days after transplanting; GA, gibberellic acid; GEO, Gene Expression Omnibus; hpi, hour post inoculation; IAA, indole-3-acetic acid; JA, jasmonic acid; M2H, melatonin 2-hydroxylase; SNAT, serotonin *N*-acetyltransferase; T3S, type III secretion system; T5H, tryptamine 5-hydroxylase; TDC, tryptophan decarboxylase; tZ, trans-zeatin; Xoo, *Xanthomonas oryzae* pv. *oryzae*.

cherry, coriander, cucumber, fennel, fenugreek, flax, green cardamom, milk thistle, oranges, poppy, potato, rice, sunflower, tobacco, tomato, white mustard, wolf berry, etc. (Manchester et al., 2000; Zhao et al., 2013; Shi and Chan, 2014). In addition, the endogenous melatonin concentration can also be modulated through genetic transformation in tomato and rice (Okazaki and Ezura, 2009; Okazaki et al., 2009, 2010; Byeon et al., 2012, 2013, 2014; Byeon and Back, 2014a,b).

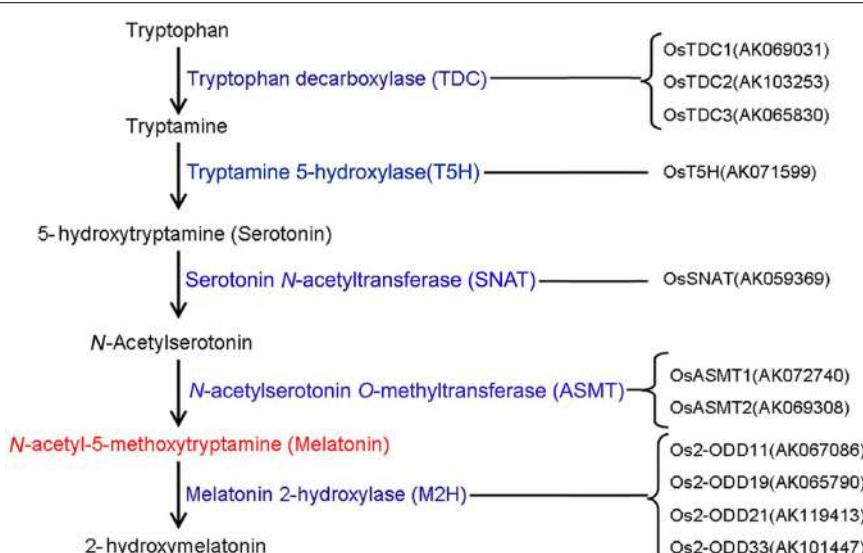
To date, the biosynthesis and metabolic pathways of melatonin in plants have been established (**Figure 1**). Melatonin in plants can be synthesized by four sequential enzymes from tryptophan (Kang et al., 2011), including TDC, T5H, SNAT, and *N*-acetylserotonin *O*-methyltransferase (ASMT) (Arnao and Hernández-Ruiz, 2014, 2015; Zuo et al., 2014). Thereafter, melatonin is catabolized by M2H into 2-hydroxymelatonin (Byeon and Back, 2015). In rice, gene families of TDC, T5H, SNAT, and ASMT contain 3, 1, 1, and 3 known members, respectively (Kang et al., 2007; Fujiwara et al., 2010; Kang et al., 2013; Park et al., 2013a). However, OsASMT3 is barely detectable in any of the plant organs (Park et al., 2013b). OsM2H genes belong to 2-ODD family and at least 4 of 2-ODD genes show M2H activities in rice (Byeon and Back, 2015).

Solid evidence implicates that melatonin is involved in multiple plant biological processes and various stress responses (Hardeland, 2015; Zhan et al., 2015), including circadian rhythm (Kolář and Macháčková, 2005; Arnao and Hernández-Ruiz, 2015), delayed senescence of leaves (Byeon et al., 2012; Wang et al., 2012, 2013a,b), leaf morphology (Okazaki et al., 2010), root development (Hernández-Ruiz et al., 2005; Pelagio-Flores et al., 2012; Zhang N. et al., 2014), coleoptile growth (Hernández-Ruiz et al., 2004, 2005), grain yield (Byeon and Back, 2014a), fruit ripening (Sun et al., 2015), drought stress (Wang et al., 2013a, 2014; Zhang et al., 2013; Meng et al., 2014; Zuo et al., 2014; Shi et al., 2015b), salt stress (Wei et al., 2014; Zhang H.J. et al.,

2014; Liang et al., 2015; Shi et al., 2015b), cold stress (Posmyk et al., 2009a; Arnao and Hernández-Ruiz, 2014; Bajwa et al., 2014; Shi and Chan, 2014; Turk et al., 2014; Shi et al., 2015b), high temperature (Tiryaki and Keles, 2012), copper stress (Posmyk et al., 2008, 2009b), oxidative stress (Park et al., 2013b; Shi et al., 2015d), cadmium stress (Byeon et al., 2015) and pathogen infection (Yin et al., 2013; Lee et al., 2014, 2015; Reiter et al., 2015; Shi et al., 2015a; Zhao et al., 2015).

Melatonin plays protective roles in the regulation of plant tolerance to abiotic stress and biotic stress (Yin et al., 2013; Lee et al., 2014, 2015; Zhan et al., 2015). Overexpression of *OsTDC* increases endogenous melatonin level and delays leaf senescence in rice (Kang et al., 2007, 2009; Byeon et al., 2014). The transcript of *OsT5H* can be induced by *Magnaporthe grisea* infection (Fujiwara et al., 2010). Exogenous application of serotonin, the penultimate substrate for melatonin biosynthesis, induces defense gene expression and increases resistance to rice blast infection (Fujiwara et al., 2010). Transgenic rice plants ectopically expressing the AANAT regulates cold stress resistance (Kang et al., 2010), seminal root elongation (Park and Back, 2012), oxidative stress resistance (Park et al., 2013b), and seedling growth (Byeon and Back, 2014a). The transcript of *OsASMT* can be induced by ABA and methyl JA treatments, and *OsASMT* overexpressing plants result in higher level of melatonin (Park et al., 2013b). Exogenous application of melatonin improved apple resistance to Marssonina apple blotch (*Diplocarpon malí*) (Yin et al., 2013), enhanced disease defense against *Pseudomonas syringae* DC3000 in *Arabidopsis* and tobacco (Lee et al., 2014; Shi et al., 2015a).

Rice is one of the most important crops around the world, and melatonin is also taken in from rice by many people everyday. Thus, it is very useful and important to dissect the distribution and regulation of endogenous melatonin in rice. Melatonin is widely involved in plant development, multiple



**FIGURE 1 |** The genes responsible for melatonin synthesis from tryptophan and melatonin degradation in rice.

abiotic and biotic stress responses in *Arabidopsis* (Shi and Chan, 2014), and Bermudagrass (Shi et al., 2015b). However, transcriptional profiling of rice melatonin synthesis and catabolic genes has not been systematically carried out. In this study, we analyzed the expression profiling of 11 rice melatonin synthesis and catabolic genes in development, various tissues, and in response to hormone, pathogen infection, drought, salt, and cold stresses. These results may provide insight into the link among melatonin biosynthesis and catabolic pathway, plant development and stress responses in rice. Further functional characterization of identified candidate genes with potential involvement in circadian rhythm and stress responses through overexpressing, knocking down or knocking out will give more clues to melatonin-mediated signaling as well as underlying molecular mechanism.

## MATERIALS AND METHODS

### Plant materials and Growth Conditions

Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) seeds were sown in germinating boxes. At 30 days after germination, the seedlings were transplanted in a paddy field under normal conditions of the cultivation season. Thereafter, 56 DAT, 58 DAT, and 90 DAT were considered as the stage of panicle initiation, the early stage of panicle development indicating a complete reproductive transition, the stages of flowering and early stages of seed development corresponding to the ripening-stage transition, respectively.

For hormone treatments, rice seeds were germinated, and grown hydroponically in a growth chamber at 28°C under continuous light. Seven-day old seedlings were transferred in culture solution containing 50 μM ABA, or 10 μM GA, or 10 μM IAA, or 1 μM brassinolide (BL), or 1 μM tZ, or 100 μM JA, or in culture solution without hormone to serve as control (mock treatment). Samples were collected after 0, 0.25, 0.5, 1, 3, and 6 h incubation for root, and after 0, 1, 3, 6, and 12 h incubation for shoot.

### Pathogen Infection

Rice (*O. sativa* cv Nipponbare) plants grown in the greenhouse for 42 days were inoculated with *Xoo* T7174R, a wild-type strain, and 74HrcV::Km, a T3S-defective mutant by the leaf-clipping method. Plants treated with water were used as control. Leaf sections (3–5 mm) that included the inoculation site were collected at 3, 6, and 12 hpi and 1, 2, 4, 6 dpi.

For the blast fungus infection, three lines of rice cultivar Nipponbare carrying the blast resistance genes (*Pia*, *Pish*) were inoculated with two strains of *Magnaporthe oryzae* harboring *AVR-Pia* and *AVR-Pish*. Rice seedlings at the 4-leaf stage were placed in moist chamber and sprayed with a conidial suspension of *M. oryzae* ( $1 \times 10^6$  conidia/ml). The seedlings were incubated in a moist chamber at 25°C for 24 h under dark condition, then grown in hydroponic culture under 14 h light (28°C) and 10 h dark (24°C). Leaf samples (4th leaf) from 3 individual experiments were harvested at 1, 2, 3, and 5 days post

inoculation (dpi). Rice seedlings sprayed with water were used as control.

## Development- and Pathogen Infection-Related Data Analysis

The data of spatio-temporal transcript levels in various tissues or organs (RXP\_0001), leaf and root transcriptional profile in light (RXP\_003 and RXP\_007) and dark (RXP\_004 and RXP\_008) throughout entire growth in the field, diurnal, and circadian leaf (RXP\_002) and root (RXP\_009) transcriptional profile throughout entire growth, plant hormone profile (RXP\_001 to RXP\_012), *Xoo*-treated profile (RXP\_3002), and *M. oryzae*-treated profile (RXP\_3001) were downloaded from RiceXPro<sup>1</sup> (Sato et al., 2011a,b, 2013). All samples were used for hybridization using the Agilent one-color (Cy3) microarray-based gene analysis system. As detailed described in Sato et al. (2013), all the above data were deposited in GEO through the following accession numbers: GSE21396, GSE21397, GSE36040, GSE36042, GSE36043, GSE36044, GSE39423, GSE39424, GSE39425, GSE39426, GSE39427, GSE39429, and GSE39432. All the raw data were downloaded and re-analyzed for cluster analysis of expression profile that shown as normalized data ( $\log_2$ ).

## Abiotic Stress-Related Data Analysis

As described in Jain et al. (2007), 7-day-old light-grown rice seedlings were transferred to control condition and 200 mM NaCl solution as salt stress for 3 h, were dried on tissue paper as dehydration stress for 3 h, and were kept at 4°C as cold stress for 3 h. Then the seedlings were sampled in triplicate. GEO series accession no. GPL2025 were used for microarray analysis as Jain et al. (2007) described. All the normalized data were obtained from Rice eFP Browser<sup>2</sup> (Jain et al., 2007).

## Cluster Analysis

The original data from RiceXPro and Rice eFP Browser were listed in Supplementary Table S1. Hierarchical cluster analysis of transcriptional profile was performed using CLUSTER program<sup>3</sup> (Larkin et al., 2007), and the heatmap was obtained using Java Treeview<sup>4</sup> (Saldanha, 2004) according to the instructions.

## RESULTS

### The Spatio-Temporal Transcript Levels of Rice Melatonin Synthesis and Catabolic Genes in Various Tissues or Organs

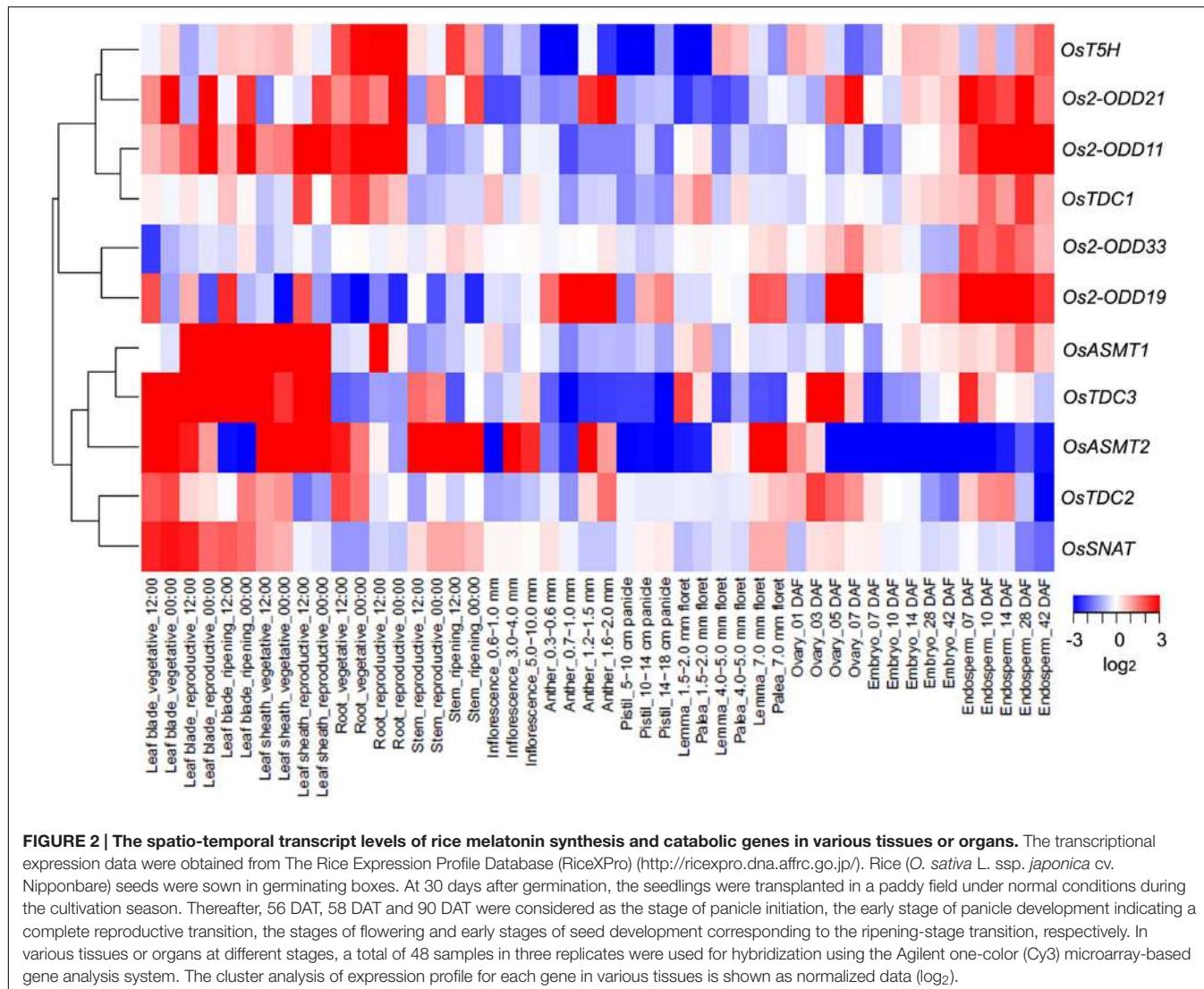
To investigate the expression profiles of rice melatonin synthesis and catabolic genes in various tissues or organs, we analyzed the expression of these genes using published microarray data (Sato et al., 2013). Eleven of rice melatonin-related genes have the

<sup>1</sup><http://ricexpro.dna.affrc.go.jp/>

<sup>2</sup><http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi>

<sup>3</sup><http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>

<sup>4</sup><http://jtreeview.sourceforge.net/>

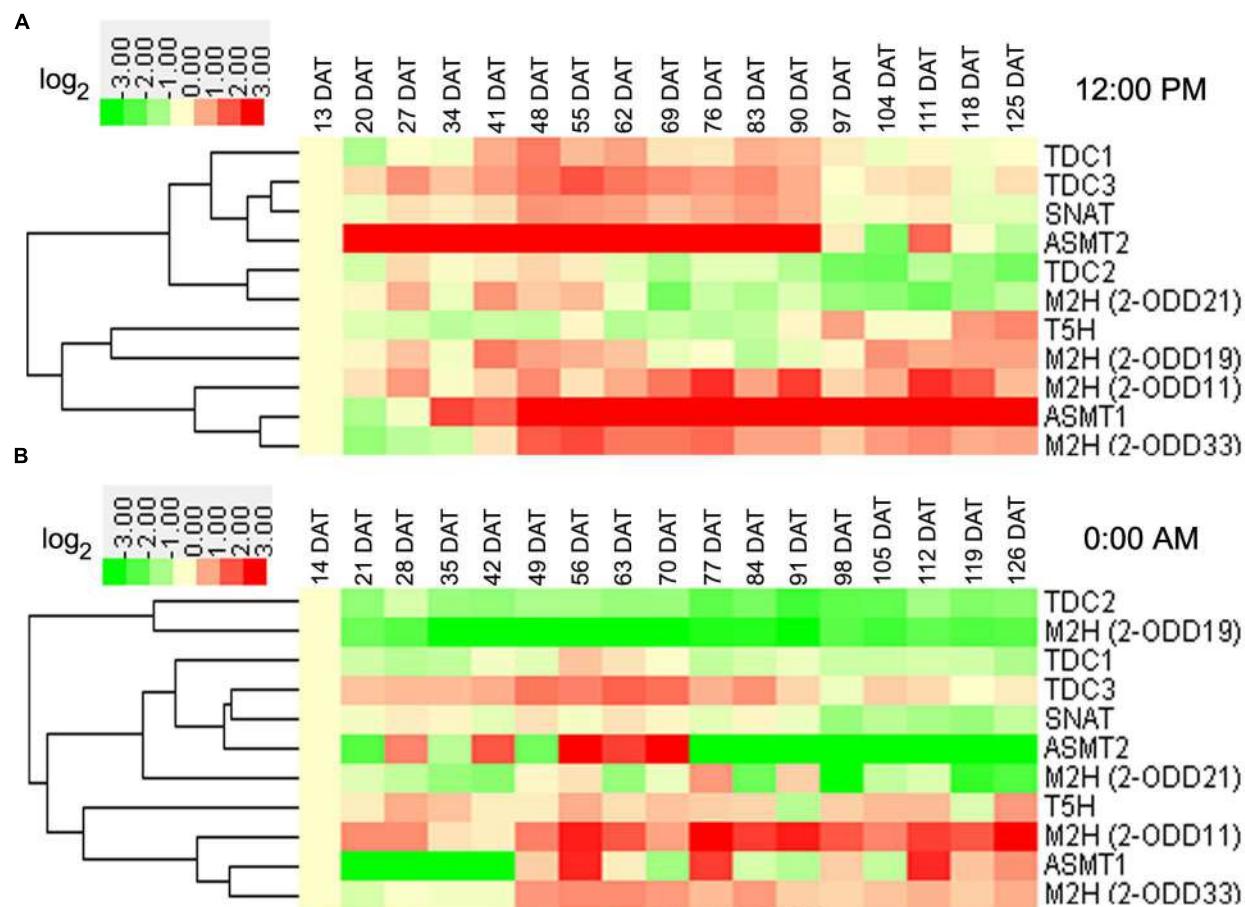


corresponding probe sets in the dataset (Figure 1). As shown in Figure 2, all genes showed different expression pattern in various tissues, indicating that these genes may play different roles in plant growth and development. Interestingly, the expression patterns of melatonin-related genes could be divided into two groups (Figure 2). One group contained six genes (*OsT5H*, *Os2-ODD11*, *-19*, *-21*, *-33*, and *OsTDC1*), and most of them showed high expression levels in endosperm tissues. The other group contained five genes (*OsASMT1*, *-2*, *OsTDC2*, *-3*, and *OsSNAT*), and most of them showed high expression levels in leaf blade and leaf sheath tissues. Moreover, *Os2-ODD19* showed high expression level in four time points of anther, while other four genes (*OsTDC1*, *-3*, *Os2-ODD11*, and *OsASMT1*) exhibited lower expression level. Similarly, six genes (*Os2-ODD11*, *-19*, *-21*, *-33*, *OsTDC1*, and *OsASMT1*) showed high expression level in five time points of endosperm, while two genes (*OsASMT2* and *OsSNAT*) with a relative low level of expression. These melatonin-related genes showed high expression levels in a special tissue indicated their possible roles of melatonin in special tissue.

Moreover, three genes (*Os2-ODD19*, *-21*, and *OsTDC2*) showed different expression level in day and night at 9 tissues (leaf blade-vegetative, leaf blade-reproductive, leaf blade-ripening, leaf sheath-vegetative, leaf sheath-reproductive, root-vegetative, root-reproductive, stem-reproductive, and stem-ripening). The results indicate that *Os2-ODD19*, *-21*, and *OsTDC2* may play some roles in circadian rhythm and may be used in further functional characterization.

## Transcriptional Profile of Rice Melatonin Synthesis and Catabolic Genes throughout Entire Growth in the Field

In rice leaves at day, the transcript levels of *OsTDC3* and *OsASMT2* were increased at vegetative stages, while that of *OsT5H* was decreased (Figure 3A). At reproductive stages, the transcripts of seven genes (*OsTDC1*, *-3*, *OsASMT1*, *-2*, *Os2-ODD11*, *-33*, and *OsSNAT*) and two genes (*OsTDC2* and *Os2-ODD21*) showed up-regulation and down-regulation,

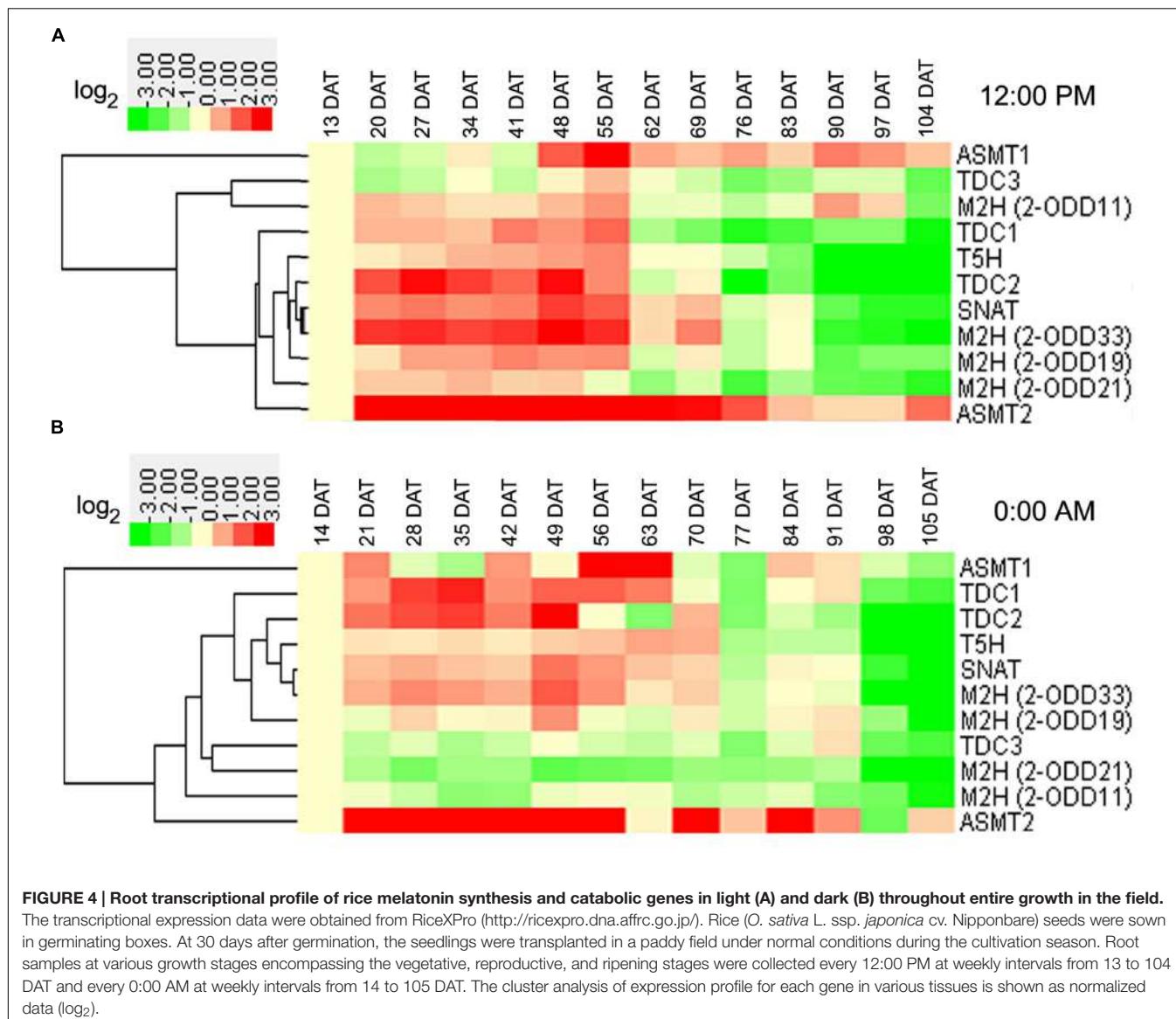


**FIGURE 3 | Leaf transcriptional profile of rice melatonin synthesis and catabolic genes in light (A) and dark (B) throughout entire growth in the field.**

The transcriptional expression data were obtained from RiceXPro (<http://ricexpro.dna.affrc.go.jp/>). Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) seeds were sown in germinating boxes. At 30 days after germination, the seedlings were transplanted in a paddy field under normal conditions during the cultivation season. Samples corresponding to the uppermost fully expanded leaves were collected every 12:00 PM at weekly intervals from 13 to 125 DAT and every 0:00 AM at weekly intervals from 14 to 126 DAT. A total of 51 samples at 12:00 PM in three replicates and 34 samples at 0:00 AM in two replicates were used for hybridization using the Agilent one-color (Cy3) microarray-based gene analysis system. The cluster analysis of expression profile for each gene in various tissues is shown as normalized data (log<sub>2</sub>).

respectively (Figure 3A). At ripening stages, the transcripts of three genes (*Os2-ODD11*, *-33*, and *OsASMT1*) and two genes (*OsTDC2* and *Os2-ODD21*) showed up-regulation and down-regulation, respectively (Figure 3A). In the leaves at night, the transcript of *Os2-ODD11* showed up-regulation at all time points, while the transcripts of *OsTDC2* and *Os2-ODD19* were obviously down-regulated (Figure 3B). At vegetative stages, the transcripts of three genes (*OsTDC3*, *OsT5H*, and *Os2-ODD11*) and two genes (*OsTDC2* and *Os2-ODD19*) showed up-regulation and down-regulation, respectively (Figure 3B). At reproductive stages, the transcripts of four genes (*OsTDC3*, *OsT5H*, *Os2-ODD11*, *-33*) and two genes (*OsTDC2* and *Os2-ODD19*) showed up-regulation and down-regulation, respectively (Figure 3B). At ripening stages, the transcripts of two genes (*Os2-ODD11*, *-33*) and five genes (*OsTDC1*, *-2*, *Os2-ODD19*, *OsSNAT*, and *OsASMT2*) showed up-regulation and down-regulation, respectively (Figure 3B).

In rice roots at day, transcript of *OsASMT2* increased in the entire growth stages in the field (Figure 4A). At vegetative stages, the transcripts of eight genes (*Os2-ODD11*, *-19*, *-33*, *OsTDC1*, *-2*, *OsT5H*, *OsSNAT*, and *OsASMT2*) showed up-regulation (Figure 4A). At reproductive stages, the transcripts of two genes (*OsASMT1*, *-2*) and four genes (*OsTDC1*, *-3*, *Os2-ODD11*, *-21*) showed up-regulation and down-regulation, respectively (Figure 4A). At ripening stages, the transcripts of two genes (*OsASMT1* and *-2*) and eight genes (*OsTDC1*, *-2*, *-3*, *Os2-ODD19*, *-21*, *-33*, *OsT5H* and *OsSNAT*) showed up-regulation and down-regulation, respectively (Figure 4A). Interestingly, the transcripts of six genes (*OsTDC1*, *-2*, *Os2-ODD19*, *-33*, *OsT5H*, and *OsSNAT*) were obviously up-regulated at vegetative stages, but down-regulated at ripening stages (Figure 4A). In the roots at night, transcript of *Os2-ODD21* decreased throughout entire growth stages in the field (Figure 4B). At vegetative stages, the transcripts of six genes



(*OsTDC1*, -2, *OsT5H*, *OsSNAT*, *Os2-ODD33*, and *OsASMT2*) and two genes (*Os2-ODD11*, -21) showed up-regulation and down-regulation, respectively (Figure 4B). Additionally, the transcripts of two genes (*OsTDC3* and *Os2-ODD21*) and five genes (*Os2-ODD11*, -21, -33, *OsTDC2*, and *OsT5H*) showed down-regulation at reproductive and ripening stages, respectively (Figure 4B). The transcripts of three genes (*OsTDC2*, *OsT5H*, and *Os2-ODD33*) showed up-regulation at vegetative stages, but down-regulation at ripening stages (Figure 4B).

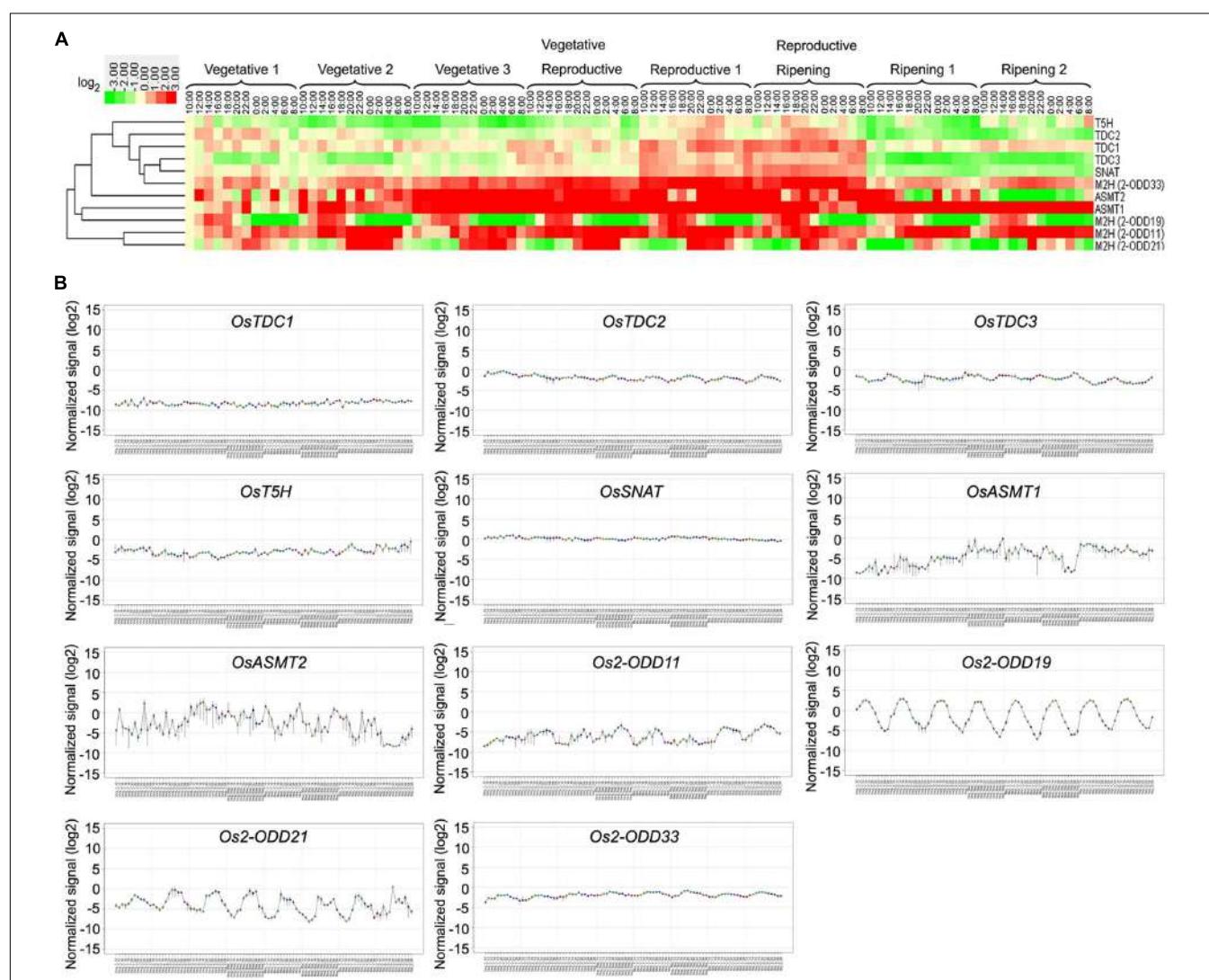
Some genes shared similar expression patterns at day and night at the same stage (Figures 3 and 4). The transcripts of *Os2-ODD11* and -33 showed up-regulation at reproductive and ripening stages in leaves, the transcripts of six genes (*OsTDC1*, -2, *OsT5H*, *OsSNAT*, *OsASMT2*, and *Os2-ODD33*) showed up-regulation at vegetative stages in roots, the transcripts of *OsTDC1* and *Os2-ODD21* shared down-regulation at reproductive and ripening stages of roots. However, there were also some genes

shared different expression patterns. For example, the transcript of *OsT5H* shared down-regulation in day at vegetative stages in leaves, but showed up-regulation in night. Moreover, some genes also shared similar expression patterns in different tissues at the same stage. At day, the transcripts of *OsASMT2* and *OsASMT1* showed up-regulation at vegetative and ripening stages in leaves and roots, respectively, while the transcript of *Os2-ODD21* shared down-regulation at reproductive and ripening stages. On the contrary, some genes shared different expression patterns in different tissues at the same stage. The transcripts of *OsT5H* showed down-regulation in day at vegetative stages in leaves, but showed up-regulation in roots. The transcripts of *Os2-ODD11* and *Os2-ODD33* showed up-regulation at ripening stages of leaves, but showed down-regulation at roots. These results suggest that the melatonin-related genes as well as endogenous melatonin may play important and dual roles in rice developmental stages.

## Diurnal and Circadian Transcriptional Profile of Rice Melatonin Synthesis and Catabolic Genes throughout Entire Growth

As shown in **Figure 5A**, *Os2-ODD11* expression was induced throughout entire growth stages, and the transcripts of four genes (*OsASMT1*, –2 *OsTDC1* and *Os2-ODD33*) were induced at most time points of growth stages, while those of *Os2-ODD19* and *Os2-ODD21* were intermittent. Before reproductive 1 stage, *Os2-ODD21* showed significant induction at night. On the contrary, *Os2-ODD19* was obviously down-regulated. This result suggested that *Os2-ODD19* and *Os2-ODD21* may play dual and

important roles in the regulation of circadian rhythm. Moreover, the transcripts of four genes (*Os2-ODD11*, –33, *OsASMT1*, and –2) and two genes (*OsASMT1* and *Os2-ODD11*) displayed significant up-regulation at four stages (vegetative 3, vegetative-reproductive, reproductive 1, and reproductive-ripening stages) and two stages (ripening 1 and 2 stages), respectively. However, *OsTDC2*, *OsTDC3*, and *OsSNAT* expressions were repressed during ripening 1 and 2 stages. Additionally, the transcripts of most genes were induced during reproductive 1 and reproductive-ripening stages. At the last two stages (ripening 1 and 2 stages), most of genes were obviously down-regulated. Interestingly, the transcripts of five genes (*OsTDC2*, –3, *OsT5H*, *OsSNAT*, and *OsASMT2*) were induced during reproductive 1 and



**FIGURE 5 |** Diurnal and circadian leaf transcriptional profile of rice melatonin synthesis and catabolic genes throughout entire growth as shown by heatmap (A) and line chart (B). The transcriptional expression data were obtained from RiceXPro (<http://ricexpro.dna.affrc.go.jp/>). Rice (*O. sativa* L. ssp. *japonica* cv. Nipponbare) seeds were sown in germinating boxes. Gene expression profile of rice plants grown under natural field conditions based on microarray analysis of leaf samples at various growth stages encompassing the vegetative, reproductive and ripening stages. Samples corresponding to the uppermost fully expanded leaves were collected in a 48-h period at 2-h intervals at eight different growth stages. The cluster analysis and normalized signal of expression profile for each gene in various tissues is shown as normalized data (log<sub>2</sub>).

reproductive-ripening stages, but exhibited down-regulation at ripening 1 and 2 stages. As shown in **Figure 5B**, the expression of *Os2-ODD19* and *Os2-ODD21* displayed obviously regular change throughout entire growth.

In roots, the transcript of *Os2-ODD11* was up-regulated at all time-points, while those of *OsTDC3*, *Os2-ODD21*, and *OsTDC2* were down-regulated (**Figure 6**). The transcriptional profile of *Os2-ODD19* was intermittent, which was consistent with the result in leaves. During 15–17 DAT, the expressions of *Os2-ODD21*, *OsTDC2*, and *OsASMT2* were repressed. The transcript of *Os2-ODD11* was significantly induced during 43–45 DAT, whereas the transcripts of *OsTDC1*, –2, –3, *OsT5H*, and *Os2-ODD21* were repressed.

### The Transcriptional Profile of Rice Melatonin Synthesis and Catabolic Genes in Response to Plant Hormones

In response to ABA and JA treatments, the transcripts of *OsT5H*, *OsTDC2*, –3, and *Os2-ODD19* displayed significantly up-regulation in root or shoot (**Figure 7**). The transcript of *Os2-ODD11* was significant up-regulated after IAA, BL, and JA treatments in root, but was strongly down-regulated after IAA, BL, and tZ treatments in shoot. The transcripts of *OsTDC1* and *OsASMT1* showed up-regulation after ABA, GA<sub>3</sub>, IAA, BL, and tZ treatments in shoot, while *OsTDC3* expression was induced after ABA, IAA, BL, tZ, and JA treatments in shoot (**Figure 7**).

Generally, melatonin-related genes showed different expression profiles in root or shoot tissues for the same treatment (**Figure 7**). The transcripts of *OsTDC1*, –3, *Os2-ODD33*, and *OsASMT1* were up-regulated in the shoots, but were not significantly regulated or down-regulated in the roots after ABA and tZ treatments. The expression of *Os2-ODD11* was increased in roots, but was decreased in shoots after ABA and BL treatments. Although some melatonin-related genes were from the same family, they exhibited different responses to plant hormones treatments, such as *OsTDC2* and *OsTDC3* in roots, *Os2-ODD11* and *Os2-ODD21* in roots, *OsASMT1* and *OsASMT2* in shoots. Thus, the transcriptional response of melatonin-related genes to plant hormones treatments in roots and shoots may provide new insight into crosstalk between melatonin and plant hormones, as well as mechanism underlying melatonin-mediated signaling in rice.

### Gene Expression Profile in Whole Leaf of Rice Melatonin Synthesis and Catabolic Genes Inoculated with Pathogen Infection

Because melatonin plays important roles in response to pathogen infection (Yin et al., 2013; Lee et al., 2014, 2015; Reiter et al., 2015; Shi et al., 2015a; Zhao et al., 2015), so we analyzed the expression profile of rice melatonin synthesis and catabolic genes in response to pathogen inoculation to identify the candidate genes for further analysis.

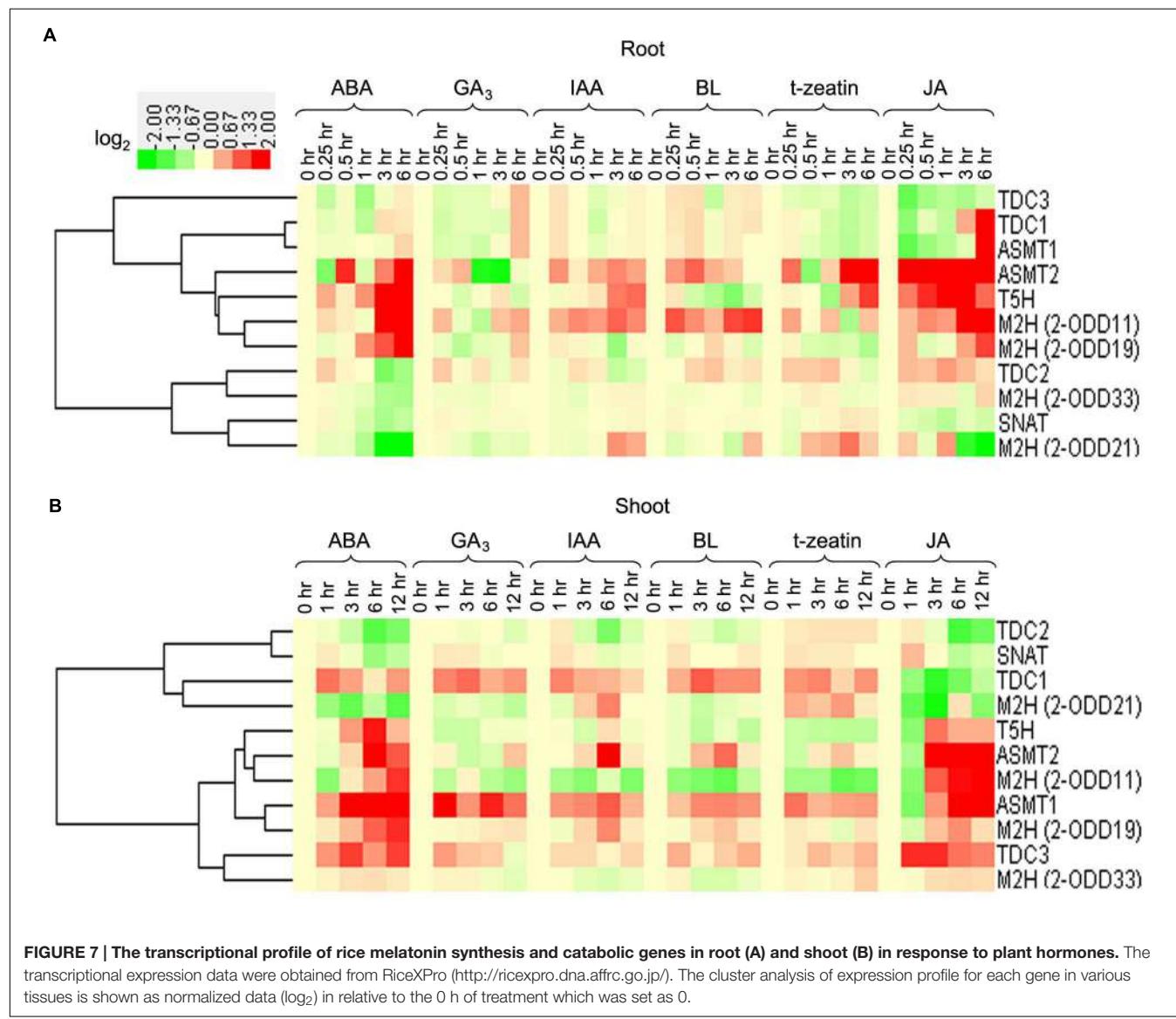
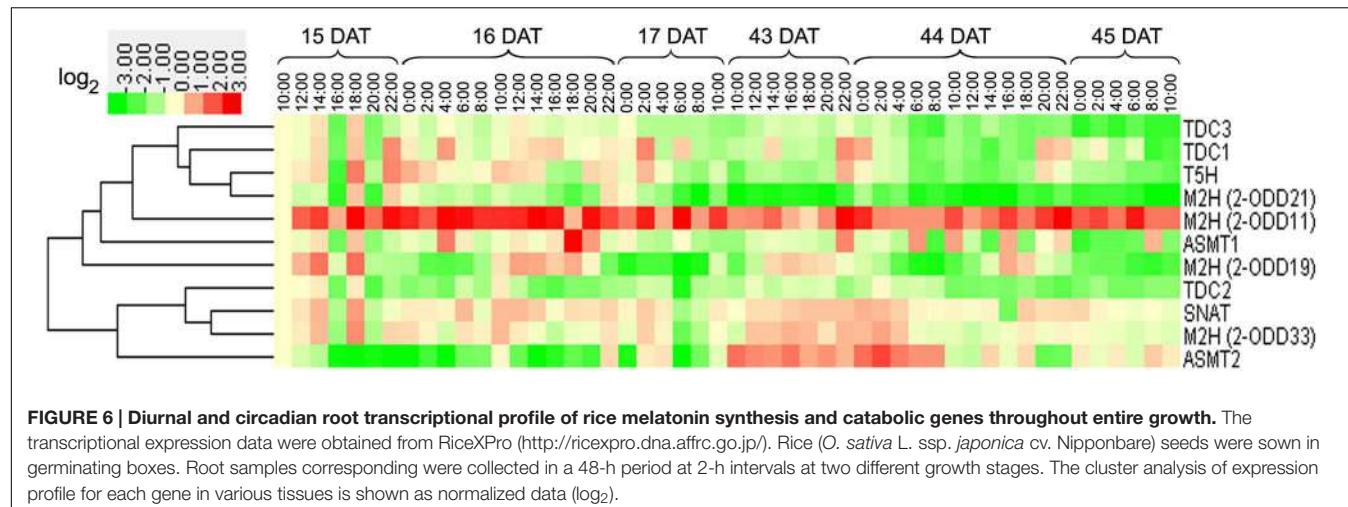
After inoculation with *Xoo*, *OsASMT2* expression was induced during almost all the time-points, while *OsASMT1* transcript was decreased at these time points (**Figure 8**). The transcripts of

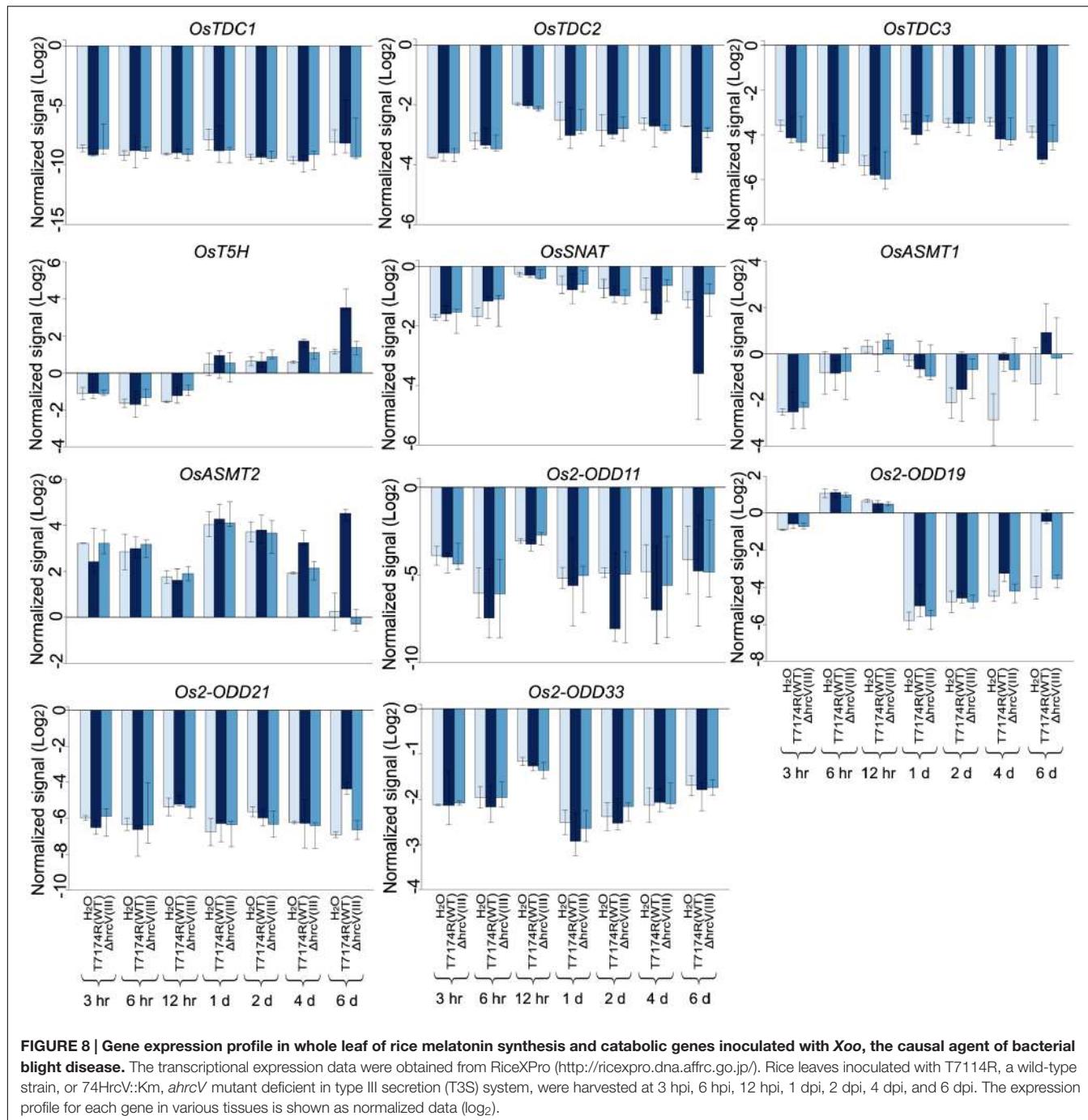
*OsT5H* and *Os2-ODD19* were induced during 1 to 6 dpi and 6 to 12 hpi, respectively (**Figure 8**). *OsTDC1*, –2, –3, *Os2-ODD11*, –21, –33, and *OsSNAT*, expressions were decreased during all the treated time points (**Figure 8**). Interestingly, the transcripts of *OsASMT1* and *OsASMT2* were increased after 6 dpi of wild-type strain T7114R, but were decreased after 6 dpi of  $\Delta ahrcV(III)$  strain. T3S is essential for *Xoo*T7174R conferred plant disease, and  $\Delta ahrcV(III)$  resulted in less plant disease in rice leaves (Sato et al., 2011b, 2013). Thus, the results indicate the possible role of *OsASMT1* and *OsASMT2* in immune response to *Xoo*.

After inoculation with the blast fungus (*M. oryzae*), the transcript levels of three genes (*OsT5H*, *OsASMT1*, and *Os2-ODD11*) showed up-regulation at most treated time points, while those of four genes (*OsTDC1*, –3, *OsSNAT*, and *Os2-ODD21*) showed down-regulation (**Supplementary Figure S1**). In response to inoculation with the three strains of *M. oryzae* harboring *AVR-Pia* and *AVR-Pish* (*Pia/Pish* × P91-15B, *Pish* × P91-15B, and *Pish* × Kyu77-07A), the transcript levels of *OsASMT1*, *Os2-ODD11*, –19, –33, and *OsT5H* showed significant up-regulation, while those of *OsTDC3*, *OsSNAT*, and *Os2-ODD21* were seriously down-regulated at all time points (**Supplementary Figure S1**). *OsASMT2* expression showed no obvious trends in response to inoculation with the two former, while was induced at all the treated time-points after inoculation with *Pish* × Kyu77-07A strain (**Supplementary Figure S1**). In response to inoculation with the *pish* mutant of *M. oryzae* ( $\Delta Pish$  × Kyu77-07A), the expressions of six genes (*OsTDC1*, *OsT5H*, *OsASMT1*, –2, *Os2-ODD11*, and 19) were significantly induced at all time points, while four genes (*OsTDC2*, –3, *OsSNAT*, and *Os2-ODD33*) were seriously down-regulated (**Supplementary Figure S1**). *Os2-ODD21* expression was strongly repressed at 2 dpi. Notably, *Os2-ODD33* expression was induced at all the treated time-points after inoculation with the three strains of *M. oryzae* harboring *AVR-Pia* and *AVR-Pish*, but was repressed in response to inoculation with  $\Delta Pish$  × Kyu77-07A strain (**Supplementary Figure S1**).

### The Transcriptional Profile of Rice Melatonin Synthesis and Catabolic Genes in Response to Abiotic Stress Treatments

Melatonin is widely involved in plant stress responses (Shi et al., 2015b,d). Thus, investigation of the expression profiles of melatonin-related genes of rice in response to various abiotic stresses is needed. After drought treatment, the transcripts of *OsTDC1*, *OsASMT1*, and *Os2-ODD19* were found to be up-regulated between 1.2 and 1.8-folds, whereas those of *OsTDC2*, –3, *Os2-ODD11*, –21, *OsT5H*, and *OsSNAT* were strongly repressed in comparison to the control (**Figure 9**). After salt treatment, the transcripts of *OsTDC1*, –3, *OsASMT1*, and *Os2-ODD19* were increased between 1.3 and 4.5-fold, whereas NaCl strongly repressed *OsTDC2*, *OsT5H*, *OsSNAT*, and *Os2-ODD11* expressions (**Figure 9**). After 4°C treatment, *OsTDC1* and *OsASMT1* transcript levels were slightly increased, whereas the expressions of six genes (*Os2-ODD11*, –19, –21, *OsTDC3*, *OsT5H*, and *OsSNAT*) were obviously down-regulated (**Figure 9**).



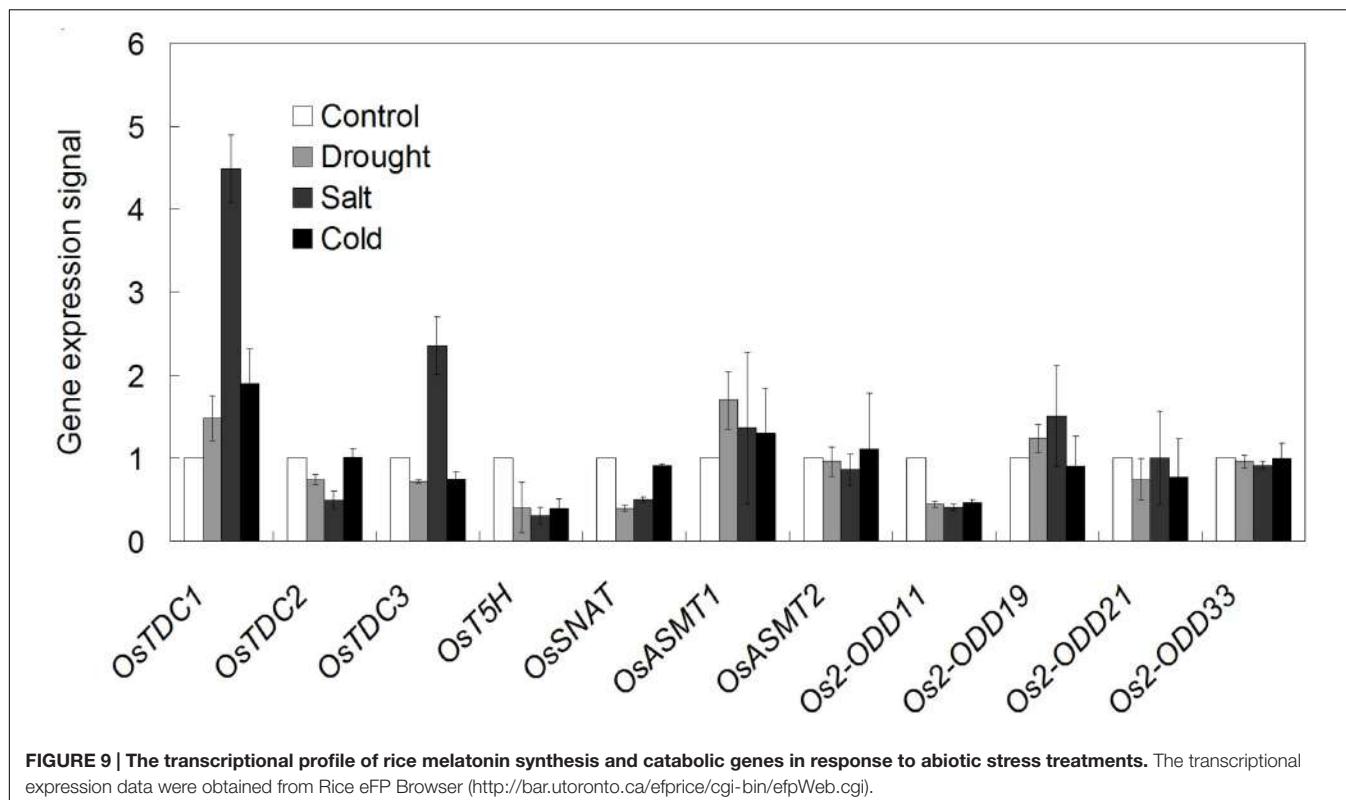


Notably, the transcript of *OsTDC3* was significantly increased after salt treatment, but was seriously decreased in response to drought and cold treatments (Figure 9). The result indicated that *OsTDC1* and *OsTDC3* may be involved in salt stress response.

## DISCUSSION

To our knowledge, this is the first study systematically analyzed the diurnal and circadian transcriptional profile of melatonin

synthesis and catabolic genes throughout the entire growth stages in rice. Our study confirmed that *OsTDCs* showed higher expression level at reproductive 1 and reproductive ripening stages than other stages (Figure 5). In cherry fruit (*Prunus avium*), the expression level of *PaTDC* was positively correlated to melatonin concentration throughout the entire period, and showed regularly circadian rhythm during a 24 h period with two peaks at 5:00 and 14:00 (Zhao et al., 2013), indicating that the melatonin concentration was higher in those two stages than others. The expression pattern of *OsTDC3* also showed circadian



**FIGURE 9 | The transcriptional profile of rice melatonin synthesis and catabolic genes in response to abiotic stress treatments.** The transcriptional expression data were obtained from Rice eFP Browser (<http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi>).

rhythm on 16 DAT (Figure 6). Similarly, this expression pattern was also existed at *Os2-ODD19* during the entire development (Figure 5). These results suggested that *OsTDC3* and *Os2-ODD19* may be involved in modulating endogenous during the entire development in rice.

Melatonin is widely involved in plant growth and development, as well as stress responses (Bajwa et al., 2014; Meng et al., 2014; Wang et al., 2014; Zuo et al., 2014; Liang et al., 2015; Shi et al., 2015b). In apple (*Malus prunifolia*), the transcripts of melatonin synthesis genes (*MdTDC1*, *MdAANAT2*, *MdT5H4*, and *MdASMT1*) was induced after drought treatment (Li et al., 2014). The concentration of melatonin was increased in barley roots and lupin after cold, drought and salt treatments (Arnao and Hernández-Ruiz, 2009, 2013). Consistently, *OsTDC1* and *OsASMT1* transcript levels were increased after drought, salt and cold treatments (Figure 9), indicating their possible involvement in abiotic stress response.

Recently, melatonin was shown to function as positive modulator against plant pathogen infection (Yin et al., 2013; Lee et al., 2014, 2015; Reiter et al., 2015; Shi et al., 2015a; Zhao et al., 2015). Exogenous application of melatonin improved apple resistance to *D. mali*, the pathogen of Marssonina apple blotch (Yin et al., 2013), enhanced *Arabidopsis* and tobacco defense against *P. syringae* pv. tomato DC3000 (Lee et al., 2014). Moreover, *Arabidopsis snat* knockout mutants exhibited increased susceptibility to the avirulent pathogen *P. syringae* pv. tomato DC3000 with decreased SA levels and reduced defense genes expression compared with wild-type (Lee et al., 2015). However, whether *OsSNAT* also involves in the

regulation of plant immunity remains unknown. Treatments with melatonin significantly enhanced antioxidant protection in rice, suggesting that melatonin plays a major role in regulating pathogen infection (Liang et al., 2015). In response to bacterial pathogen infection, some genes showed similar expression profiles (Figure 8). The differential response of melatonin-related genes to pathogen infection in different kinds implied the dual mechanisms underlying melatonin-related genes mediated pathogens responses.

Previous studies have revealed that melatonin had significant effect in regulating ABA and GA<sub>4</sub> in plant response to salinity and drought stress (Li et al., 2014; Zhang H.J. et al., 2014). Additionally, melatonin shared the common substrate (tryptophan) with IAA (Arnao and Hernández-Ruiz, 2014), and AXR3/IAA17 is involved in *Arabidopsis* melatonin signaling underlying senescence (Shi et al., 2015c). Thus, genome-wide expression analysis of melatonin-related genes in response to plant hormones may provide new insight into crosstalk between melatonin and plant hormones. Plant hormones, such as ABA, SA, and GA, related with most of the plant physiological responses, including water logging, drought and salt stress responses (Yang et al., 2004; Kim et al., 2011, 2014; Shimamura et al., 2014). Melatonin is also a regulator of ABA and GA<sub>4</sub> in plant response to salinity and drought stress (Li et al., 2014; Zhang H.J. et al., 2014). Moreover, the expression of *OsASMT2* and *OsASMT3* were induced after ABA and JA treatments at 1-month-old detached rice leaves, while were down-regulated in response to ethephon, zeatin, and SA stress. *OsASMT1* also showed up-regulation upon ABA and JA stress treatment, but

did not display obvious trends during ethephon, zeatin, and SA treatments (Park et al., 2013a). In this study, the transcripts of four genes (*Os2-ODD11*, *-19*, *OsASMT2*, and *OsT5H*) and five genes (*OsASMT1*, *-2*, *OsT5H*, *Os2-ODD19*, and *OsTDC3*) were increased in response to ABA stress during 3 h to 6 h treatment in root and during 3 to 12 h in shoot, respectively. Under JA stress, the transcripts of three genes (*OsASMT2*, *OsT5H*, and *Os2-ODD11*) and three genes (*OsASMT1*, *-2*, and *Os2-ODD11*) were significantly increased during 0.25 to 6 h in root and 3 h to 12 h in shoot, respectively. Thus, different transcriptional responses of melatonin-related genes in hormone specific manners, suggested the dual role and crosstalk between melatonin and various hormones.

It is widely known that ABA is the most important regulator of abiotic stress (Kim et al., 2011, 2014; Shimamura et al., 2014), and JA serves as the major defense hormone that are associated with pathogen infection (Van der Ent et al., 2009; Ballaré, 2011; Xie et al., 2011; Yamada et al., 2012; Yang et al., 2013; Campos et al., 2014). More recently, the crucial role of ABA in virulence of rice blast fungus *M. oryzae* is confirmed (Spence et al., 2015), the involvement of JA in abiotic stress response is also largely confirmed (Riemann et al., 2015; Wasternack and Strnad, 2016). We highlight the commonly regulation of rice melatonin-related genes by ABA, JA, pathogen infection and various abiotic stresses (Figures 7–9), indicating the possible role of these genes in multiple stress responses and underlying crosstalks of plant hormones, especially ABA and JA. Weeda et al. (2014) identified 1308 differentially expressed genes (566 up-regulated genes and 742 down-regulated genes) exhibiting at least of twofold change by exogenous melatonin treatment in *Arabidopsis*, and many of them are enriched in plant hormone signaling. These differentially expressed genes include 52 genes in auxin signaling, 50 genes in ABA signaling, 67 genes in JA pathway, and 42 genes in ET pathway. Our studies together with the data of Weeda et al. (2014) further indicate the interaction among melatonin, ABA and JA pathways.

We have to pointed out the possible limitation of this study, since the different changes in expression levels of the various genes do not always explain in a simple way why melatonin concentrations increase or decrease under the different conditions. On one hand, there may be difference between expression level and enzyme activity, such as the posttranslational regulation of AANAT in primates via phosphorylation/dephosphorylation and association/dissociation of a 14-3-3 protein, which is decisive for the melatonin rhythm in those organisms (Ganguly et al., 2001, 2005). On the other hand, incomplete knowledge of rate-limiting enzymes or isoenzymes may also lead to the difference. Further studies by other methods may give more clues.

Taken together, the expression patterns of 11 melatonin related genes from rice were synthetically analyzed at different periods and after different treatments in this study. These information may provide abundant resources for functional characterization of melatonin related genes. The differential expression patterns of melatonin related genes in different tissues throughout entire growth stages and stress responses will be useful to investigate *in vivo* role of specific gene in

rice development and circadian rhythm. Thus, this study will contribute to better understand the melatonin biosynthesis and catabolic pathway as well as their association with development and stress responses in rice. Further functional analysis of identified candidate genes with potential involvement in circadian rhythm and stress responses will give shed more lights in melatonin-mediated signaling as well as underlying molecular mechanism.

## AUTHOR CONTRIBUTIONS

HS conceived and directed this study, analyzed the data, wrote, and revised the manuscript; YW and HZ performed the experiments, analyzed the data, wrote, and revised the manuscript; WH and LC analyzed the data and revised the manuscript; CH provided suggestions and revised the manuscript. All authors approved the manuscript and the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00676>

**TABLE S1 | The detailed data used for Figures 3–7 and Figure 9.** The original data were obtained from RiceXPro (<http://ricexpro.dna.affrc.go.jp/>) and Rice eFP Browser (<http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi>).

**FIGURE S1 | Gene expression profile in whole leaf of rice melatonin synthetic and degraded genes inoculated with the blast fungus, *M. oryzae*.** The transcriptional expression data were obtained from RiceXPro (<http://ricexpro.dna.affrc.go.jp/>). Three lines of rice cultivar Nipponbare carrying the blast resistance genes (*Pia*, *Pish*) were inoculated with two strains of *M. oryzae* harboring AVR-*Pia* and AVR-*Pish*. Rice seedlings at the 4-leaf stage were inoculated with the conidial suspension of *M. oryzae* and the leaves were harvested at 1, 2, 3, and 5 dpi. The expression profile for each gene in various tissues is shown as normalized data ( $\log_2$ ).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# A Simple, Rapid Method for Determination of Melatonin in Plant Tissues by UPLC Coupled with High Resolution Orbitrap Mass Spectrometry

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Melatonin (MLT) was involved in regulating various stages of plant growth and development. However, due to the low concentration and complex matrixes of plant, the analysis of MLT is a challenging task. In this study, we developed a rapid and efficient method with simplified sample preparation by employing UPLC coupled with a high resolution Orbitrap mass spectrometry, and stable isotope-labeled MLT (MLT-d<sub>4</sub>) was first used as internal standard in the developed analytical method. In the developed method, we used one-step liquid–liquid extraction to purify the crude extracts both from shoot and root of rice for the analysis, which remarkably simplify the sample preparation process. The method exhibits high specificity and recovery yield (>96.4%). Good linearities were obtained for MLT ranging from 0.01 to 20 ng/ mL with determination coefficient ( $R^2$ ) of 0.9991. The limit of detection for MLT was 0.03 pg. Reproducibility of the method was evaluated by intra-day and inter-day measurements and the results showed that relative standard deviations were less than 7.2%. Moreover, MLT quantification was accomplished by using only 100 mg fresh plant tissues. Additionally, the established method was successfully applied to investigate the spatiotemporal distributions of MLT in rice under cadmium (Cd) stress condition. We found that the content of MLT in shoot and root of rice increased under Cd stress, suggesting that MLT would play a crucial role in modulating the responses to Cd stress in different plant tissues.

**Keywords:** LC-MS, melatonin, Orbitrap, rice, stable isotope

## INTRODUCTION

Studies on melatonin (*N*-acetyl-5-methoxytryptamine, MLT) in plants have attracted great attention of researchers in recent years. MLT is an indoleamine that has been found to widely distribute in the animal kingdom (Arnao and Hernández-Ruiz, 2015). As an animal hormone, especially a neurohormone (Reiter, 1991), it plays a vital role in vertebrates. Since the first discovery and detection of MLT in plants by Dubbels et al. (1995) and Hattori et al. (1995), successive studies have been invested in its possible physiological functions in plants. In the past 20 years, MLT has been found in more than 140 plant species

(Hardestrand et al., 2007; Zohar et al., 2011; Gomez et al., 2013; Arnao, 2014; Feng et al., 2014). Moreover, it was reported to be involved in almost all of the plant growth and development stages, from seeds germination to the senescence (Hardestrand et al., 2007; Paredes et al., 2009; Arnao and Hernández-Ruiz, 2015; Reiter et al., 2015; Hardestrand, 2016). Additionally, many studies have shown that MLT could effectively protect plant tissues from the damage caused by a variety of abiotic and biotic stresses, such as drought stress (Shi et al., 2015b,c), salt stress (Shi et al., 2015b,c), cold stress (Bajwa et al., 2014; Shi et al., 2015b,c), heat stress (Byeon and Back, 2014; Shi et al., 2015d), and pathogen infection (Yin et al., 2013; Shi et al., 2015a).

Accurate and sensitive determination of endogenous MLT enabled us to better understand its physiological functions, biological synthesis and metabolic pathways, and the regulatory networks. Various quantitative approaches have been developed to analyze MLT content in plants. Immunoassays such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) have long been applied to MLT quantification (Dubbels et al., 1995; Hattori et al., 1995; Pape and Lüning, 2006; Gomez et al., 2013). ELISA kits are commercially available, along with its ease of operation make it a convenient and common tool for MLT analysis (Pape and Lüning, 2006). However, the cross-reactivity of antibodies is inevitable, which leads to reduced specificity and accuracy (Huang and Mazza, 2011a; Gomez et al., 2013). Gas chromatography coupled to mass spectrometry (GC-MS) has also been used to the qualitative and quantitative analysis of MLT, although it has improved sensitivity and specificity, the requirement for sample volatile derivatization presents a disadvantage for its application to MLT determination (Badria, 2002; Ragab et al., 2006). The sensitivity of capillary electrophoresis (CE) methods has also been reported to be similar to the high performance liquid chromatography (HPLC) approaches (Gomez et al., 2015). HPLC is the most commonly used technique for the

analysis of MLT. In this case, different detectors have been used, such as fluorescence detector (FD) (Lavizzari et al., 2006; Pape and Lüning, 2006; Arnao and Hernández-Ruiz, 2007; Padumanonda et al., 2014), electrochemical detector (ECD) (Hernández-Ruiz et al., 2004, 2005; Reiter et al., 2005; Zettersten et al., 2009), and UV (Hosseini et al., 2008; Ly et al., 2008). However, compared to the MS detector, these detectors exposed their shortcomings of low sensitivity and specificity. LC coupled with MS has been proved as a powerful method for MLT analysis because of its high sensitivity, reproducibility and accuracy (Cao et al., 2006; Hernández-Ruiz and Arnao, 2008; Huang and Mazza, 2011b; Gomez et al., 2012, 2013).

Unfortunately, it is unpractical to directly analyze endogenous MLT in plant tissues by LC-MS due to its low concentration and the complicated matrix of plant extracts. To overcome the difficulty, different sample purification techniques such as liquid-liquid extraction (LLE) (Arnao and Hernández-Ruiz, 2009; Huang and Mazza, 2011a,b; Byeon and Back, 2014), solid phase extraction (SPE) (Huang and Mazza, 2011a; Gomez et al., 2015), microextraction by packed sorbent (MEPS) (Mercolini et al., 2012), HPLC purification (Pape and Lüning, 2006) were commonly employed to enrich MLT from plant tissues. As the classical techniques, LLE and SPE are the most used methods for purification of MLT (Huang and Mazza, 2011a,b; Gomez et al., 2015). At the previous stage, a large volume of solvent and a large amount of plant tissues were needed when LLE was utilized. Thanks to the development of detection techniques, the extraction solvent volume for LLE and the plant amount needed could be decreased, and the LLE merits of fast and easy operation could be kept.

Moreover, matrix effects caused by endogenous interferents often occur during the analysis of low abundant metabolites in the complicated matrix of plant extracts using LC-MS. Nevertheless, these effects can be compensated by using isotope

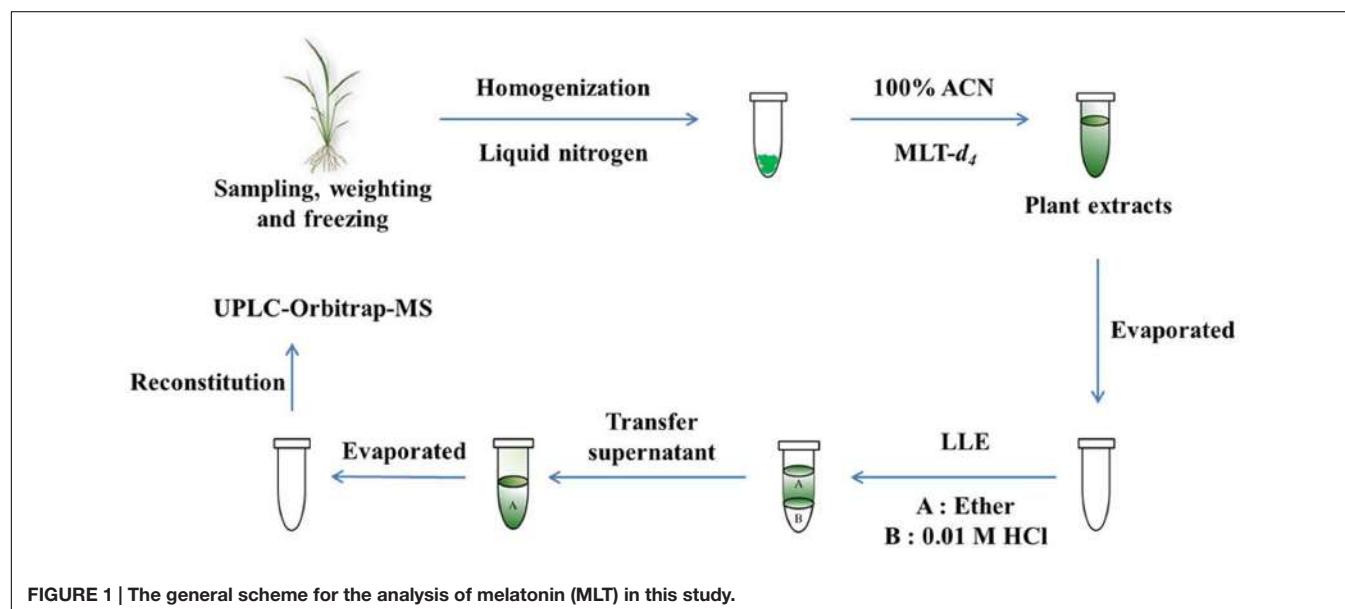
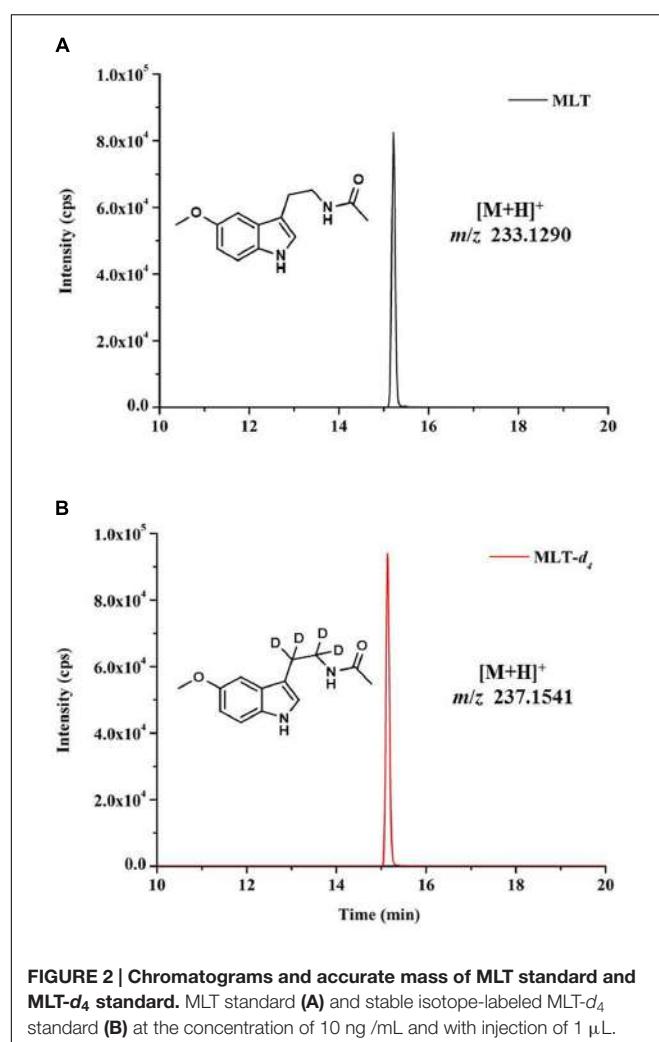
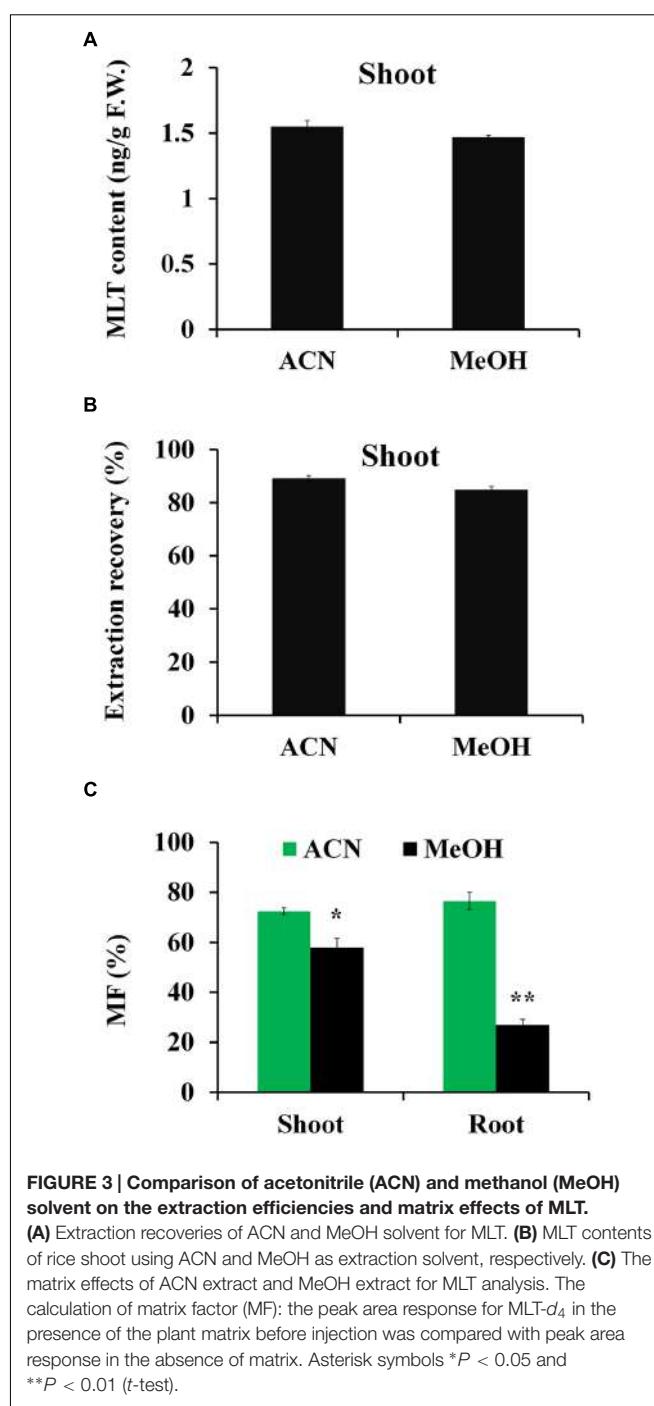


FIGURE 1 | The general scheme for the analysis of melatonin (MLT) in this study.



of dilution techniques (Barkawi et al., 2010; Porfírio et al., 2016). Stable isotope-labeled compound(s) is (are) essential internal standard(s) for their physical and chemical similarities with the original analyte(s), providing correction for loss analyte during the experiment procedures and matrix effects during ionization (Barkawi et al., 2010; Porfírio et al., 2016). However, isotope dilution technique has not been reported to be applied to the analysis of MLT content in plants.

Additionally, a highly sensitive and selective MS method was crucial to identify analytes of interest from complicated matrix of plant. The higher specificity of the technique also leads to a higher sensitivity due to the reduced background noise. Multi reaction monitoring (MRM) (Gomez et al., 2012, 2013; Kocadali et al., 2014) and selective reaction monitoring (SRM) modes (Huang and Mazza, 2011b) have been used previously to the quantification of MLT. And they have been reported to be powerful tools to improve the sensitivity and specificity of analytes. Ions at  $m/z$  216 and 174 were chosen as MS/MS signatures for MLT and have proven particularly useful for quantification (Huang and Mazza, 2011b; Gomez et al., 2012; Gomez et al., 2013; Kocadali et al., 2014). However, the low mass



resolution used in these studies could result in false positives, specifically in case of trace metabolites like MLT. Therefore, high-resolution LC-MS is of utmost importance in identification of trace metabolites like MLT or IAA allowing for high sensitivity and appropriate specificity even from crude plant extracts (Yu et al., 2014).

Herein, we developed a rapid, sensitive, and efficient method for the analysis of MLT content by employing a high resolution Orbitrap MS. It enables us to minimize the steps of sample

preparation. For both shoot and root of rice, just one-step LLE was added to purify the crude extracts for the analysis. Furthermore, to correct the loss analyte and matrix effects, stable isotope-labeled MLT ( $\text{MLT}-d_4$ ) was added as internal standard to plant samples prior to extraction. Using the developed method, we successfully investigated the dynamic distributions of MLT in response to Cd stress in shoot and root of rice.

## MATERIALS AND METHODS

### Chemicals and Reagents

Melatonin standard was purchased from Sigma Chemical (St. Louis, MO, USA). Stable isotope-labeled standard, [ $^2\text{H}_4$ ] MLT, was purchased from C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). Chromatography grade acetonitrile (ACN) and methanol (MeOH) were obtained from TEDIA Co. (Fairfield, OH, USA). Ultra-pure water used in the study was purified with Milli-Q system (Milford, MA, USA). Hydrochloric acid (HCl) was purchased from Sinopharm Chemical Reagent (Shanghai, China).

### Plant Materials

Rice (*Oryza sativa* ssp. *japonica* cv. Nipponbare) seeds were germinated and then transplant under hydroponic conditions in Hoagland's nutrient solution in a growth chamber with 70–80% relative humidity under photoperiods of 16 h light (28°C)/8 h dark (25°C). After 10 days growing under normal conditions, the seedlings were subjected to Cd stress with a series concentration of  $\text{CdNO}_3$  (0, 100, 200, and 500  $\mu\text{M}$ ). Shoot and root of rice

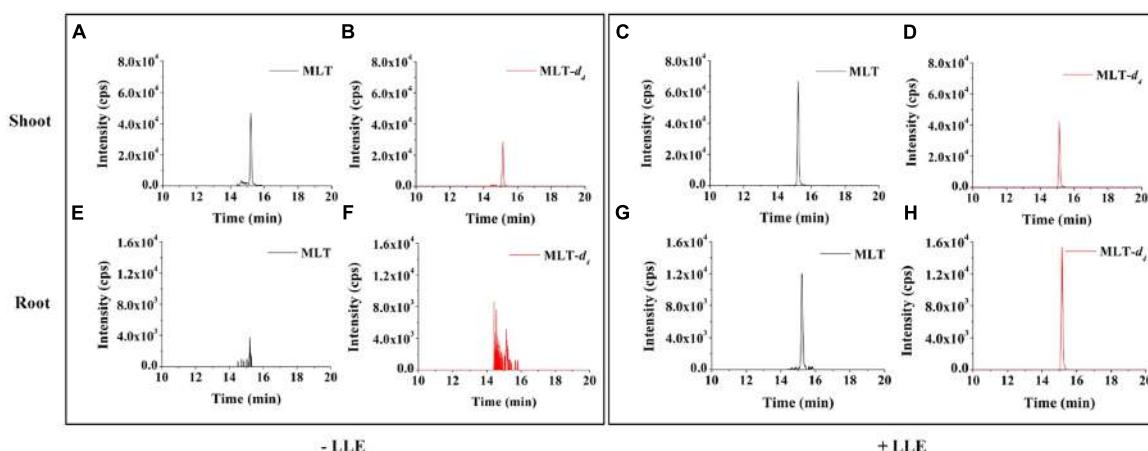
were harvested after 5, 10, 15 days stresses separately, weighted and then stored at -80°C immediately after freezing in liquid nitrogen.

### Preparation of Plant Samples

Rice samples (100 mg F.W.) were frozen in liquid nitrogen and finely ground, followed by extraction with 1 mL ACN at 4°C for 12 h in dark. [ $^2\text{H}_4$ ] MLT (1.0 ng/g) were added to the samples as internal standards (IS). After centrifugation at 12,000 rpm and 4°C for 10 min, the supernatants were sequentially evaporated under mild nitrogen stream. The evaporated samples followed by re-dissolving in 100  $\mu\text{L}$  0.1 M HCl, and then extracted with ether (1 mL). The ether phase was collected, dried under nitrogen gas and reconstituted in 100  $\mu\text{L}$   $\text{H}_2\text{O}/\text{MeOH}$  (80/20, v/v) for further analysis."

### Instruments and Analytical Conditions

Analysis of MLT was performed on an UltiMate 3000 UHPLC System (Thermo-Dionex) equipped with LTQ Orbitrap MS (Thermo-Fisher Scientific, Waltham, MA, USA). The separation of MLT was achieved on an Acquity UPLC BEH phenyl column (2.1 mm × 100 mm, 1.7  $\mu\text{m}$ , Waters) with a flowrate of 0.2 mL/min. The column oven temperature was set at 35°C. Water (solvent A) and acetonitrile (solvent B) were employed as mobile phase. The gradient was started at an initial composition of 95% A and 5% B, 2–20 min, 40% B, then 23 min linear gradient to 90% B, held for 5 min. A return to the initial conditions was accomplished by a 2 min gradient to 95% B, it was held for 10 min, total chromatographic run time was 40 min.



**FIGURE 4 | Comparison of chromatograms with or without LLE purification for MLT analysis.** Chromatograms of MLT standard and  $\text{MLT}-d_4$  standard in an extract of 100 mg rice shoot (A–D) and root (E–H) samples with or without purification of LLE.

**TABLE 1 | Calibration characteristics of melatonin (MLT) standard spiked in solution of rice seedling samples.**

Analyte	Linear range (ng/mL)	Regression data			LOD (pg)	LOQ (pg)
		Slope	Intercept	$R^2$ value		
Melatonin	0.01–20	0.9538	0.0566	0.9991	0.03	0.11

**TABLE 2 |** Accuracy and precision (intra- and inter-day) for the determination of MLT in shoot and root tissues of rice (100 mg F.W.).

Analyte	Tissues	Intra-day precision (RSD, %, n = 5)			Inter-day precision (RSD, %, n = 5)			Recovery (%), n = 5)		
		Low (1 ng/mL)	Medium (5 ng/mL)	High (20 ng/mL)	Low (1 ng/mL)	Medium (5 ng/mL)	High (20 ng/mL)	Low (1 ng/mL)	Medium (5 ng/mL)	High (20 ng/mL)
Melatonin	Shoot	3.76	3.13	2.89	1.28	0.47	1.11	101.35 ± 3.97	96.47 ± 4.07	96.42 ± 1.68
	Root	1.97	6.15	7.23	3.17	5.75	4.71	98.09 ± 6.15	97.33 ± 6.33	105.15 ± 2.98

The MS was set to acquire full MS scan in positive ion mode with a mass range of m/z 150–300 at a resolution of 120,000. Ion source conditions were as follows: heater temperature, 300°C; capillary temperature, 350°C; sheath gas flow, 35 arbitrary; auxiliary gas flow, 15 arbitrary; spray voltage, 3.5 kV; S-lens RF level, 60%. Data acquisition and analysis was performed using Xcalibur v3.0.63 (Thermo Fisher Scientific) and SIEVE v2.2 (ThermoFisher Scientific, USA).

## Determination of Electrolyte Leakage (EL)

Electrolyte leakage (EL) was determined according to the previously reported method (Ye et al., 2015). In brief, the initial conductivity was determined after gently shaking at room temperature for 6 h at 150 rpm using a conductivity meter (Leici-DDS-307A, Shanghai, China). The fully releasing conductivity was measured after boiling at 121°C for 20 min using previous samples. The percentage of EL was determined as the ratio of the initial conductivity to fully releasing conductivity.

## Statistical Analysis

The results were shown as means ± standard errors ( $n = 3$ ), SPSS 13.0 software was used for statistical analysis. Analyses of variance (ANOVA) for variables from measurements were used for testing the species and treatment differences according to Duncan's method. Different letters above the columns in each figure indicate significant differences at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### General Design of Strategy

Generally, each additional step would result in loss of analyte to some extent, especially for MLT with the antioxidant capability and light-sensitive characteristic. So, minimizing the number of sample preparation steps is essential. Moreover, high-resolution and accurate MS would benefit for simplifying procedures for sample preparation procedure and reducing analysis time, so an UPLC-high resolution Orbitrap MS was applied to the analysis of MLT in our study. Additionally, stable isotope-labeled MLT (MLT-d<sub>4</sub>) was added as internal standard, providing correction for loss analyte during the experiment procedures and matrix effects during ionization. The whole schematic diagram for the analysis of MLT in this study was shown in Figure 1.

### Comparison of Different Extraction Solvent

Melatonin is amphipathic substances, with antioxidant capabilities and poor ionization efficiency (Huang and Mazza, 2011a). And concentration of MLT in complicated plant matrix is fairly low, whereas numerous of other substances with far greater amounts are present. Therefore, it is important to choose an extraction method that yields good recovery. To achieve high extraction efficiency, two frequently used organic solvents, ACN and MeOH were chosen for the optimization of MLT extraction

from plant tissues. The retention time and accurate mass of MLT standard and MLT-*d*<sub>4</sub> standard were shown in **Figure 2**. Our result showed the extraction efficiencies of MLT by ACN and MeOH were similar, and there is no significant different of MLT content in shoot (MLT signal was severely suppressed in crude extract of root in both solvents, and it will be discussed in next section) of rice using ACN (1.55 ng/g) or MeOH (1.47 ng/g), and the both solvents exhibited good extraction recoveries (>80%) (**Figure 3**).

In plant samples, matrix often play negative roles in MS signals. Due to the complicated matrix of plant extract, the abundant interferences from the plant extract might depress the MS response of MLT. So, the matrix effects were further assessed between ACN extract and MeOH extract. The MS response (peak area) for MLT-*d*<sub>4</sub> in the presence of the plant matrix was compared to peak area in the absence of matrix to calculate matrix factor (MF) according to Yu et al. (2016). For shoot samples, MF from ACN extract was slightly higher than MeOH extract. But for root sample, a strong matrix effect was observed for MeOH extract (MF, 30%), while approximately more than two times lower than ACN extract (MF, 76%) (**Figure 3**). Therefore, ACN was selected as appropriate solvent for extracting MLT from rice tissues.

## Optimization of Sample Preparation Procedure

In the previous section, we mentioned that MLT signal was strongly suppressed in crude extract of root. To solve this problem, further purification step was needed. Taking advantage of the analyte solubility differences in different solvents, LLE is an effective way to purify the MLT in plants (Chen et al., 2012). Furthermore, LLE can improve the ionization efficiency by decreasing the influence of impurities (Chen et al., 2012). The evaporated sample followed by re-dissolving in 100  $\mu$ L 0.1 M HCl, and then extracted with ether (1 mL). The results showed that with the purification of LLE, the signal intensity of MLT in root samples was significantly increased, while the intensity of MLT was also increased slightly in shoot samples (**Figure 4**). Therefore, LLE was adopted to eliminate the polar impurities and improve the ionization efficiency of MLT in rice.

## Method Validation

To evaluate the quantitative aspect of the developed method, calibration curve of MLT was established. The MLT standard (0.01–20 ng/mL) was spiked in shoot and root samples

(100 mg F.W.). The internal standard MLT-*d*<sub>4</sub> was added to the sample prior to extraction step. The calibration curves were plotted by the peak area ratio (MLT labeled with MLT-*d*<sub>4</sub>) against the MLT concentrations. As shown in **Table 1**, good linearity was obtained for MLT with coefficient of determination ( $R^2$ ) of 0.9991. The LODs and the LOQs were calculated as the concentration of the analyte at a Signal/Noise (S/N) ratio of 3 and 10, respectively. The results showed that LOD and LOQ for MLT were 0.03 and 0.11 pg, respectively.

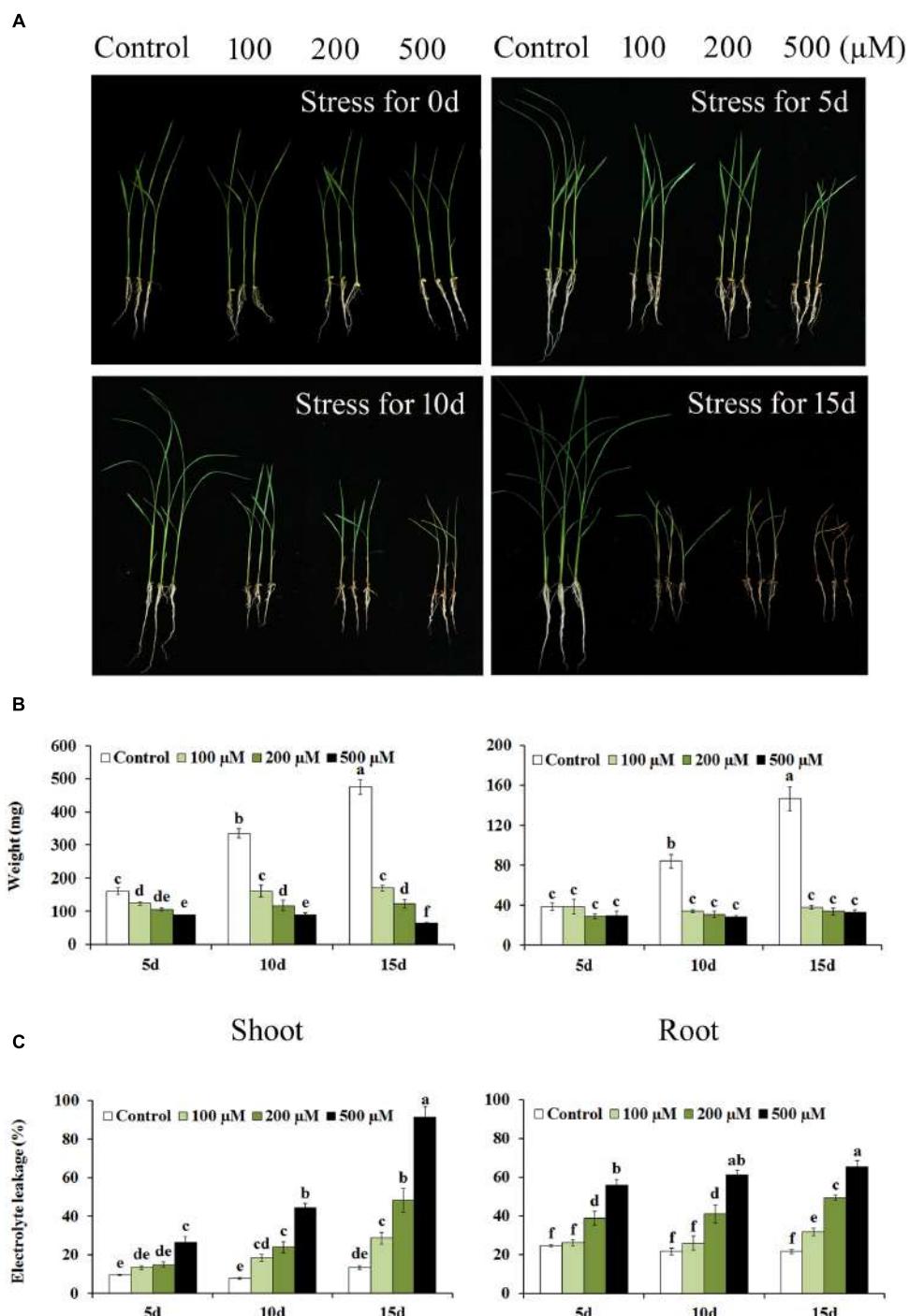
The accuracy and precision of the developed method were further assessed, respectively, by the recoveries and by the relative standard deviations (RSDs) of intra- and inter-day. Both recoveries and intra- and inter-day RSDs were calculated with MLT standard spiked in rice samples at three different concentrations. The intra-day variation was evaluated by repeating the process for five times within one day and the inter-day variation was investigated on five successive days. For each concentration, five replicated measurements were performed in both shoot and root samples. The relative recoveries were calculated by comparing the peak area ratios of MLT from the spiked rice samples to those from the standard solutions. As shown in **Table 2**, good precisions were obtained with RSD values below 7.2%, and the relative recoveries were in the range of 96.4 to 105.1%, indicating the good reproducibility and accuracy of developed method.

Furthermore, to assess the general utility of proposed method for determination of MLT in other plant species or different tissues of plant, the method was used to detect MLT in *whole seedlings and leaves of Arabidopsis* (Col-0), rice leaves (*Oryza sativa* ssp. *japonica* cv. Nipponbare), cotton leaves and fibers (*Gossypium hirsutum*), rape leaves (*Brassica napus* L. cv. Zhongshuang 11). As shown in **Table 3**, the content of MLT varies in different plant species and tissues. The MLT content in leaves of one-month-old *Arabidopsis* and *Brassica napus* was similar. In rice leaves (10 days after transplant under hydroponic conditions), the MLT reached 0.58 ng/g F.W., which was about threefolds higher than in *Arabidopsis* leaves. The MLT content in *Gossypium* sp of maturity date was much higher than rice. The relative recoveries from different plant tissues were found to be in the range of 84.3 to 104.2%, indicating the universal applicability of the developed method.

Compared with previous LC-MS methods, our proposed method showed the following improvements. Firstly, high resolution MS was used for the detection of MLT. Accurate m/z

**TABLE 3 | General utility assessment of proposed method for determination of MLT in different plant species and tissues.**

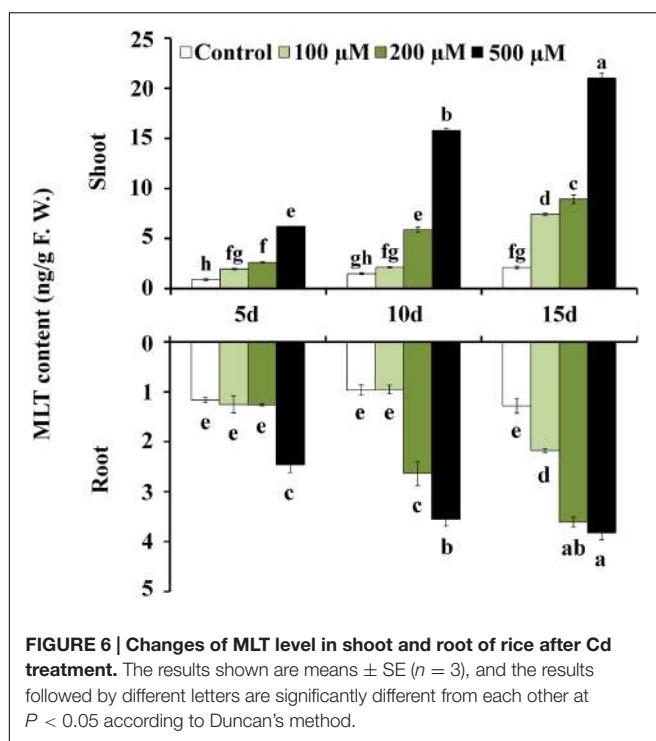
Analyte	Plant species	Plant tissues	Content (ng/g F.W.)	Recovery (%), n = 3
Melatonin	<i>Arabidopsis</i>	Whole seedling	0.22 ± 0.01	88.84 ± 5.42
	<i>Gossypium</i> sp.	Fibers	0.34 ± 0.01	99.11 ± 9.76
	<i>Arabidopsis</i>	Leaf	0.19 ± 0.03	104.21 ± 5.98
	<i>Brassica campestris</i> L.	Leaf	0.23 ± 0.02	91.88 ± 3.59
	<i>Gossypium</i> sp.	Leaf	0.74 ± 0.01	102.89 ± 2.01
	<i>Oryza sativa</i>	Leaf	0.58 ± 0.01	84.28 ± 7.27



**FIGURE 5 | Comparison of physiological responses to cadmium (Cd) stress in shoot and root of rice. phenotypes (A), weights (B), EL (C) of rice shoot and root under control and stressed condition at designated time intervals. The data represent the means of three independent experiment  $\pm$  SE, and data followed by different letters are significantly different from each other at  $P < 0.05$  according to Duncan's method.**

(5 ppm) of the target analytes was monitored with high selectivity. The high specificity of the m/z value lead to the decreased matrix interference and improved sensitivity of analytes by reducing the background noise. Secondly, we employed a simple and effective

LLE procedure to purify MLT in plant tissues, and the matrix interference got further decreased. Thirdly, the stable isotope dilution strategy was first used for absolute quantification of MLT in plant tissues, and worked well to correct the loss of analyte



**FIGURE 6 | Changes of MLT level in shoot and root of rice after Cd treatment.** The results shown are means  $\pm$  SE ( $n = 3$ ), and the results followed by different letters are significantly different from each other at  $P < 0.05$  according to Duncan's method.

during the sample pretreatment procedures and signal variation in MS detection.

### MLT Distribution under Cd Stress in Shoot and Root of Rice

Cadmium is one of most toxic pollutions in the present. Unlike the other heavy metal pollutions, Cd is a non-essential nutrient, and seriously affects plant growth and development. Recently, Cd has been reported to regulate MLT content in rice leaves by coordinating the synthesis and degradation genes of MLT (Byeon et al., 2015). And then Hasan et al. found, exogenous application of MLT could mitigate phytotoxicity induced by Cd stress in *Solanum lycopersicum* L., by modulation of phytochelatins biosynthesis, vacuolar sequestration, and antioxidant potential (Hasan et al., 2015). However, our knowledge of MLT for regulating response of plant to Cd stress is still fragmentary (Arnao and Hernández-Ruiz, 2014).

In this study, we investigate the distributions of MLT both in shoot and root of rice under Cd stress condition using the established method. 10-day-old rice seedlings were subjected to Cd stress with a series concentration of CdNO<sub>3</sub> (0, 100, 200, and 500 μM). When exposed to 15 day treatments, significant differences in the growth of rice were observed, suggesting that Cd stress caused serious damages (growth inhibition, wilted leaves) on rice (Figure 5). The shoot weight only reached 63.5 mg at 15 day under Cd condition of 500 μM, which were only 13% of control seedlings, while the growth of root was almost completely inhibited with the Cd treatment of 200 and 500 μM (Figure 5). Additionally, Cd stress progressively increased the EL with the duration of the treatment. The EL reached 91% at 15 day under Cd treatment of 500 μM (Figure 5). These result

indicated Cd stress caused severe cell membrane damages and severely inhibited rice growth. Nevertheless, the MLT content in rice shoot was increased with the increasing of Cd concentration and the stress time. After 15 day treatment of 500 μM Cd, the MLT content in rice shoot was up to 21.0 ng/g, which was about 10-folds higher than control (Figure 6). Additionally, the MLT levels in rice root under Cd stress were detected as well (Figure 6). The results showed that MLT content was also gradually increased over time after Cd treatment in root. Notably, higher concentration of Cd treatment induced higher level of MLT. Under Cd condition of 500 μM at 15 day, the MLT content in root was up to 3.9 ng/g, which was over three times higher than control. Our results suggested that MLT might play a critical role in rice tolerance to Cd stress both in shoot and root tissues. The MLT content was also induced by Cd treatment in root, indicating MLT might be involved in preventing translocation of Cd from root to shoot, and might modulate the responses to Cd stress through the different mechanisms in shoot and root. Further studies are still needed to provide the evidence in support of the involvement of melatonin in Cd tolerance and detoxification, and to illuminate signaling pathway in different tissues of plants.

### CONCLUSION

In this study, we developed a rapid, sensitive and efficient sample preparation method to accurate measure MLT in crude plants extracts down to 100 ng F.W. by employing a high resolution Orbitrap MS and using stable isotope-labeled MLT (MLT-d<sub>4</sub>) as internal standard. Using this approach, we successfully minimize sample purification process into one-step LLE procedure to purify the crude extracts in rice, which greatly simplified the sample preparation procedure and improved the analytical throughput. The established method was successfully applied to investigate the dynamic distributions of MLT responses to Cd stress in shoot and root of rice. The developed method may facilitate to better understanding the physiological functions and regulatory mechanism of MLT in plant.

### AUTHOR CONTRIBUTIONS

TY designed and performed the experiments, analyzed the data, and wrote the manuscript; Y-HH performed the experiments and analyzed the data; LY performed the experiments; HS and RR revised the manuscript; Y-QF designed the experiments and revised the manuscript; and all authors approved the manuscript.

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# Comparative Transcriptomic Analyses of Differentially Expressed Genes in Transgenic Melatonin Biosynthesis Ovine *HIOMT* Gene in Switchgrass

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Melatonin serves pleiotropic functions in prompting plant growth and resistance to various stresses. The accurate biosynthetic pathway of melatonin remains elusive in plant species, while the N-acetyltransferase and O-methyltransferase were considered to be the last two key enzymes during its biosynthesis. To investigate the biosynthesis and metabolic pathway of melatonin in plants, the RNA-seq profile of overexpression of the ovine *HIOMT* was analyzed and compared with the previous transcriptome of transgenic *oAANAT* gene in switchgrass, a model plant for cellulosic ethanol production. A total of 946, 405, and 807 differentially expressed unigenes were observed in *AANAT* vs. control, *HIOMT* vs. control, and *AANAT* vs. *HIOMT*, respectively. Two hundred and seventy-five upregulated and 130 downregulated unigenes were detected in transgenic *oHIOMT* line comparing with control, including the significantly upregulated (F-box/kelch-repeat protein, zinc finger BED domain-containing protein-3) genes, which were potentially correlated with enhanced phenotypes of shoot, stem and root growth in transgenic *oHIOMT* switchgrass. Several stress resistant related genes (SPX domain-containing membrane protein, copper transporter 1, late blight resistance protein homolog R1A-6 OS etc.) were specifically and significantly upregulated in transgenic *oHIOMT* only, while metabolism-related genes (phenylalanine-4-hydroxylase, tyrosine decarboxylase 1, protein disulfide-isomerase and galactinol synthase 2 etc.) were significantly upregulated in transgenic *oAANAT* only. These results provide new insights into the biosynthetic and physiological functional networks of melatonin in plants.

**Keywords:** switchgrass, melatonin, transgene, *oHIOMT*, RNA-seq

## INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) was first discovered in vertebrates (Lerner et al., 1958), then detected in higher plants (Dubbels et al., 1995; Hattori et al., 1995), and now is widely accepted of its distribution in all kingdoms, from prokaryotes to eukaryotes, from animals to plants (Manchester et al., 2000; Hardeland and Poeggeler, 2003; Simopoulos et al., 2005; Hardeland, 2015), and functions as a direct scavenger of reactive oxygen species (ROS), a mediator hormone of circadian rhythms and an activator of antioxidant enzymes (Arnao and Hernández-Ruiz, 2015; Reiter et al., 2015; Bai et al., 2015, 2016; Gao et al., 2016; Shi et al., 2016a). In plants, melatonin regulated the seed germination, growth of roots, and shoots, and development of flowering (Hernández-Ruiz et al., 2005; Zhao et al., 2013; Arnao and Hernández-Ruiz, 2015). Moreover, the responses to abiotic stresses, including extreme temperature, drought, salt, radiation, make melatonin a concerned candidate as a natural stimulator for crop cultivation (Li et al., 2012; Zhang et al., 2014a; Fan et al., 2015; Shi et al., 2015a). However, the involved regulations of the functional gene expression and physiological mechanism of melatonin biosynthesis and metabolic pathways remains poorly understood in plants (Hardeland, 2015; Zhang et al., 2014b).

The classic pathway of melatonin biosynthesis comprises four steps beginning with tryptophan, firstly decarboxylation by tryptophan decarboxylase (TDC), then *N*-acetylation by arylalkylamine *N*-acetyltransferase (AANAT) in animals/serotonin *N*-acetyltransferase (SNAT) in plants, and the final *O*-methylation by *N*-acetylserotonin methyltransferase (ASMT) in animal/hydroxyindole-*O*-methyltransferase (HIOMT) in plants (Tan et al., 2016). The last two steps are presumed as rate-limiting which are catalyzed by AANAT/SNAT and HIOMT/ASMT (Morton and Forbes, 1988; Byeon et al., 2015, 2016). Furthermore, the overexpression of rice caffeic acid *O*-methyltransferase (COMT) exhibited upregulation of melatonin contents in transgenic rice plants, indicating that the *N*-acetylserotonin methyltransferase activity was required for melatonin biosynthesis (Byeon et al., 2015). Recently, genetic engineering modifications of coding genes for melatonin biosynthesis and metabolism enzymes were applied to alter the melatonin contents in transgenic rice, tomato, *Arabidopsis thaliana*, and *Nicotiana sylvestris* (Kang et al., 2010; Okazaki et al., 2010; Park et al., 2012; Zhang et al., 2012; Wang et al., 2014; Zuo et al., 2014).

Switchgrass (*Panicum virgatum*) is a Poaceae warm season C4 perennial grass native to the U.S., and is regarded as a model plant of cellulosic biofuel production for its desirable characteristics, such as large biomass and strong ability to thrive in marginal areas (McLaughlin and Kszos, 2005; Keshwani and Cheng, 2009; Brown et al., 2016). Molecular breeding has made much progress toward improving biomass yield, biofuel quality, and stress resistance in switchgrass, which is the model plant for cellulose ethanol production (Bouton, 2007; Fu et al., 2011; Shen et al., 2013; Poovaiah et al., 2014; Wuddineh et al., 2015). Recently, a series of transgenic overexpression of transcriptional factor (*NAC*, *AP2/ERF*, *MYB4*), sucrose synthesis gene (*SUS*) to prompt growth (Shen et al., 2013; Poovaiah et al., 2014; Yang

et al., 2014; Wuddineh et al., 2015), the inhabitation of cinnamyl alcohol dehydrogenase (*CAD*) to reduce the lignin content (Fu et al., 2011), and the exploration of abiotic stress related genes and miRNA (Sun et al., 2012; Sharma et al., 2015; Liu et al., 2016) were produced in switchgrass. In addition, the activities of antioxidant and free radical scavenger for melatonin provide opportunities for prompting growth and development in plants (Arnao and Hernández-Ruiz, 2015; Reiter et al., 2015; Shi et al., 2016a). The exogenous applications of melatonin exhibited the enhanced seed germination in cucumber (Zhang et al., 2014a), lateral root formation in both *Brassica juncea* (Chen et al., 2009) and cucumber (Zhang et al., 2014c), and salt resistance in soybean plants (Wei et al., 2015) etc. Other studies revealed that melatonin also exercised some control over root architecture as observed in St. John's Wort, wild leaf mustard, sweet cherry root stocks, and lupin (Murch et al., 2001; Arnao and Hernández-Ruiz, 2007; Chen et al., 2009; Sarropoulou et al., 2012). Moreover, the endogenous modifications of related genes to gain the melatonin-rich plants displayed cold resistance in rice (Kang et al., 2010), the drought-tolerant phenotypes of tomato (Wang et al., 2014) and *A. thaliana* (Zuo et al., 2014). Here, the *HIOMT* gene encoding the last enzyme in melatonin biosynthesis pathway was overexpressed in switchgrass, and the transcriptomic profile was analyzed in order to disentangle the melatonin biosynthesis pathway and also the potential functions of melatonin in plants.

## MATERIALS AND METHODS

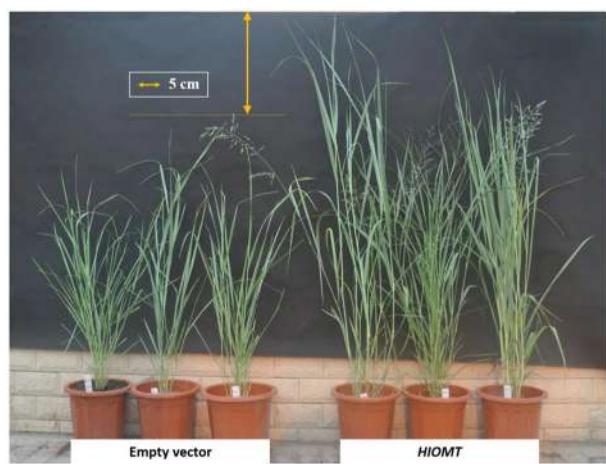
### Plant Materials and Morphological Traits

Transgenic switchgrass (cultivar Alamo) lines expressing ovine *HIOMT* gene (ID: JF815374.1) were grown under 16 h light (26°C, 120 μmol/m<sup>2</sup>/s) and 8 h dark (18°C) conditions in greenhouse. Fully matured plants were chosen from each genotype for molecular characterization and transcriptome sequencing. Three replicates of stems for each transgenic line and in total six (three transgenic *HIOMT* (H) lines: H1, H2, H3, and three transgenic empty vector (EV): EV1, EV2, and EV3) were collected and frozen in liquid nitrogen and stored at 80°C until analysis. The *t*-test was applied to compare the differences of the morphological traits between transgenic *oHIOMT* lines and control.

At the transgenic reproductive third (R3) stage (Hardin et al., 2013), the tiller number, plant height, stem node number, internode length, internode diameter, leaf blade length, leaf blade width, root number, root length, root diameter, and spike length were determined (Figure 1). The third internode was chosen for measuring internode diameter. Leaf blade length and leaf blade width of third internode were measured. Twelve replicates were randomly sampled for each transgenic line.

### RNA Isolation and Qualification

Total RNA was extracted from the sampling stems using Trizol method (Invitrogen, Carlsbad, CA, USA). RNA purity and integrity were assessed using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA),



**FIGURE 1 |** Phenotypes of the transgenic *HIOMT* switchgrass comparing with the transgenic empty vector (EV).

respectively. RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer.

## Transcriptome Preparation

The 1.5 µg RNA per sample was prepared for the RNA-seq. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). The cDNA fragments of 150~200 bp were selected from the library by purification with AMPure XP system (Beckman Coulter, Beverly, USA).

## Clustering

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, paired-end reads were generated by sequencing with the library preparations on an Illumina Hiseq platform.

## Validation of RNA-seq Data by Real-Time Quantitative

RNA-seq results were validated by Real-time quantitative PCR of 16 different genes using 7500 Real-Time PCR System (Applied Biosystems) (primer sequences please see Supplementary Table 1). Gene expression levels were calculated by the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). Each plate was repeated three times in independent runs for all reference and selected genes.

## Data Analysis

### Quality Control

After removing the adapter containing reads, ploy-N containing reads and low quality reads, clean data/clean reads were obtained from raw data. Meanwhile, the Q20, Q30 values, GC-contents, and sequence duplication level of the clean data were calculated (Supplementary Table 2). The RNA-seq raw data have been

submitted to NCBI SRA dataset (<http://www.ncbi.nlm.nih.gov/bioproject>), and the project accession number is PRJNA322585.

## Transcriptome Assembly and Gene Functional Annotation

*De novo* transcriptome assembly was constructed and accomplished for the incomplete genome of switchgrass using Trinity with min\_kmer\_cov set to 2 and all other parameters set default (Grabherr et al., 2011). Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology).

## Differential Expression Analysis

Gene expression levels were calculated by RSEM (Li and Dewey, 2011). To reveal the transcriptionally regulatory event occurring during the transgenic process, comparative transcriptomic analysis was performed among the pools of control and the two transgenic (*oAANAT* and *oHIOMT*) RNA samples. Only the genes with a *P*-value-adjusted (*padj*) < 0.05 were identified as being significantly changed by the Benjamini and Hochberg's approach.

## GO Enrichment Analysis and KEGG Pathway Enrichment Analysis

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can adjust for gene length bias in DEGs. KEGG (Kanehisa et al., 2008) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism, and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (<http://www.genome.jp/kegg/>). The KOBAS software was performed to test the statistical enrichment of differential expression genes in KEGG pathways (Mao et al., 2005).

## RESULTS

### Promoted Vegetative and Reproductive Growth

To compare the effects of *HIOMT* gene during the biosynthesis processes, the phenotypic traits were measured. Transgenic *HIOMT* switchgrass exhibited significant promotion of growth comparing with EV (Figure 1; Supplementary Table 3). Average plant height and internode length were 35.6 and 52.9% higher in *HIOMT* (90.60 and 13.43 cm) than that of EV (66.81 and 8.78 cm, respectively, *P* < 0.05). Stem node number of *HIOMT* was 15.6% higher than that of EV (*P* > 0.05). There was no significant difference in internode diameter between the two groups (*P* > 0.05). Average leaf blade length was 20.7% longer in *HIOMT* (52.53 cm) than that of EV (43.53 cm, *P* < 0.05). Root number,

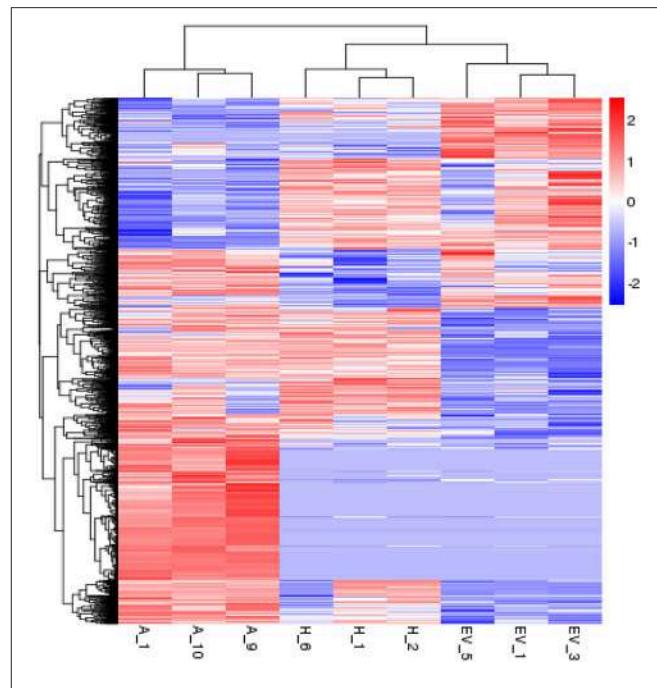
root length and root diameter of *HIOMT* were 75.8, 18.3, and 39.4% larger than those of EV, respectively ( $P < 0.05$ ). Spike length in *HIOMT* (19.5 cm) was nearly 4-fold of the EV (5.4 cm,  $P < 0.01$ ).

## Differential Expression Profiling between Transgenic *oHIOMT* and EV Lines

To analyze the similarities and differences in the transgenic transcriptome, a hierarchical clustering was represented and indicated the significant differences in transcripts of all of the DEGs in the three replicates of the control and two transgenic groups (Figure 2). After calculating the expression level of each mapped unigene, a total of 1556 unigenes were detected that had levels of expression that were significantly different among the two transgenic and control libraries. The 946, 405, and 807 differentially expressed unigenes were observed in *AANAT* vs. EV, *HIOMT* vs. EV, and *AANAT* vs. *HIOMT*, respectively (Figure 3). A total of 183 genes were differentially expressed in both *AANAT* vs. EV and *HIOMT* vs. EV, but not significantly different in *AANAT* vs. *HIOMT*. Three unigenes overlapped with all three groups, including c52804\_g3 (hypothetical protein), c56995\_g3 (O-methyltransferase), c56995\_g4 (ribosomal protein). One hundred twenty nine unigenes were upregulated in both transgenic *oAANAT* and *oHIOMT* comparing with EV, while 55 unigenes were downregulated. There were 145 and 76 genes that significantly upregulated and downregulated in transgenic *oHIOMT*, respectively, but not differentially expressed in transgenic *oAANAT*. Comparatively, there were 607 and 153 genes that significantly upregulated and downregulated in transgenic *oAANAT*, while not differentially expressed in transgenic *oHIOMT* (Supplementary File 1). Twenty-five unigenes specifically and differentially expressed in *HIOMT*, including 13 upregulated (c64524\_g1, c52718\_g1: glycine-rich domain-containing protein 1-like; c43728\_g1: Bowman-Birk type wound-induced proteinase inhibitor WIP1-like; c62742\_g1: SPX domain-containing membrane protein) and 12 downregulated ones (c46269\_g1: NAC domain-containing protein 67-like; c61012\_g1: protein tyrosine kinase) comparing with *AANAT* and EV (Supplementary File 1).

## Functional Classification of the DEGs by Gene Ontology Analysis

Among all differentially expressed unigenes, 1071 were upregulated among the three libraries, and 513 were downregulated. However, only 511, 193, and 504 unigenes were functionally annotated with GO terms in *AANAT* vs. EV, *HIOMT* vs. EV, and *AANAT* vs. *HIOMT*, respectively, revealed by DEG analysis were functionally assigned to the relevant terms in three categories (Biological Process, Cellular Component, and Molecular Function) of the GO database (Figure 4). The GO terms “organonitrogen compound biosynthetic process,” “oxidoreductase activity,” “NAD(P)H oxidase activity,” growth factor activity, and “hyperosmotic response” were highly enriched in the DEGs. At the same time, other terms that were related to the response to various other types of abiotic and biotic stresses, such as SOS response “phototropism,” “cellular response



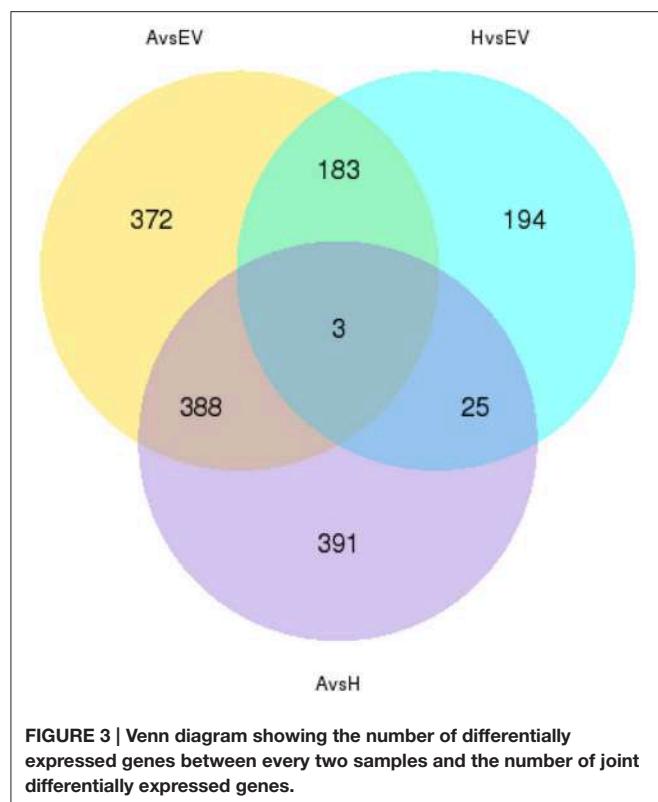
**FIGURE 2 |** Hierarchical clustering of the differentially expressed genes, using the RNA-seq data derived from three groups (A: *AANAT*, H: *HIOMT*, and EV: empty vector, hereafter) based on log<sub>10</sub> RPKM values.

to external stimulus,” and “response to external stimulus,” were also highly enriched in the DEGs.

By comparing transgenic *AANAT* vs. EV, 2012 GO terms were DEGs, including 1687 upregulated and 1094 downregulated GO items. The GO terms of the “peptide metabolic process” and “amide biosynthetic process” in Biological Process and “structural molecule activity” in Molecular Function were significantly overrepresented (Figure 4). By comparing transgenic *HIOMT* vs. EV, 1314 DEGs, including 724 downregulated GO items and 1062 upregulated GO items, were functionally assigned to the relevant terms; “metabolic process” in Biological Process was significantly overrepresented, followed by “oxidation-reduction process” in Biological Process and “oxidoreductase activity” in Molecular Function. Notably, none of the GO terms was significantly enriched after multiple testing corrections (corrected  $P > 0.05$ ) in *HIOMT* vs. EV. Considering the *AANAT* vs. *HIOMT*, 1904 DEGs, including 1493 upregulated and 1126 downregulated GO items, were significantly overrepresented of the “macromolecule biosynthetic process” and “cellular macromolecule biosynthetic process” in Biological Process, “cytoplasm,” and “cytoplasmic part” in Cell Component, and “structural molecule activity” and “structural constituent of ribosome” in Molecular Function (Figure 4).

## KEGG Pathway Analysis of the Melatonin-Related Genes

The 50 pathways were identified between the *HIOMT* vs. EV from the KEGG database, with 36 upregulated and



17 downregulated pathways (**Figure 5**). The DEGs were significantly enriched in “homologous recombination,” “mineral absorption,” “isoquinoline alkaloid biosynthesis,” “tropine, piperidine, and pyridine alkaloid biosynthesis,” “nitrogen metabolism,” “flavonoid biosynthesis,” “steroid biosynthesis,” “alpha-Linolenic acid metabolism,” “tyrosine metabolism,” and “beta-Alanine metabolism.” The top 20 obviously enriched pathways are shown in **Figure 5**. Specifically, the photosynthesis-antenna proteins pathway, MAPK signaling pathway and oxidative phosphorylation were significantly upregulated in KEGG pathway maps during the transgenic *oAANAT* line. While nitrogen metabolism, flavonoid biosynthesis, beta-Alanine metabolism, Brassinosteroid biosynthesis, Phenylalanine metabolism, and Ascorbate and aldarate metabolism were significantly upregulated in transgenic *oHIOMT* line comparing with EV (Supplementary File 2). Furthermore, a total of 166 pathways, with 141 upregulated and 66 downregulated pathways, represented the DEGs between *AANAT* and *HIOMT* groups, including “ribosome,” “oxidative phosphorylation,” “photosynthesis,” “DNA replication” and “calcium signaling pathway.”

## Validation of Gene Expression Profiles Using RT-qPCR

The 16 DEGs were selected to evaluate the different expressions for qRT-PCR. Total RNA samples extracted from switchgrass leaves at reproductive stages were used as templates. Histograms were generated by comparing the FPKM determined by

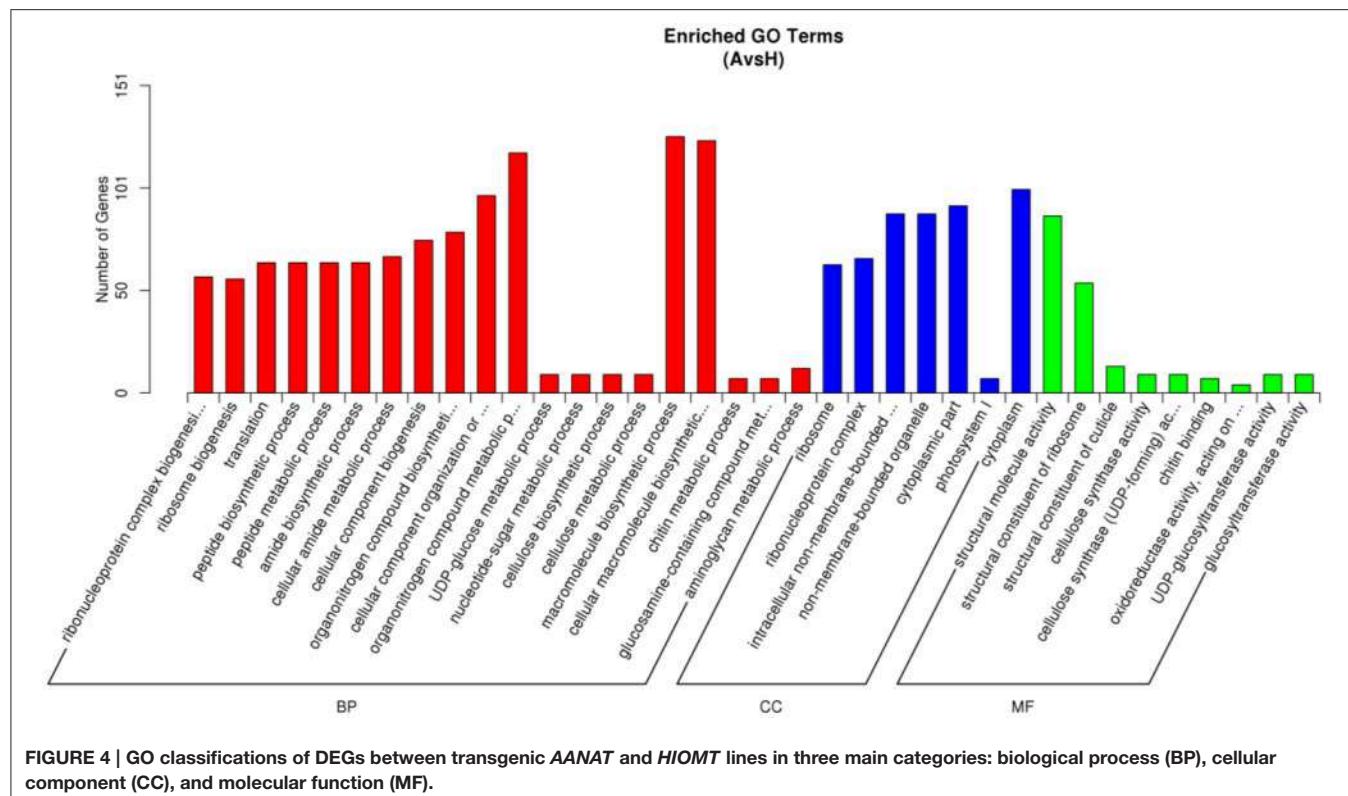
transcriptome analysis and qRT-PCR. Expression quantities of the selected genes using RT-qPCR were consistent with the results obtained with RNA-Seq analysis, which means the RNA-seq data were credible. ( $R^2 = 0.861$  for *AANAT*,  $R^2 = 0.933$  for *HIOMT*,  $P < 0.01$ ) (**Figure 6**).

## DISCUSSION

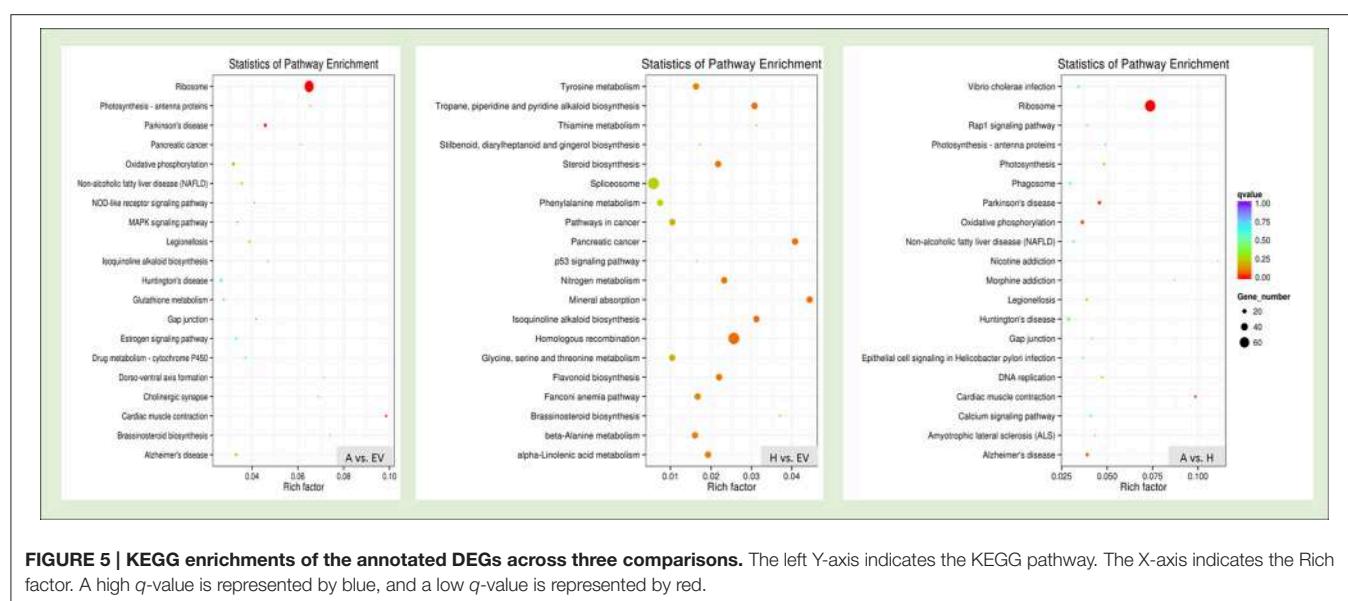
The catalytic reactions of the two key enzymes (*AANAT* and *HIOMT*) are the last two steps during the melatonin biosynthesis processes, and the overexpression of the two genes significantly enhanced the melatonin levels in rice (Kang et al., 2010) and tomato (Wang et al., 2014). In our study, the average melatonin contents of transgenic *oAANAT* and *oHIOMT* lines was 12 and 36% ( $P < 0.05$ ) higher than that of EV control, respectively. Melatonin aids plants in terms of root growth, leaf morphology, chlorophyll preservation, and fruit development (Reiter et al., 2015). The enhanced phenotypic growth traits in transgenic *oHIOMT* switchgrass were in accordant with the increasing of the melatonin contents, and average shoot height, leaf length, root number, root length, and spike length of transgenic *HIOMT* lines surpassed those of EV control (**Figure 1**; Supplementary Table 3). The transcriptional level of the related genes was significantly upregulated, for instance, F-box/kelch-repeat protein, zinc finger BED domain-containing protein 3, RAX2-like protein and so on (**Table 1**). Other researches demonstrated that increasing of melatonin significantly stimulate vegetative growth by both exogenous and endogenous applications in several plant species (Hernández-Ruiz et al., 2005; Wang et al., 2014).

Furthermore, the transgenic *oHIOMT* switchgrass exhibited earlier flowering than that of the EV, suggesting that the reproductive development was expedited by the endogenous addition of melatonin contents (**Figure 1**). The reduction and delay of flowering were reported under the exogenous melatonin treatment of *Chenopodium rubrum* (Kolář et al., 2003) and transgenic melatonin-rich rice (Byeon and Back, 2014), respectively. Melatonin is potentially involved in the regulation of flowering process by the similar pattern of the antioxidant ascorbic acid (Kotchoni et al., 2009) or the impediment of the floral transition from vegetative growth to reproductive growth by the repressor of gibberellic acid (GA) pathway (Shi et al., 2016b). However, other researches implied that melatonin was transiently induced with a peak level during flower development in rice (Park et al., 2013). In our study, one of the flowering regulator encoding gene *APETALA2* significantly upregulated in transgenic *oHIOMT* line ( $\log_2\text{FoldChange} = 6.11$  for *c52062\_g5*,  $P < 0.01$ , **Table 1**) comparing with the EV control, which acts on the regulation of establishment of the floral meristem (Chen, 2004). The accumulation of melatonin represented the resistance for internal and external oxidative stress during reproductive development (Park et al., 2013).

Melatonin also plays innate immune responses to the complex of biotic and abiotic stresses in various plants species, dicots and monocots (Lee et al., 2014; Shi et al., 2015b; Hardeland,



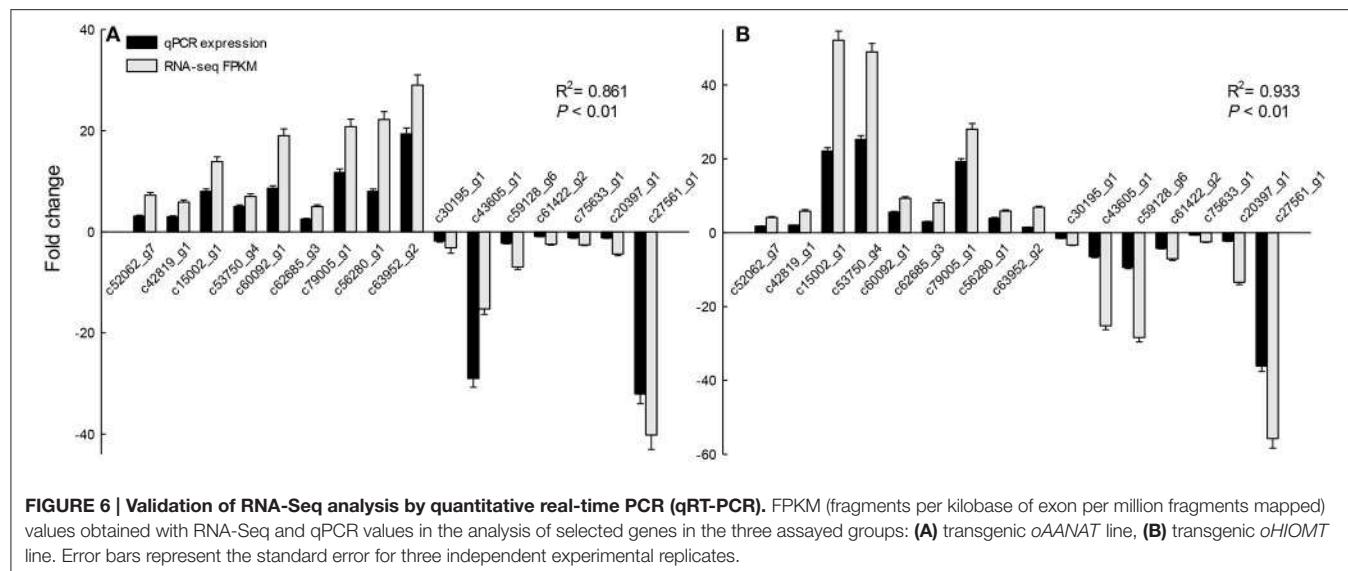
**FIGURE 4 |** GO classifications of DEGs between transgenic *AANAT* and *HIOMT* lines in three main categories: biological process (BP), cellular component (CC), and molecular function (MF).



**FIGURE 5 |** KEGG enrichments of the annotated DEGs across three comparisons. The left Y-axis indicates the KEGG pathway. The X-axis indicates the Rich factor. A high q-value is represented by blue, and a low q-value is represented by red.

2016). As summarized in recent publications, increases of melatonin were typically induced by diverse forms of stresses, including extreme temperatures, drought, salinity, and oxidant (Li et al., 2012; Zhang et al., 2014a; Fan et al., 2015). From our results, the overexpression of *oHIOMT* gene also drives a series of defense genes activated, for instance, c43076\_g1 gene (annotated to “response to stress”) was significantly

upregulated comparing with the EV (Supplementary File 1). In addition, the several amino acid and secondary metabolite related pathways were significantly upregulated, including flavonoid biosynthesis, tyrosine metabolism, beta-Alanine metabolism, glycine, serine, and threonine metabolism, brassinosteroid biosynthesis, phenylalanine metabolism, suggesting the activated growth and stress responses in transgenic *oHIOMT* lines



(Supplementary File 2). Other researches exhibited similar tolerance to drought in transgenic *MzASMT* *A. thaliana* (Zuo et al., 2014), and the resistance to herbicide-induced oxidative damages in transgenic melatonin-rich rice (Park et al., 2012). The transcriptional regulation of defense genes alleviate the oxidative damages driven by various stresses (Wei et al., 2016).

Notably, the differentially expressed unigenes in *HIOMT* vs. EV (405) was less than those of *AANAT* vs. EV (946), suggesting that the overexpression of *oAANAT* could affect more growth-related genes than that of *oHIOMT*. In contrast, the significantly enriched pathways of the DEGs in *HIOMT* were more than those of *AANAT*, indicating that the alterations of involved processes driving by the O-methyltransferase were more complex (Figures 3, 5). The previous RNA-seq analysis of overexpression of *oAANAT* gene reported a large number of differentially expressed unigenes, which were majorly involved in various compound biosynthetic processes and organelle component (Yuan et al., 2016). Here, the synchronously upregulated transcriptional factors and growth related genes in transgenic *oAANAT* and *oHIOMT* implied the prompting roles of melatonin in plant growth and development comparing with EV. In addition, a number of differentially expressed genes specifically driving by transgenic *oAANAT* or *oHIOMT* potentially indicated the typical functions of each enzyme. For instance, several stress resistant related genes (SPX domain-containing membrane protein, copper transporter 1, late blight resistance protein homolog R1A-6 OS etc.) were specifically and significantly upregulated in transgenic *oHIOMT* only, while metabolism-related genes (phenylalanine-4-hydroxylase, tyrosine decarboxylase 1, protein disulfide-isomerase, and galactinol synthase 2 etc.) were significantly upregulated in transgenic *oAANAT* only (Table 1). The previous transgenic tomato represented the branching phenotype in overexpressing of *oAANAT* and *oHIOMT* and drought tolerance in transgenic *oHIOMT* lines (Wang et al., 2014). These indicated the both similarity and differences of the functions of the two key

enzymes in the melatonin biosynthesis and metabolic networks. Furthermore, several researches regard the *AANAT/SNAT* is the rate-limiting enzyme during the melatonin biosynthesis (Morton and Forbes, 1988), while the other recent studies consider that the *HIOMT/ASMT* is the functional one (Byeon et al., 2015, 2016). The previous observation of the nearly two times of leaf melatonin level in *oHIOMT* line of transgenic tomato than those in *oAANAT* lines, provided the proof that ASMT, the homologous to *HIOMT*, was possibly the rate-limiting enzyme in plants (Wang et al., 2014). Although the similar patterns of the more melatonin accumulation and metabolic pathways were involved in the overexpression of the *oHIOMT* lines than those from the *oAANAT* ones in switchgrass seem to support the *HIOMT* is the rate-limiting enzyme (Figure 5), the large amount of KEGG pathways related to animal diseases possibly indicated the limited knowledge of melatonin functions in plants through the animal-oriented transgene. Therefore, further investigations from plant-oriented transgenic donor will be expected to provide valuable information for rate-limiting enzyme during the melatonin biosynthetic process.

The multiple membrane receptors and signal transduction mechanism were clarified in animals (Reppert et al., 1994). However, no high-affinity melatonin receptor was identified in plants to date (Park, 2011). Therefore, the receptor-independent mechanism of melatonin in plants was proposed as the neurons (Jan et al., 2011). In our study, the c58996\_g3 unigene significantly downregulated ( $\log_2\text{FoldChange} = -1.768$ ,  $\text{padj} < 0.01$ ), which was annotated as G-protein coupled receptor signaling pathway by GO classification. The discrepancies between increasing melatonin contents and decreasing signaling receptor roughly sustain the hypothesis that melatonin might act via a receptor independent mechanism.

In conclusion, the transcriptomic data from transgenic *oHIOMT* gene switchgrass revealed amount of differentially expressed unigenes comparing with transgenic *oAANAT* and

**TABLE 1 |**The summary list of differentially expressed unigenes in transgenic *oH1OMT* (H), *oAANAT* (A), and empty vector (EV) lines.

No.	Gene_id	H vs. EV	log2ratio (H vs. EV)	A vs. EV	log2ratio (A vs. EV)	A vs. H	log2ratio (A vs. H)	Annotation
1	c43728_g1	U	2.362	F	0.5403	D	-1.814	wound induced proteinase inhibitor WIP1
2	c45282_g1	U	2.381	F	-0.340	D	-2.700	Phage protein C
3	c52718_g1	U	1.698	F	0.459	D	-1.224	glycine-rich domain-containing protein 1
4	c58236_g6	U	2.025	F	0.5410	D	-1.469	copper transporter 1
5	c60917_g13	U	4.696	F	0.706	D	-3.976	late blight resistance protein homolog R1A-6 OS
6	c62742_g1	U	1.351	F	0.301	D	-1.039	SPX domain-containing membrane protein
7	c64524_g1	U	2.476	F	1.112	D	-1.350	glycine-rich domain-containing protein 1
8	c65280_g2	U	1.618	F	0.329	D	-1.278	U-box domain-containing protein 4
9	c56995_g3	U	9.160	D	-3.093	D	-12.245	Acetylserotonin O-methyltransferase
10	c52804_g3	D	-2.202	U	1.583	U	3.794	plasma membrane
11	c46269_g1	D	-2.273	F	-0.125	U	2.159	NAC domain-containing protein 67
12	c58750_g3	D	-2.518	F	-0.360	U	2.169	ATP-dependent RNA helicase DHX36
13	c59344_g7	D	-10.20	F	0.981	U	11.200	VQ motif-containing protein 8
14	c61012_g1	D	-1.754	F	0.443	U	2.206	lectin-like receptor protein kinase family protein
15	c93391_g1	D	-6.149	F	-0.206	U	5.953	structural protein 2
16	c32847_g1	F	NA	U	Inf	U	Inf	protein disulfide-isomerase
17	c33992_g1	F	-0.496	U	3.440	U	3.942	cytochrome c oxidase subunit 3
18	c35379_g1	F	NA	U	Inf	U	Inf	phenylalanine-4-hydroxylase
19	c35977_g1	F	NA	U	Inf	U	Inf	serine protease family S01A
20	c38813_g1	F	NA	U	Inf	U	Inf	SWIB domain-containing protein 1
21	c39636_g1	F	NA	U	Inf	U	Inf	leucine aminopeptidase 1
22	c40464_g1	F	NA	U	Inf	U	Inf	trypsin-like serine protease
23	c41376_g1	F	NA	U	Inf	U	Inf	NADH dehydrogenase subunit 1
24	c44489_g1	F	NA	U	Inf	U	Inf	acid phosphatase
25	c45327_g1	F	NA	U	Inf	U	Inf	heat shock protein 83
26	c47781_g1	F	NA	U	Inf	U	Inf	cytochrome P450
27	c49171_g2	F	0.477	U	1.748	U	1.284	F-box protein At5g51370
28	c49204_g1	F	NA	U	Inf	U	Inf	tyrosine decarboxylase 1
29	c50619_g1	F	NA	U	Inf	U	Inf	ATP synthase F0 subunit
30	c51184_g2	F	0.344	U	1.443	U	1.112	chlorophyll a-b binding protein 8
31	c51629_g1	F	0.020	U	1.278	U	1.265	glucan endo-1,3-beta-glucosidase
32	c52480_g1	F	0.966	U	2.506	U	1.548	galactinol synthase 2
33	c53871_g2	F	0.080	U	1.261	U	1.189	zinc finger protein CONSTANS-LIKE 3
34	c57096_g2	F	0.517	U	1.752	U	1.240	F-box protein PP2-A13
35	c30658_g1	F	-0.304	D	-2.256	D	-1.943	phytosulfokines 5
36	c40152_g1	F	0.738	D	-7.143	D	-7.865	cationic peroxidase SPC4-like isoform X3
37	c46161_g1	F	-0.122	D	-1.485	D	-1.352	Cysteine-rich receptor-like protein kinase 36
38	c50940_g1	F	-0.126	D	-1.370	D	-1.234	ABC transporter G family member 3
39	c53039_g1	F	1.590	D	-2.496	D	-4.072	ent-copalyl diphosphate synthase 1
40	c54359_g2	F	0.978	D	-2.271	D	-3.233	disease resistance protein RPM1-like
41	c54403_g1	F	0.635	D	-2.400	D	-3.026	L-ascorbate oxidase
42	c62341_g2	F	0.140	D	-3.478	D	-3.602	lysine-specific demethylase JMJ25-like
43	c63308_g5	F	-0.818	D	-3.649	D	-2.824	disease resistance protein RGA3

U, significantly up-regulated (corrected  $P < 0.05$ ); D, significantly down-regulated (corrected  $P < 0.05$ ); and F, not differentially expressed (corrected  $P > 0.05$ ). No. 1–8 and No. 11–15 genes indicated the specific up-regulation and down-regulation in H; No. 9 gene was upregulated in H but downregulated in A line, while No. 10 was oppositely downregulated in H but upregulated in A line; No. 16–34 and No. 35–43 showed the specific up-regulation and down-regulation in A line. NA or Inf presented if the adjusted readcount is zero.

control lines, implying that the last step of catalytic reaction probably is the rate-limiting step during the melatonin biosynthesis in plants. The potential roles of melatonin in plants

were indicated by the upregulation of a series of transcriptional factors and functional genes involving growth and resistance for various stresses. Moreover, additional experiments under adverse

environmental stresses will confirm the melatonin physiological functions in plants. The definitions of the membrane receptors and signal transduction will largely drive the explanation of the melatonin biosynthesis processes and functional metabolic pathways.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: YZ and FY. Performed the experiments: SY, CG, SL, and YH. Analyzed the data: SY, CG, YH, XC, and DT. Wrote the paper: SY, CG, and YZ. All authors reviewed and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

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# RNA-seq Analysis of Overexpressing Ovine AANAT Gene of Melatonin Biosynthesis in Switchgrass

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Melatonin serves important functions in the promotion of growth and anti-stress regulation by efficient radical scavenging and regulation of antioxidant enzyme activity in various plants. To investigate its regulatory roles and metabolism pathways, the transcriptomic profile of overexpressing the ovine arylalkylamine *N*-acetyltransferase (*oAANAT*) gene, encoding the penultimate enzyme in melatonin biosynthesis, was compared with empty vector control using RNA-seq in switchgrass, a model plant of cellulosic ethanol conversion. The 85.22 million high quality reads that were assembled into 135,684 unigenes were generated by Illumina sequencing for transgenic *oAANAT* switchgrass with an average sequence length of 716 bp. A total of 946 differentially expression genes in transgenic line comparing to control switchgrass, including 737 up-regulated and 209 down-regulated genes, were mainly enriched with two main functional patterns of melatonin identifying by gene ontology analysis: the growth regulator and stress tolerance. Furthermore, KEGG maps indicated that the biosynthetic pathways of secondary metabolite (phenylpropanoids, flavonoids, steroids, stilbenoid, diarylheptanoid, and gingerol) and signaling pathways (MAPK signaling pathway, estrogen signaling pathway) were involved in melatonin metabolism. This study substantially expands the transcriptome information for switchgrass and provides valuable clues for identifying candidate genes involved in melatonin biosynthesis and elucidating the mechanism of melatonin metabolism.

**Keywords:** switchgrass, melatonin, overexpression, *oAANAT*, RNA-seq

## INTRODUCTION

Since melatonin (*N*-acetyl-5-methoxytryptamine) was first discovered in plant in Dubbels et al. (1995) and Hattori et al. (1995), varying concentrations of melatonin have been found in many other plant species (Murch et al., 1997; Manchester et al., 2000; Simopoulos et al., 2005). The fundamental issues of melatonin biosynthetic pathways and physiological functions still need to be deciphered to utilize in plants (Tan et al., 2003, 2012; Arnao and Hernández-Ruiz, 2009; Arnao, 2014; Wei et al., 2016). Both exogenous melatonin treatments (Kolář et al., 2003; Arnao and Hernández-Ruiz, 2007; Sarropoulou et al., 2012; Zhang et al., 2013) and melatonin-rich transgenic plants (Kang et al., 2010; Park et al., 2012; Byeon and Back, 2014; Wang et al., 2014) have been carried on in order to determine its potential functional roles. The physiological role of

melatonin in plants involved growth regulation, scavenging reactive oxygen species and increases of antioxidant enzyme activities (Paredes et al., 2008; Arnao and Hernández-Ruiz, 2014; Hardeland, 2016). Through the encoding genes for the catalytic reactions during melatonin biosynthesis have been identified and cloned recently, its biosynthetic pathways still not clearly deciphered yet in plants (Kang et al., 2011, 2013; Byeon et al., 2013a; Hardeland, 2016). The classic pathways of melatonin biosynthesis consist of the four catalytic reactions from tryptophan: decarboxylation by tryptophan decarboxylase (TDC), hydroxylation by tryptamine 5-hydroxylase (T5H) to serotonin, N-acetylation by serotonin N-acetyltransferase (SNAT) and the final O-methylation to melatonin by N-acetylserotonin O-methyltransferase (ASMT). The penultimate step catalyzes the same reaction as the non-homologous AANAT of vertebrate, which catalyzes conversion of serotonin into N-acetylserotonin (Byeon et al., 2013a). Overexpression of AANAT gene can promote the ability of biosynthesis of melatonin, and significantly improve the melatonin content in plants (Kang et al., 2010; Wang et al., 2014; Zhang et al., 2014).

Switchgrass (*Panicum virgatum* L.) is a perennial C4 grass native to North America, and is well-researched on germplasm collection, cultivation, genetic breeding as a model plant for cellulosic bioethanol production over the past several decades (Sanderson et al., 1996; Parrish and Fike, 2005). Aiming at the production demand of large biomass, the traditional breeding techniques have been challenged by its nature of outcrossing and polyploidy, as well as the general infertile environments for the biomass grass cultivation. Fortunately, modification of the functional genes that related to the growth and resistance could effectively assist the breeding process of switchgrass (Fu et al., 2011; Poovaiah et al., 2014; Baxter et al., 2015; Wuddineh et al., 2015). Several transgenic plants expressing the key genes for melatonin biosynthesis enzymes have been demonstrated to increase the resistance to environmental stresses (Kang et al., 2010; Park et al., 2012).

In this study, the transgenic melatonin-rich switchgrass (overexpressing of the *oAANAT* gene) and the transgenic control with empty vector (EV) were used to conduct a RNA-seq and analyze the effects of melatonin on gene expression. This research intends to provide differentially expressed genes (DEGs) in the melatonin-rich switchgrass and mechanism information on the biosynthesis, regulation, metabolism of melatonin for further investigations in plants.

## MATERIALS AND METHODS

### Plant Materials and Cultivation

The switchgrass (*Panicum virgatum* L. var. Alamo) plants expressing sheep AANAT gene (ID: 25120) were used for this study. The transgenic plants were grown under 16 h light (26°C, 120  $\mu\text{mol}/\text{m}^2/\text{s}$ ) and 8 h dark (18°C) conditions with watered every other day. Fully matured plants were chosen from each genotype for molecular characterization and transcriptome sequencing, which were all phenotypically identical with respect

to overall size, tiller number and leaf pattern. Three replicates of relative high melatonin contents for each transgenic stem were frozen in liquid nitrogen and stored at 80°C until further analysis.

### Characterization of Growth and Development of Transgenic Switchgrass

Tiller number, plant height, internode number, internode length, internode diameter, leaf blade length, leaf blade width, root number, root length, root diameter, and spike length were measured at the transgenic reproductive third (R3) stage (Figure 1), a stage with fully emerged spikelets and an emerged peduncle (Hardin et al., 2013). Internode 3 (I3) was used for measuring internode diameter. The leaves of I3 were used to measure leaf blade length and leaf blade width. Twelve replicates were measured for each transgenic line.

### RNA Isolation and Qualification

Total RNA was extracted from the stems using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA) and was treated with RNase-free DNase or 30 min at 37°C to remove residual DNA. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (Implen, Westlake Village, CA, USA). RNA concentration was determined by Qubit RNA Assay Kit in Qubit 2.0 Fluorometer. RNA integrity was assessed with the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

### Transcriptome Sample Preparation for Sequencing

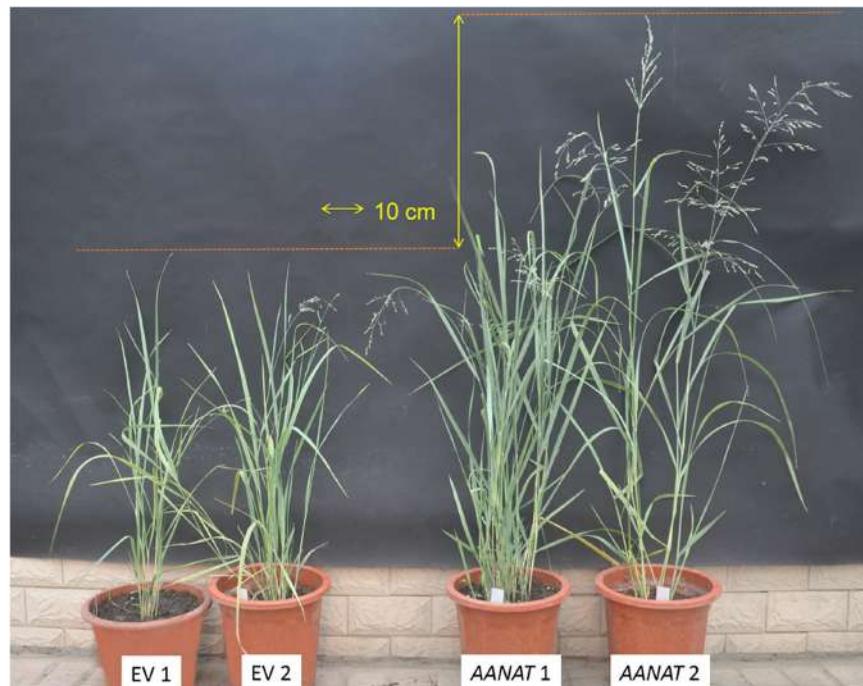
The total amount of 1.5  $\mu\text{g}$  RNA per sample was prepared for the RNA-seq. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA). The cDNA fragments of 150~200 bp were preferentially selected from the library by purification with AMPure XP system (Beckman Coulter, Beverly, MA, USA).

### Clustering and Sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, paired-end reads were generated by sequencing with the library preparations on an Illumina Hiseq platform.

### Validation of RNA-seq Data by Real-Time Quantitative

Real-time quantitative PCR validation of RNA-seq data for 12 random genes was performed in 20  $\mu\text{l}$  of reaction mixture (Supplementary Table S1). The amount of the amplified DNA was monitored by fluorescence at the end of each cycle using 7500 Real-Time PCR System (Applied Biosystems). Each plate was repeated three times in independent runs for all reference and selected genes. Gene expression was evaluated by the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).



**FIGURE 1 |** The phenotypes of transgenic *oAANAT* line and the control switchgrass in reproductive third stage.

## Data Analysis

### Quality Control

Clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. Q20, Q30, GC-contents and sequence duplication level of the clean data were calculated. Our raw data have been uploaded to NCBI<sup>1</sup>, and the accession number is PRJNA322585.

### Transcriptome Assembly

The complete genome of switchgrass has not been released through the draft version was released in 2012 and updated to V3. *De novo* transcriptome assembly was accomplished using Trinity (Grabherr et al., 2011) with min\_kmer\_cov set to 2 by default and all other parameters set default.

### Gene Functional Annotation

Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology).

### Differential Expression Analysis

Gene expression levels were estimated by RSEM (Li and Dewey, 2011). Differential expression analysis of two conditions/groups was performed using the DESeq R package (1.10.1). Genes with

an adjusted *P*-value < 0.05 found by DESeq were assigned as differentially expressed using the Benjamini and Hochberg's approach.

### GO Enrichment Analysis and KEGG Pathway Enrichment Analysis

GOseq R packages were implemented to define the DEGs based Wallenius non-central hyper-geometric distribution (Young et al., 2010). We used KOBAS (Mao et al., 2005) software to test the statistical enrichment of differential expression genes in KEGG pathways<sup>2</sup> (Kanehisa et al., 2008).

## RESULTS

### Morphological Characterization of Transgenic Plants

The transgenic switchgrass exhibited the largely enhanced growth condition (shoot, leaf, and root) compared with the EV (Figure 1; Table 1). Average tillers, shoot height, stem nodes, third internode length, and stem diameter in transgenic line were 45.5, 80.2, 48.3, 63.3, and 22.99% higher than those of EV, respectively (*P* < 0.05). Leaf blade length was 23.4% longer in transgenic switchgrass (52.72 cm) than that of EV (43.53 cm, *P* < 0.05). There was no significant difference on leaf width between the two groups (*P* > 0.05). Average roots, root length, and root diameter were 24.2, 25.6, and 41.5% higher in melatonin-rich switchgrass than those of control groups. Mean spike length in AANAT

<sup>1</sup><http://www.ncbi.nlm.nih.gov/bioproject>

<sup>2</sup><http://www.genome.jp/kegg/>

transgenic switchgrass (23.17 cm) was more than fourfold of the control (5.4 cm,  $P < 0.05$ ; **Table 1**).

## Illumina Paired-End Sequencing and Assembly

Total RNA was extracted from transgenic and control switchgrass in order to sequence using Illumina paired-end sequencing technology. In this study, exceeded 74G clean bases were acquired, and the average GC-rich content and the Q30 level of the six samples was 58.45 and 93.64%, respectively (Supplementary Table S2). After removal of adaptors and low-quality reads, average of 85,227,460 and 81,104,143 clean reads (95.9 and 94.2% of the raw data) of transgenic line and control were obtained, respectively (Supplementary Table S2). These reads were assembled into 264,869 transcripts with an average length of 1,052 bp and an N50 of 1,792 bp. After compared the different transcripts representing one unigene, the longest length transcript for each unigene was extracted. A total of 135,684 unigenes were obtained. The average length was 716 bp, and transcripts with lengths of more than 500 bp accounted for about 37.65% of all transcripts (Supplementary Table S3).

## Annotation of All Non-redundant Unigenes

For the validation and annotation of the assembled unigenes, all assembled unigenes were submitted to a BLASTx search with an E value threshold of 1e-5 against the following databases: Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), Swiss-Prot (a manually annotated and reviewed protein sequence database), GO (Gene Ontology), KOG (eukaryotic orthologous groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes). The unigenes were subjected to public databases for similarity searching. Among 135,684 unigenes, 56,968 (41.98%), 605,126 (44.59%) and 38,266 (28.2%) unigenes showed homology with sequences in the NCBI Nr, Nt and SwissProt databases, respectively (Supplementary Table S4; Supplementary Figure S1).

## Functional Classification by GO and KOG

Gene ontology (GO, a standardized classification system for gene function) was assigned to classify the functions of predicted switchgrass unigenes. In total, 1,931 functional GO terms were assigned among 41,494 unigenes with BLAST matching to known proteins (Supplementary Table S4). The majority of the unigenes were assigned to the categories of biological processes (100,887, 47.61%), followed by cellular components (63,207, 29.83%) and molecular functions (47,821, 22.57%; Supplementary Figure S2). Under the category of biological processes, cellular processes (22,564, 22.37%) and metabolic processes (21,358, 21.17%) were prominently represented. Under the classification of molecular functions, the binding (22,620, 47.3%) and catalytic activities (17,760, 37.1%) represented the two largest categories, while other categories, such as those for transporter activities, structural molecule activity, molecular function regulator, and others, together contained only 7,441 unigenes representing 15.56% of the total number of unigenes. As for the cellular component,

**TABLE 1 | Morphological characterization of transgenic switchgrass plants in reproductive third stage.**

Tiller number	Plant height (cm)	Stem node number	Internode length (3) (cm)	Internode diameter (cm)	Leaf blade length (cm)	Leaf blade width (cm)	Root number	Root length (cm)	Root diameter (cm)	Spike length (cm)
EV	6.1 ± 0.4b	66.81 ± 4.15b	3.2 ± 0.44b	8.78 ± 0.61b	2.95 ± 0.23b	43.53 ± 2.76b	1.17 ± 0.10a	16.5 ± 1.35a	41.95 ± 0.35b	0.99 ± 0.06b
A	8.9 ± 0.4a	120.37 ± 8.94a	4.8 ± 0.38a	14.33 ± 0.81a	3.63 ± 0.30a	53.72 ± 0.51a	1.13 ± 0.11a	20.5 ± 1.71a	52.68 ± 3.51a	1.4 ± 0.10a

EV: expressing the empty vector only; A: transgenic oAANAT line. Different letters indicate significant differences ( $P < 0.05$ ).

two categories, pertaining to cells and cell parts, accounted for approximately 40.38% of the cellular components that were identified; the organelle category accounted for approximately 13.53% of the cellular component unigenes, and the membrane and membrane part categories accounted for 19.43%.

In order to predict and classify possible functions, all unigenes were aligned to the euKaryotic Ortholog Groups (KOG) database in which orthologous gene products were classified. Out of 56,968 unigenes with significant similarity to nr proteins in this study, 21,165 sequences were assigned to KOG classifications. Among the 26 KOG categories, the cluster related to general function prediction (3,674, 17.36%) was the largest group, followed by those for post-translational modification (2,670, 12.62%); translation, translation, ribosomal structure, and biogenesis (1,693, 8.00%); and signal transduction mechanisms (1,605, 7.58%).

## Functional Classifications Using KEGG Pathways

All the unigenes were analyzed with respect to the KEGG pathway database to further examine the transcriptome of transgenic switchgrass. Out of the 135,684 identified unigenes, 16,222 (11.96%) with significant matches were assigned to five main categories that included 131 KEGG pathways (Figure 2; Supplementary file-KEGG). Among the five main categories that were identified, metabolism held the greatest number of unigenes (9,257, 57.06%), followed by genetic information (4,484, 27.64%), cellular processes (1,000, 6.17%), organismal systems (816, 5.03%), and environmental information processing (665, 4.10%). These results indicate that active metabolic processes were occurring in transgenic switchgrass. As shown in Supplementary file-KEGG, the metabolism category contained 19 sub-categories, including environmental adaptation, nucleotide metabolism, metabolism of terpenoids and polyketides, energy metabolism, carbohydrate metabolism, folding, sorting and degradation, membrane transport, biosynthesis involved in other secondary metabolism, amino acid metabolism, transport and catabolism, signal transduction and (Figure 2).

## Differential Gene Expression in Melatonin-Rich Transgenic Switchgrass

To reveal the molecular events occurring during the transgenic process, the digital gene expression libraries were constructed using RNA from the pools of control and the transgenic RNA samples and sequenced using Illumina technology. After Illumina sequencing and the removal of adaptors and low-quality reads, approximately 76,976,922,81,777,632, and 84,557,876 reads were obtained for the three control replicates, and 76,912,922, 88,415,412, and 90,354,046 reads were obtain for the three replicates for the transgenic lines. We then mapped the clean reads to the transcriptome reference data, and a total of 54,349, 56,895 and 73,352 unigene sequences were identified for the control replicates, and 53,595, 56,080 and 57,158 unigene sequences were identified for the drought replicates. After calculating the expression level of each mapped unigene, a total of 946 unigenes were detected that had levels of expression that

were significantly different between the transgenic and control libraries, including 737 up-regulated and 209 down-regulated unigenes (Figure 3). The significantly higher average FPKM of AANAT gene of transgenic lines (648.53) than that of control group (0.11).

The FPKM values were used to normalize gene expression levels and to compare their differences among transgenic lines and control. The percentage of high level expressed genes (FPKM beyond 15) in transgenic lines was slightly greater than in control samples, while that of low level expressed genes (FPKMs in the interval 0.1–3.57) in transgenic lines was relatively smaller than control samples (Supplementary Table S5).

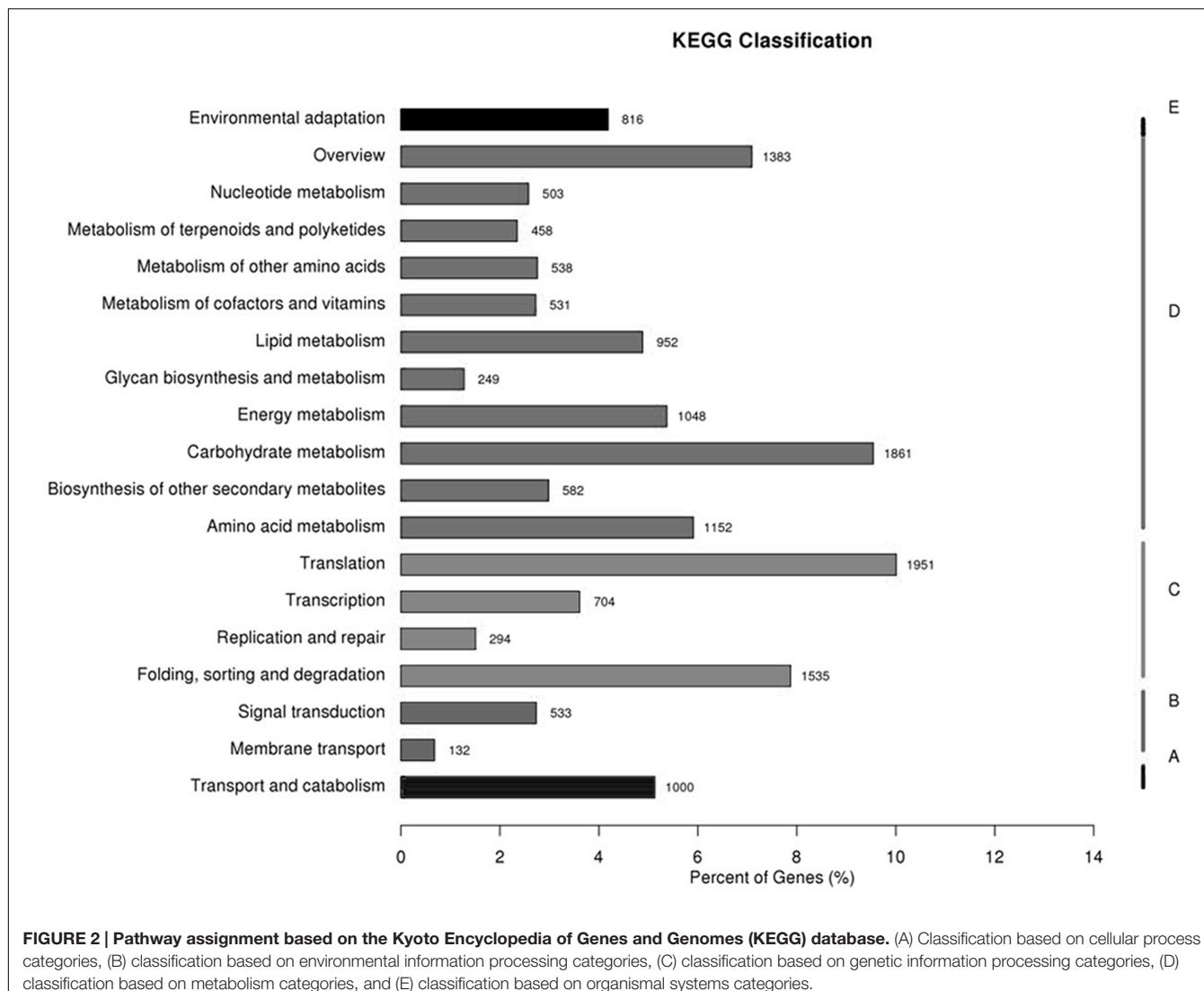
## Functional Annotation and Classification of the DEGs by Gene Ontology Analysis

Among the differentially expressed unigenes, 558 genes were significantly up-regulated in the melatonin-rich switchgrass by more than twofold of the levels in the EV ( $P < 0.05$ ). These genes included F-box protein (a gene for the controlled degradation of cellular protein); disease resistance protein (a resistance protein guard the plant against pathogens); abscisic stress-ripening protein 3 (an abscisic acid-, stress-, and ripening-induced protein); heat shock protein 83 (a gene for promoting the maturation), structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction.

To identify the genes that are differentially expressed in transgenic lines, a functional categorization was carried out by GO analysis. A total of 511 unigenes, including 415 up-regulated genes and 96 down-regulated genes, were functionally assigned to the three categories of the GO database (Figure 4). The 2012 GO terms were functionally annotated with GO terms in transgenic and control groups. The GO terms of 'organonitrogen compound biosynthetic process,' 'cellular amide metabolic process,' 'amide biosynthetic process,' 'aminoglycan metabolic process,' and 'peptide biosynthetic process' in biological process were highly enriched in the DEGs (corrected  $P$ -value  $< 0.05$ ), further suggesting the efficiency of the melatonin biosynthesis and the reliability of the gene expression data. Other terms, such as 'cytoplasmic part,' 'non-membrane-bounded organelle,' 'ribonucleoprotein complex' in cellular component, 'structural molecule activity' and 'chitin binding' in molecular function, were also significantly overrepresented (corrected  $P$ -value  $< 0.05$ ). By comparing transgenic and control groups, 1687 up-regulated and 1094 down-regulated GO items.

## KEGG Pathway Analysis of the Melatonin-Related Genes

To determine whether the melatonin-related genes engaged in specific pathways, the DEGs were used as objects to search against the KEGG pathway database. The top 20 obviously enriched pathways are shown in Figure 5. By comparing transgenic with control, 'ribosome' pathway enriched the most DEGs (Figure 6), followed by 'oxidative phosphorylation,' 'Glutathione metabolism,' 'MAPK signaling pathway,' 'photosynthesis – antenna proteins' and other pathways, suggesting that these



**FIGURE 2 | Pathway assignment based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.** (A) Classification based on cellular process categories, (B) classification based on environmental information processing categories, (C) classification based on genetic information processing categories, (D) classification based on metabolism categories, and (E) classification based on organismal systems categories.

pathways and processes might participate in the melatonin synthesis and metabolism (Supplementary file-KEGG).

## Transcription Factors

In this study, a total of 2214 transcription factors (TFs) were identified and classified into 79 different families, and the largest group of TFs was the MYB family (147, 6.64%), followed by NAC (108, 4.79%), Orphans (108, 4.88%), C<sub>2</sub>H<sub>2</sub> (102, 4.62%), GRAS (101, 4.56%), C<sub>3</sub>H (95, 4.29%), bZIP (94, 4.29%), and WRKY (103, 6.71%; Table 2). These results further suggested that 33 DEGs encoding known or putative TFs were changed among transgenic and control groups, including the MYB, NAC, SNF2, and FAR1 TFs (Table 2).

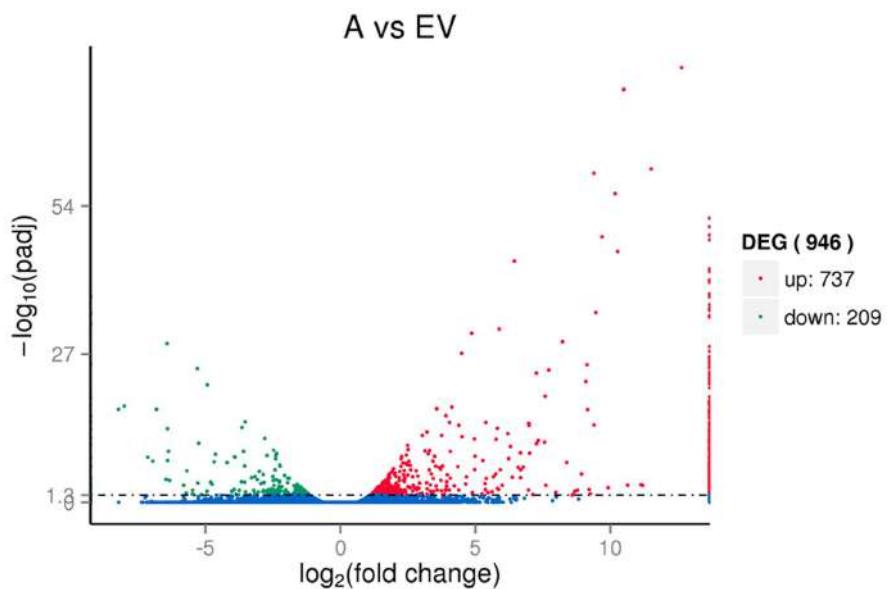
## Validation of Gene Expression Profiles Using RT-qPCR

The 12 DEGs were randomly selected for qRT-PCR. Histograms were generated by comparing the FPKM determined by

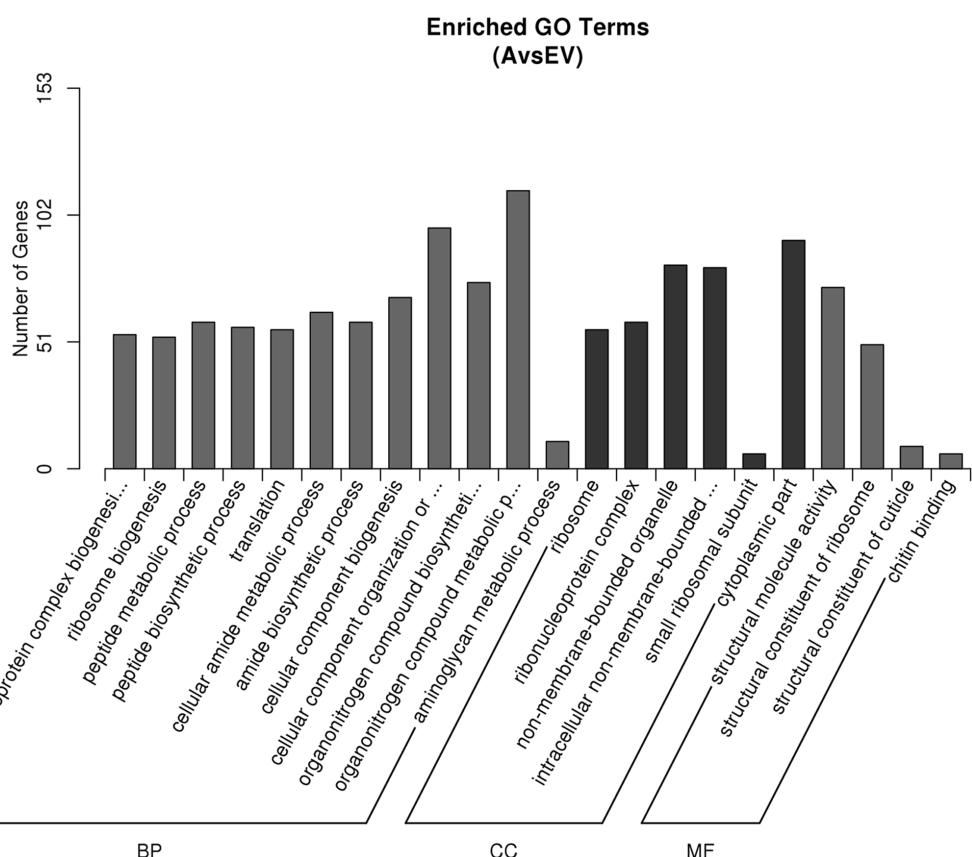
transcriptome analysis and qRT-PCR. A highly significant correlation ( $R^2 = 0.703, P < 0.01$ ) was found between the qPCR and RNA-Seq, indicating reproducibility and credible RNA-seq data (Supplementary Figure S3).

## DISCUSSION

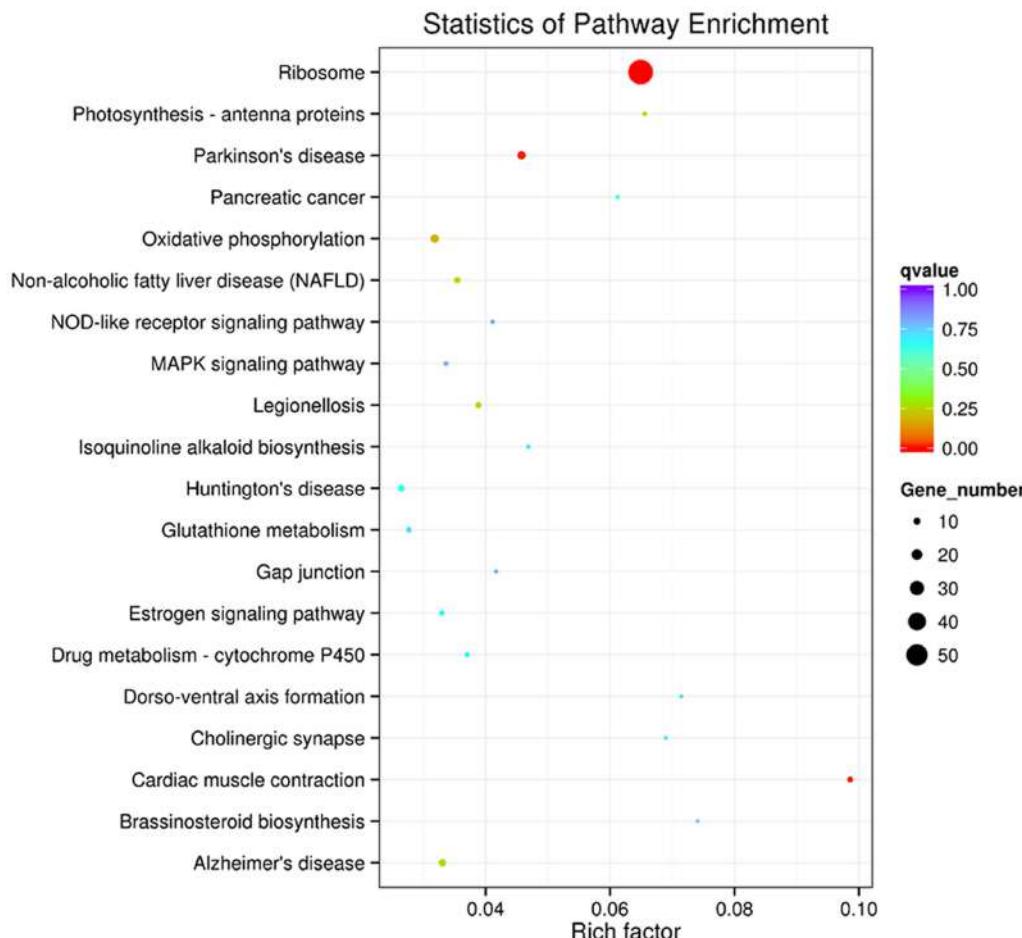
Although, a growing body of molecular and gene expression research regarding melatonin has been documented on model plants (Kang et al., 2010; Park et al., 2012; Byeon et al., 2013b; Weeda et al., 2014), the expression profiling of genes on melatonin biosynthesis and metabolism in forage grass has not yet been investigated, which was potentially valuable for the molecular breeding of the switchgrass as an superior bioethanol grass. In this study, to offer an initial insight into the related genes of melatonin in plants, we conducted a RNA-seq analysis to generate the global transcriptomic profile in transgenic switchgrass plants overexpressing the sheep *AANAT* gene.



**FIGURE 3 |** Volcano plot of the DEGs between the transgenic *AANAT* and control switchgrass.



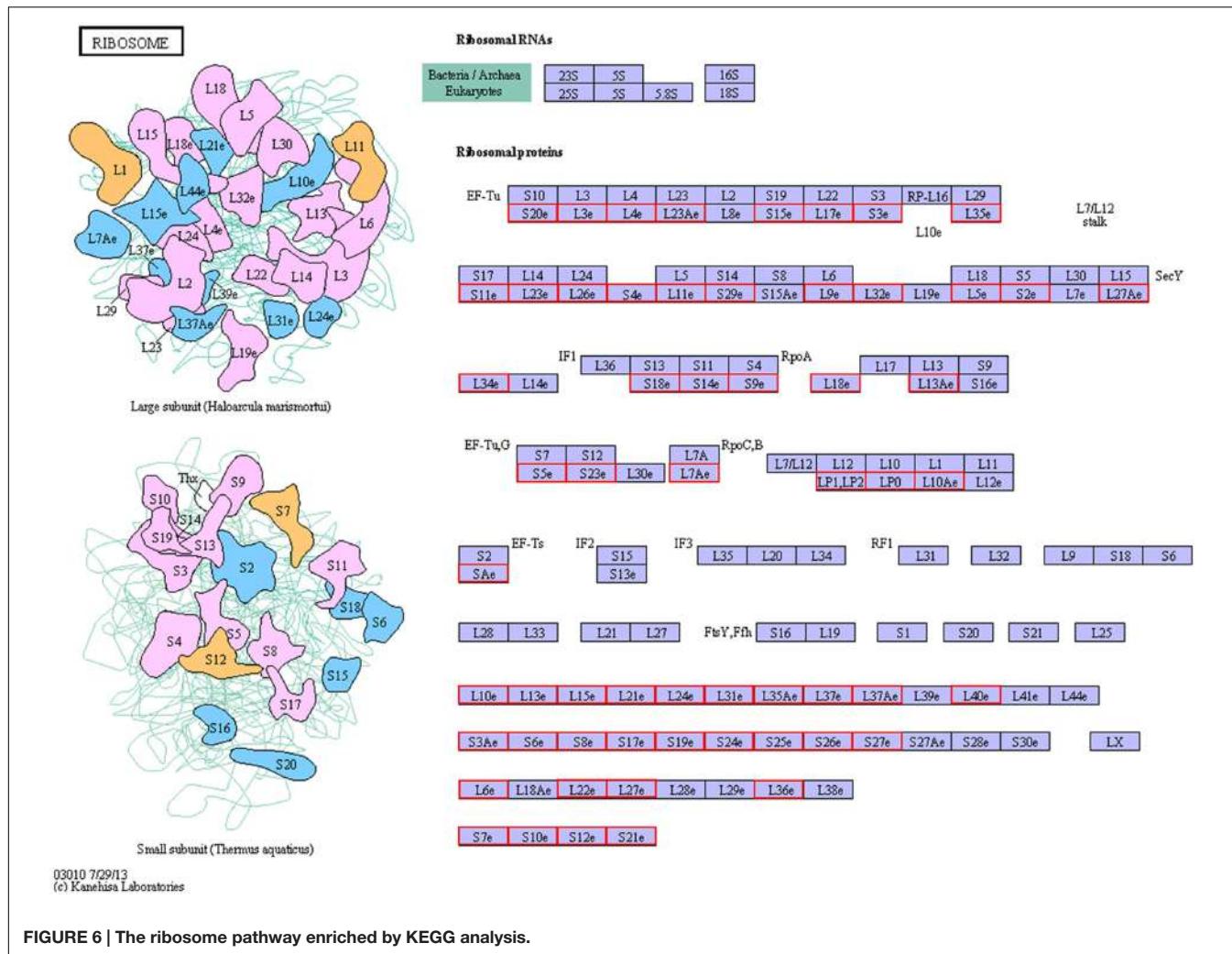
**FIGURE 4 |** Gene ontology (GO) classifications of DEGs across three comparisons. The Y-axis represents the number of DEGs in a category. The results of transgenic A line vs. EV (control) are summarized in three main categories: biological process (BP), cellular component (CC), and molecular function (MF).



**FIGURE 5 | Kyoto Encyclopedia of Genes and Genomes enrichments of the annotated DEGs across three comparisons.** The left Y-axis indicates the KEGG pathway. The X-axis indicates the Rich factor. A high q value is represented by blue, and a low q value is represented by red. (a) transgenic A line vs. EV (control); (b) transgenic H line vs. EV (control); (c) transgenic A vs. H line.

Overexpression of the *oAANAT* gene, encoding the key enzyme during melatonin biosynthesis process, significantly enhanced melatonin contents (over threefold of the EV from our previous work,  $P < 0.01$ ) and promoted growth (shoot height, root length, stem diameter, leaf size) and reproductive (spike length) processes in transgenic switchgrass (**Table 1**). Through the classic view supports the last HIOMT/ASMT enzyme is rate-limiting during the biosynthesis of melatonin, the promotional roles of AANAT/SNAT were also reported in other species, e.g., the *oAANAT* transgenic 'micro-tom' tomato have higher melatonin levels than control (Wang et al., 2014). The positive regulatory role of melatonin on growth and development was confirmed in other transgenic melatonin-rich plants (Byeon and Back, 2014; Wang et al., 2014). For example, the transgenic *oAANAT* rice exhibited altered height, biomass and panicle numbers per plant, suggesting that melatonin took part in plant growth and reproduction (Byeon and Back, 2014). Moreover, exogenous melatonin treatments also enhanced root regeneration, photosynthetic pigments, total carbohydrates and biomass in the *Chenopodium rubrum* (Kolár

et al., 2003), cherry rootstock (Sarropoulou et al., 2012); cucumber (Zhang et al., 2013), and soybean (Wei et al., 2015). The low concentrations melatonin increases photosynthetic activity by inducing porphyrin and chlorophyll biosynthesis, in contrast, high concentrations of melatonin induces the synthesis of proline and carbohydrate, which are beneficial for the osmoregulation of plants under stresses (Sarropoulou et al., 2012). Moreover, the stimulation of root generation and vitality and addition of the root/shoot ratio under melatonin treatment supported its effect on strengthening cucumber roots (Zhang et al., 2013). Furthermore, we detected the earlier flowering of transgenic *oAANAT* switchgrass than EV. Several flower-specific genes, *FLC* (Flowering Locus C, c52347\_g1), *AP2* (APETALA2, c34036\_g2, c48942\_g1), *DELLA* (c47591\_g2), differentially expressed between transgenic *oAANAT* and EV plants ( $P < 0.05$ ). However, more flowering related genes did not show significant regulation by overexpression of *oAANAT* genes (*LFY*, *CO*, *TFL*, *AG*, *AP3*). This might be attributed by the discrepancies of the gene expressions between plant stems and inflorescences, considering that the large



number of putative floral-specific transcripts were detected in flowers of sweet potato using RNA-seq (Tao et al., 2013). The striking promotions on development patterns of stems, leaves and roots and alteration of flowering suggested the possible involvement of the melatonin in these physiological actions (Park, 2011; Arnao and Hernández-Ruiz, 2014; Hardeland, 2016).

Transcriptomic analysis exhibited that the large number of 946 DEGs in the melatonin-rich switchgrass comparing to the control (Figure 3). Among the DEGs, functional patterns of melatonin were divided into two main aspects identifying by gene ontology analysis: the growth regulator and stress tolerance. The growth related function of melatonin was supported by the GO terms of plant development ('post-embryonic root development,' 'seed germination,' 'seedling development,' and 'pollen tube development') and biosynthetic processes ('organonitrogen compound biosynthetic process,' 'amide biosynthetic process,' and 'peptide biosynthetic process'). The genes encoding for signaling regulation and TFs such as MYB domain-containing protein, NAC and C<sub>2</sub>H<sub>2</sub> type protein, leucine-rich repeat and zinc-finger genes were involved in

plant growth and metabolisms (Table 2). These differentially expressed TFs were also detected in the other transcriptome analysis of growth related phenotypes in switchgrass (Palmer et al., 2012; Wang et al., 2012). The DREB subfamily, AP2 TFs, several different classes of zinc finger TFs (C<sub>2</sub>H<sub>2</sub>), auxin responsive TFs (ARF), Myb family TFs, and NAC were identified in the *Arabidopsis* dormancy related gene set (Wang et al., 2012). The APETALA2/ethylene response factor (AP2/ERF) superfamily of TFs plays essential roles in the regulation of various growth and developmental programs including stress responses (Wuddineh et al., 2015). This may be closely associated with the gene-regulation capacity of melatonin as a plant growth regulator (Park, 2011; Arnao and Hernández-Ruiz, 2014).

Furthermore, the stress tolerance function of melatonin was evidenced by the GO terms of 'response to oxidative stress' and 'cellular response to stress.' The F-box protein (the controlled degradation of cellular protein); disease resistance protein (a resistance protein guard the plant against pathogens); abscisic stress-ripening protein 3 (an abscisic acid-, stress-, and ripening-induced protein); heat shock protein 83 (a gene for promoting

**TABLE 2 | Selected genes about transcription factor with altered expression ( $P < 0.05$ ) in the two groups.**

Transcription factors	Genes	Description	FPKM	
			EV	A
AP2-EREBP	c52062_g7	Ethylene-responsive transcription factor RAP2-13-like	4.28	7.41
ARF	c53113_g1	Auxin response factor 16	14.39	22.89
ARR-B	c52945_g1	Two-component response regulator ARR10-like	1.79	3.08
C <sub>2</sub> H <sub>2</sub>	c52453_g3	C <sub>2</sub> H <sub>2</sub> -type zinc finger	11.57	28.20
CSD	c22802_g1	Cold shock domain-containing protein E1	0.00	0.32
CSD	c30734_g1	'Cold-shock' DNA-binding domain	0.00	1.15
FAR1	c30195_g1	Protein FAR1-Related sequence 5 OS	0.71	0.01
G2-like	c50135_g1	GLK2 transcription factor	20.91	53.32
GRAS	c52194_g5	Heme binding	0.97	3.51
GRAS	c53142_g4	Heme binding	85.42	30.81
GRAS	c58702_g2	Heme binding	0.21	0.86
HMG	c33183_g2	HMG box-containing protein Drosophila melanogaster	0.00	0.40
Jumonji	c62341_g2	Putative transcription factor 5qNCA, contains JmjC domain	4.48	0.41
LIM	c52258_g1	LIM domain-containing protein WLIM1 OS	20.25	24.29
LOB	c57227_g1	LOB domain-containing protein 37-like	62.87	162.85
MYB	c55204_g2	MYB44-like	49.42	18.80
MYB	c60941_g1	MYB superfamily, myb proto-oncogene protein	101.44	26.08
MYB	c63952_g2	MYB DNA-binding domain superfamily protein	0.03	0.33
MYB	c50835_g3	MYB proto-oncogene protein	183.08	213.97
MYB	c61614_g1	MYB proto-oncogene protein	137.17	153.77
MYB	c64075_g2	MYB proto-oncogene protein	6.36	10.69
NAC	c58322_g4	NAC domain transcription factor superfamily protein	68.51	189.30
NAC	c46269_g1	NAC domain-containing protein	5.72	1.39
Orphans	c53871_g2	Zinc finger protein CONSTANS-LIKE 3-like	180.04	388.03
Orphans	c57774_g5	Zinc finger protein CONSTANS-LIKE 16-like	94.83	212.30
Orphans	c61529_g5	B-box zinc finger protein 25-like	109.68	502.47
Orphans	c87095_g1	B-box zinc finger	0.00	2.01
SNF2	c50837_g2	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 3-like 3	1.27	0.13
SNF2	c42819_g1	SNF2 domain-containing protein	0.13	0.78
SWI/SNF-BAF60b	c38813_g1	SWIB domain-containing protein 1	0.00	0.83
TAZ	c57461_g1	BTB/POZ and TAZ domain-containing protein 1	6.89	23.44
TRAF	c5721_g1	BTB/POZ domain//Zinc finger, C <sub>2</sub> H <sub>2</sub> type	0.00	0.45
WRKY	c56069_g1	WRKY transcription factor 53 isoform X2	6.72	1.77

the maturation) involving for cell cycle control and signal transduction were differentially expressed in transgenic *oAANAT* switchgrass (Table 2). Similarly, the oxidative resistances were also detected in transgenic rice (Kang et al., 2010) and agrochemicals (Park et al., 2012) owing to the increased levels of endogenous melatonin. The promotion of the maturation, structural maintenance and proper regulation of specific target proteins was consistent with the result of a previous study in which a microarray analysis on the *AANAT* overexpressed rice, suggesting that melatonin is involved with stress responses (Byeon et al., 2013b). In addition, exogenous melatonin enhances abiotic tolerance (water, cold, salt stresses) in various plants (Zhang et al., 2013; Fan et al., 2015; Wei et al., 2015). The inhibitory effects of salt stress on gene expressions related to binding, oxidoreductase activity/process, and secondary metabolic processes under salt stress were alleviated by melatonin

in soybean (Wei et al., 2015). Melatonin achieves its promotional roles by influencing gene expressions involved in metabolic activities: nitrogen metabolism, major carbohydrate metabolism, hormone metabolism, and secondary metabolism (Paredes et al., 2008; Nawaz et al., 2015).

Kyoto Encyclopedia of Genes and Genomes maps provided much information on exploration of metabolic pathways involved in melatonin and deconstruction of its biological functions. Many differentially expressed unigenes were enriched into the biosynthetic pathways of secondary metabolite (phenylpropanoids, flavonoids, steroids, stilbenoid, diarylheptanoid, and gingerol). Plant steroid is used as signaling molecules for physiological and developmental regulation and offers exciting potentials for enhancing crop yield (Vriet et al., 2012). In addition, signaling pathways (MAPK signaling pathway, estrogen signaling pathway) were significantly

unregulated as well (**Figure 5**). Taking the MAPK (Mitogen-activated protein kinase) pathway as example, responses to various biotic and abiotic stresses as an integral component of cellular signaling during mitogenesis and differentiation of mitotic cells (Atkins et al., 1998; Group et al., 2002; Supplementary file-KEGG). The decades of secondary metabolic process and signal pathways related genes were differentially expressed in both endogenous melatonin-rich transgenic rice (Byeon et al., 2013b) and exogenous melatonin treatment (Zhang et al., 2014). These significantly altered expressions of metabolic pathways were not completely consistent between endogenous and exogenous melatonin treatment: the method of spraying leaves or coating seeds with melatonin merely drives short-term changes in morphological and physiological traits, while overexpression of endogenous melatonin gene enables sustainable synthesis of melatonin during the lifespan (Park, 2011). Besides, overexpression genes of the melatonin biosynthesis key enzyme participates in the *de novo* synthesis and is possibly involved in much more metabolism pathways than those from exogenous treatment, thus providing valuable information for the elucidation the biosynthesis pathway and functional mechanism of melatonin in plants (Kang et al., 2011, 2013; Byeon et al., 2013a,b; Zhang et al., 2016).

Previous studies have revealed that melatonin had significant effect in regulating hormones (such as ABA, GA<sub>4</sub>) in plant response to salinity and drought stress (Li et al., 2012; Zhang et al., 2016). Melatonin shared the common substrate (tryptophan) with IAA, however, the relationship of biosynthesis between melatonin and IAA is still in suspense and the independent relationship has been proposed in *Arabidopsis* root system architecture (Pelagio-Flores et al., 2012; Arnao and Hernández-Ruiz, 2014; Wei et al., 2016). In our study, the auxin-responsive protein IAA genes differentially expressed in the melatonin-rich switchgrass comparing with the control, and possibly resulted from the competitive relation for the same biosynthesis substrate. Thus, genome-wide expression analysis of melatonin-related genes in response to plant hormones supported the crosstalk between melatonin and IAA biosynthesis processes, and the intern mechanism of the relative expression needs more clues from other direct analysis of hormones treatments (Zhang et al., 2014; Wei et al., 2016).

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## CONCLUSION

This study is the first report of the transcriptomic profile of endogenous melatonin effects on in bioenergy crop switchgrass using RNA-seq technology. Switchgrass is an identified model species for bioethanol but the genetic background and genome information are not well-established, and these transcriptomic datasets will provide fundamental information and serve as new tools to genetically dissect melatonin-mediated pathways in other common grasses. The analysis based on DEGs reveals broad roles of melatonin in regulating plant growth, development and defense systems. Furthermore, the expression of many genes involved in signaling regulation such as MAPK signaling pathway, was also altered in response to the transgenic switchgrass of overexpression of melatonin biosynthesis gene. Taken together, studies on sequencing of transgenic melatonin-rich switchgrass suggests that melatonin plays a critical role in promotion of plant growth and may facilitate identification of melatonin's functions in plants.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: YZ, FY. Performed the experiments: SY, YH, CG, SL, DT. Analyzed the data: SY, CG, XC. Wrote the paper: SY, YH, YZ. All authors reviewed and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01289>

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# Genistein: A Novel Anthocyanin Synthesis Promoter that Directly Regulates Biosynthetic Genes in Red Cabbage in a Light-Dependent Way

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Genistein (GNT), an isoflavone, is used in the clinical treatment of various health disorders. GNT is found in primary food source plants and some medical plants. However, studies on the functions of GNT in plants are rarely reported. In this study, we demonstrated that GNT plays an important role in promoting anthocyanin accumulation in red cabbage. GNT solutions (10, 20, 30, 40, and 50 mg/L) as foliar fertilizers were applied to red cabbage. Consequently, anthocyanin accumulation in red cabbage increased in a light-dependent manner. GNT solution at 30 mg/L exhibited the optimal effect on anthocyanin accumulation, which was twice that of the control. Quantitative real-time PCR analysis indicated that GNT application upregulated the expression of all structural genes, contributing to anthocyanin biosynthesis under light conditions. Under dark conditions, GNT exerted no significant promotive effect on anthocyanin accumulation; only early biosynthetic genes of anthocyanin biosynthesis responded to GNT. The promotive effect of GNT on anthocyanin biosynthesis is directly attributable to the regulation of structural gene expression. Transcription factors exhibited no response to GNT. The levels of anthocyanin in red cabbage positively correlated with the enzyme activities of antioxidant systems. This finding correlation suggested that the promotive effect of GNT on anthocyanin levels was correlated with improved antioxidant activity in the red cabbage.

**Keywords:** anthocyanin, antioxidant activity, gene expression, GNT, red cabbage

## INTRODUCTION

Genistein (GNT) is the common name of 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one. This compound belongs to the group of isoflavones, heterocyclic polyphenols that naturally occur in plants; thus, this compound is also referred to as 4', 5, 7-trihydroxyisoflavone. GNT is a soy-derived biologically active isoflavone that exerts diverse health-promoting effects. It exhibits numerous biological activities: tyrosine kinase inhibition, chemoprotective activity against cardiovascular disease, and phytoestrogen activity. GNT can impede cancer progression by promoting apoptosis, inducing cell cycle arrest, modulating intracellular signaling pathways, as well as inhibiting angiogenesis and metastasis of neoplastic cells (Wei et al., 1995;

Xia and Weng, 2010). A micronutrient with multifaceted effects, GNT should be developed further for its clinical use in the prevention and treatment of various health disorders.

Flavonoids comprise a major class of secondary plant metabolites. Among these metabolites, anthocyanins are the most conspicuous class because of the wide range of colors resulting from their synthesis (Tahara, 2007). Anthocyanins are commonly found in the red, blue, and purple colors of fruits, vegetables, flowers, and other plant tissues (Mateus et al., 2001). As a group of flavonoid compounds, anthocyanins protect plants against various biotic and abiotic stresses, as well as provide flowers and fruits with distinct colors to attract insects and animals for pollination and seed dispersal (Harborne and Williams, 2000). Numerous simple flavonoid compounds also exhibit antioxidant properties and can thus be potentially used as dietary nutraceuticals for human health (Winkel-Shirley, 2001). Anthocyanin biosynthesis has been studied extensively (Grotewold, 2006), and its biosynthetic pathway is well described in *Arabidopsis* and other plants, including *Antirrhinum majus* (snapdragon) and *Petunia hybrid* (petunia). Early biosynthetic genes are in the upstream of late biosynthetic genes. Early biosynthetic genes include chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and flavonoid 3'-hydroxylase (F3'H), which are common to different flavonoid subpathways (Zhang et al., 2016). Late biosynthetic genes include dihydroflavonol 4-reductase (DFR), leucoanthocyanidin oxygenase (LDOX), and UDP-glucose: flavonoid 3-O-glucosyltransferase (Pelletier et al., 1997). The expression of these structural genes is closely related to anthocyanin levels. Regulatory genes influence the intensity and pattern of anthocyanin biosynthesis by regulating the expression of the structural genes. Many regulatory genes, such as transcription factors R2R3 MYB, basic helix-loop-helix (bHLH), and WD40 proteins, have been cloned from many plants. R2R3 MYB is a transcription factor family carrying the highly conserved R2R3 DNA-binding domain. WD40 is a protein family with WD-repeat sections. They form MBW (MYB-bHLH-WD40) complexes to activate the expression of genes along anthocyanin biosynthesis (Broun, 2005). MYB and bHLH transcription factors contribute differently in activating anthocyanin biosynthetic genes among different plant species (Gonzalez et al., 2008). This variation suggests that unique species-specific regulation of structural genes R2R3 MYB and bHLH transcription factors represent the two major families of anthocyanin regulatory proteins. In *Arabidopsis*, anthocyanin accumulation has been shown to be mediated by 4 MYB proteins and 3 bHLH proteins (Yuan et al., 2009).

Anthocyanin biosynthesis is also influenced by many environmental factors, such as drought (Yuan et al., 2009), temperature (Ubi et al., 2006), hormone (Jeong et al., 2010), or light (Tan, 1980). Red cabbage (*Brassica oleracea* L. var. *capitata*) grows worldwide as a fresh market vegetable and is a native crop in the Mediterranean region in Europe. Red cabbage is distinct in its high anthocyanin content. It serves as a functional vegetable and is very popular in salad (Charron et al., 2007). To promote anthocyanin accumulation, application of plant growth regulators has been proposed as an

economically viable alternative. Many plant growth regulators have been evaluated for regulating anthocyanin biosynthesis in plant tissues. These regulators include gibberellins (Martinez et al., 1996), auxins (Jeong et al., 2010), cytokinins (Kim et al., 2006), ethylene (El-Kereamy et al., 2003), and jasmonate (Ayala-Zavala et al., 2005). Both genetic and physiological approaches have verified that abscisic acid (ABA) positively modulates, whereas gibberellic acid (GA) negatively modulates anthocyanin accumulation on hormone mutants and exogenous applications (Carvalho et al., 2010). In seed germination, ABA and GA were also described as a pair of antagonists (Ho et al., 2003). GA can induce the degradation of DELLA proteins, whereas ABA can cause the stabilization of DELLA proteins (Achard et al., 2006). Meanwhile, a positive role for DELLA proteins in anthocyanin accumulation was demonstrated during phosphorus starvation in *Arabidopsis* (Jiang et al., 2007). DELLA proteins may mediate the antagonism between ABA and GA in anthocyanin biosynthesis. ABA is proven to induce phenylalanine ammonialyase (PAL), a key enzyme for anthocyanin biosynthesis. However, this process does not occur on every species (Guo and Wang, 2009). In addition to these hormones, several chemicals have been found to increase anthocyanin biosynthesis. In the current study, we selected 3 chemicals that naturally exist in plants. 5-Aminolevulinic acid (ALA) is the first compound in the porphyrin synthesis pathway. ALA promotes anthocyanin accumulation in apple (Xie et al., 2013). Guanosine 3', 5' -cyclic monophosphate (cGMP) is an important signaling molecule that, as a second messenger in plants, controls various cellular functions. Cyclic GMP regulates the transcriptional activation of the anthocyanin biosynthetic pathway in soybeans (Suita et al., 2009). Melatonin is a chemical that benefits stress tolerance in plants (Zhang et al., 2014, 2015; Arnao and Hernández-Ruiz, 2015; Shi et al., 2016). In our previous study, melatonin treatment increased anthocyanin accumulation in cabbage, *Arabidopsis*, and tomato (Sun et al., 2015, 2016; Zhang et al., 2016).

The effects of GNT in plants remain poorly understood. The mechanism underlying the regulation of anthocyanin accumulation by GNT remains unknown. In the current study, we applied GNT as a foliar fertilizer to evaluate the effect of GNT applications on red cabbage. To determine how GNT improves anthocyanin accumulation, we evaluated the expression of structural and regulatory genes that contribute to anthocyanin synthesis. Light is an essential factor in anthocyanin biosynthesis. Thus, we evaluated the gene expression under light and dark conditions and found that light is an essential factor in GNT-induced anthocyanin accumulation.

## MATERIALS AND METHODS

### Plant Materials

The following experiments were conducted at China Agricultural University, Beijing (39.9° N, 116.3° E). Cabbage (*Brassica oleracea* var. *capitata* L.) seeds were obtained from Chinese Academy of Agricultural Sciences. After germination, seeds were sown and grown in pots (20 cm in diameter) filled with soil (peat:

vermiculite = 2:l) in a growth chamber at 25°C for 10 h during the day and 15°C for 12 h during the night. The light intensity was 600  $\mu\text{mol s}^{-1} \text{m}^{-2}$ .

## Reagents

All chemicals used in the experiments were of analytical grade. GNT, 5-aminolevulinic acid, and ABA were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were supplied by Sinopharm Chemical Reagent Beijing Co., Ltd. in China.

## Plant Treatments

For the pre-experiment, solutions of GNT (25 mg/L), 5-aminolevulinic acid (300 mg/L), ABA (600 mg/L), cGMP (80 mg/L), and melatonin (200 mg/L) as foliar fertilizers were applied to 4-week-old red cabbage. The experiment was repeated three times. For the experiments involving GNT, the concentrations of GNT solutions were 10, 20, 30, 40, and 50 mg/L; water was used as the control (with 0.01 % Tween-20). Each treatment consisted of 20 pots, with each pot having 1 plant. All treatments were conducted in triplicate. For the dark treatment, we turned off the light in chamber after the chemical treatment. For the light treatment, the light intensity was 600  $\mu\text{mol s}^{-1} \text{m}^{-2}$ . The first and the second leaves were collected 5 days post-treatment. All samples were frozen in liquid nitrogen and stored at -80°C for anthocyanin measurement, RNA extraction, and other analyses.

## Analyses of Total Anthocyanin and Total Chlorophyll

Total anthocyanins were measured using a slightly modified differential pH method, a spectrophotometric technique (Rapisarda et al., 2000). Frozen samples (100 mg) were ground into powder in a mortar. Anthocyanin was separately extracted in a pH 1.0 buffer (50 mM KCl + 150 mM HCl) and a pH 4.5 buffer (400 mM sodium acetate + 240 mM HCl). The extracts were centrifuged at 12,000 g for 15 min at 4°C. Supernates were collected and diluted to measure the absorbance at 510 nm. Total anthocyanin content was calculated using the following equation.

$$\text{Anthocyanin (mg.g}^{-1} \text{FW}) = (\text{A}_{\text{pH}1.0} - \text{A}_{\text{pH}4.5}) \times 484.8 \times 1000/24,825 \times \text{dilution factor.}$$

In the formula, 484.8 represents the molecular mass of cyanidin-3-glucoside chloride, and 24,825 is equal to its molar absorptivity ( $\epsilon$ ) at 510 nm. Each sample was analyzed in triplicate, and the results were expressed as the average of the three measurements.

For chlorophyll measurement, we extract chlorophyll by 80% chilled acetone (v/v). Then we used a spectrophotometer to quantify it.

## RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated from the samples by using TRIzol Reagent according to manufacturer's protocol (Invitrogen, Burlington, ON, Canada). The cDNA was reverse-transcribed

into cDNA using the reverse transcription system (Takara Biotechnology, Japan). Quantitative RT-PCR was conducted using the Applied Biosystems 7500HT Fast Real-Time PCR System (Applied Biosystems, USA). The reaction volume was 20  $\mu\text{L}$ , containing 2  $\mu\text{L}$  of cDNA, 0.4  $\mu\text{L}$  of each 10  $\mu\text{M}$  forward and reverse primers, and 10  $\mu\text{L}$  of SYBR Premier Ex Taq mix (Takara, Japan). The PCR thermal cycling parameters were 95°C for 10 s, followed by 40 cycles at 95°C for 5 s, 60 °C for 15 s, and 72 °C for 30 s. Melting curve analysis of Quantitative real-time PCR samples revealed only one product for each gene primer reaction, confirming specific amplification. Gene expression was evaluated using the  $2^{-\Delta\Delta\text{Ct}}$  method. All Qrt-PCR reactions were normalized using the *BoActin* gene (Mittler, 2006). The DNA sequences of PCR primers are listed in Table 1.

## Evaluation of Lipid Peroxidation

The levels of lipid peroxidation in the leaves were evaluated by protocol we described before (Zhang et al., 2013). The level of lipid peroxidation was expressed as concentrations of malondialdehyde (MDA).

## Antioxidant Enzyme Extraction and Assay

Frozen samples (1 g FW) were homogenized with 0.2 g hydrated PVP (insoluble polyvinylpyrrolidone) in 10 mL of 50 mM phosphate buffer (pH 7.8) supplemented with 2 mM dithiothreitol and 0.1 mM ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 16,000 g for 15 min. The resulting supernatant was used for enzyme assays. All steps of the extraction procedure were carried out on ice to make sure the temperature is low.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to Giannopolitis and Ries (Giannopolitis and Ries, 1977), with certain modifications. You can find the detailed protocol in the paper we published in 2013 (Zhang et al., 2013).

Catalase (CAT; EC 1.11.1.6) activity was measured at 25°C according to Kato and Shimizu (Kato and Shimizu, 1987). The 3 mL reaction mixture contained 3.125 mM  $\text{H}_2\text{O}_2$  in 50 mM phosphate buffer (pH 7.8) and 0.2 mL of enzyme extract. CAT activity was estimated by the absorbance decrease at 240 nm of 0.1 unit/min and was expressed as units  $\text{g}^{-1} \text{min}^{-1}$ .

Peroxidase (POD; EC 1.11.1.7) activity was measured at 25°C according to Sceba et al. (2001). The method is based on monitoring the  $\text{H}_2\text{O}_2$  decomposition rate by POD, using guaiacol as a hydrogen donor. The reaction was initiated by adding 50  $\mu\text{L}$  of enzyme extract to 1,950  $\mu\text{L}$  phosphate buffer (65 mM, pH 5.5) containing 11 mM  $\text{H}_2\text{O}_2$  and 2.25 mM guaiacol. The rate of color development was determined by recording the absorbance of the reaction solution at 470 nm per 0.1 s. One unit of POD activity was defined as an absorbance change of 0.01 units/s; activity was expressed as units  $\text{g}^{-1} \text{s}^{-1}$ .

## Statistical Analysis

All data were subjected to one-way ANOVA with Duncan's test or *t*-test in SPSS 20.0.

**TABLE 1 | Oligonucleotide primers used for qRT-PCR analysis.**

Target gene	Primer name	Sequence of primer	Accession	<i>Arabidopsis</i> blastN AGI
BoACTIN	BoACTIN-F	CTGTGACAATGGTACCGGAATG	AF044573	AtACTIN2/AT3G18780
	BoACTIN-R	ACAGCCCTGGGAGCATCA		
BoPAL	BoPAL-F	CAGAGCAACACAACCAAGACGTGAA	BH716217	AtPAL1/AT2G37040
	BoPAL-R	TCTCCTCCAAGTGTAGATCGATG		
BoCHS	BoCHS-F	GCGCATGTGCAAGTCGAC	EF408921	AtCHS/AT5G13930
	BoCHS-R	CCTGTCGAGCGTCGAGAGAAGGA		
BoCHI	BoCHI-F	TCAAGTTGATTCCGTTACTTTCCA	EU402417	AtCHI/AT3G55120
	BoCHI-R	ATGACGGTGAAGATCACAACTTC		
BoF3'H	BoF3'H-F	TTCCGTACCTTCAGGCGGTTATCAA	BH675335	AtF30H/At5g07990
	BoF3'H-R	CTTGGGGATATGATAGCCGTTGATC		
BoDFR	BoDFR-F	GCTCTCCTATCACTCGTAACGA	AY228487	AtDFR/AT5G42800
	BoDFR-R	GTCGCATCGTGAGAGGAACAAA		
BoLDOX	BoLDOX-F	GTGGACAGCTGAGTGGGAAGATTAC	AY228485	AtLDOX/AT4G22880
	BoLDOX-R	GTACTCACTCGTAGCTTCAATGTAATCAG		
BoGST	BoGST-F	CTTGTAGCCATTGGTCAA	BH738469	AtGSTF12/AT5G17220
	BoGST-R	GAGACTTGGCCAAAAGGTTCGT		
BoTT2	BoTT2-F	AAACCAAGCTGGTCTCAAGAGGTGTG	DQ778648	AtTT2/At5g35550
	BoTT2-R	AACGACCATCTGTTCCAAGGGAGATTAT		
BoTT8	BoTT8-F	CCAATAGTTAGATACACACATGGACATG	BH450920	AtTT8/At4g09820
	BoTT8-R	TCTTGACATTCTCACTCTCCACGATAT		
BoTTG1	BoTTG1-F	AGTTGCAGTGGTCGGCTTC	BH653524	AtTTG1/AT5G24520
	BoTTG1-R	ATACGAACTCTAAACTCTAAGGAGCT		
BoEGL3	BoEGL3-F	AACTGTCAATTGCAAGCATAAGGGACA	EX078387	AtEGL3/At1g63650
	BoEGL3-R	TGTTGAATCACTGAGTTCATAGATTGGA		
BoMYB12	BoMYB12-F	TGGAACCTCATCTCCGCCGTA	BH539285	AtMYB12/At2g47460
	BoMYB12-R	CGGCGGTGCAGACGTTCTT		
BoMYB2	BoMYB2-F	GGAAACAGGTGGTCTTAATTGCT	N/A	AtMYB14/At1g66380
	BoMYB2-R	AGCTCAAATTATCATCATCTTGTTACATGTGATTA		
BoMYB4	BoMYB4-F	GGAAACAGGTGGTCTTAATTGCT	N/A	AtPAP1/At1g56650
	BoMYB4-R	ATCCAAGGCATAGGGAACAAAT		

## RESULTS

### Effects of Five Different Chemicals on Anthocyanin Accumulation in Red Cabbage

We evaluated the anthocyanin levels of red cabbage under five different chemical treatments. **Figure 1** shows the structure of the chemicals used in this study, with GNT as the star chemical. ALA is an intermediate along the chlorophyll biosynthetic chain, which is used to promote the skin color in apple fruits. ABA is an important phytohormone related to stress response, but it is widely reported as an improvement of anthocyanin biosynthesis. Guanosine 3', 5'-cyclic monophosphate (cGMP), a second messenger, also improves anthocyanin accumulation. Meanwhile, the indole melatonin can accelerate anthocyanin biosynthesis. We gathered these chemicals to evaluate their effects on anthocyanin accumulation. All anthocyanin levels were higher in these treatments than in the controls (**Figure 2**). GNT, ALA, ABA, and MT exerted similar effects on the anthocyanin levels, which were higher compared with cGMP. In our previous study, we evaluated the effect of melatonin on anthocyanin accumulation (Zhang et al., 2016). In the present study, we

selected GNT as a subject to evaluate its effect on anthocyanin accumulation.

### Improvement of Anthocyanin Accumulation with Different GNT Concentrations in Red Cabbage

To evaluate the effect of GNT on anthocyanin accumulation in red cabbage, GNT with gradient concentrations were applied to cabbage seedlings. GNT treatments under five different concentrations significantly regulated anthocyanin accumulation in red cabbage (**Figure 3**). GNT with lower concentrations showed higher anthocyanin accumulation levels, with 30 mg/L being the optimal concentration of GNT treatment. The anthocyanin level was 2.4-fold higher under the optimal concentration than in the control, with 5.24 mg/g FW. The highest concentration used in this experiment was 50 mg/L, which also improved anthocyanin accumulation; however, the increase was not as much as that obtained at optimal concentration. These results suggested that GNT could promote anthocyanin accumulation in red cabbage, and the promotive effect was concentration-dependent.

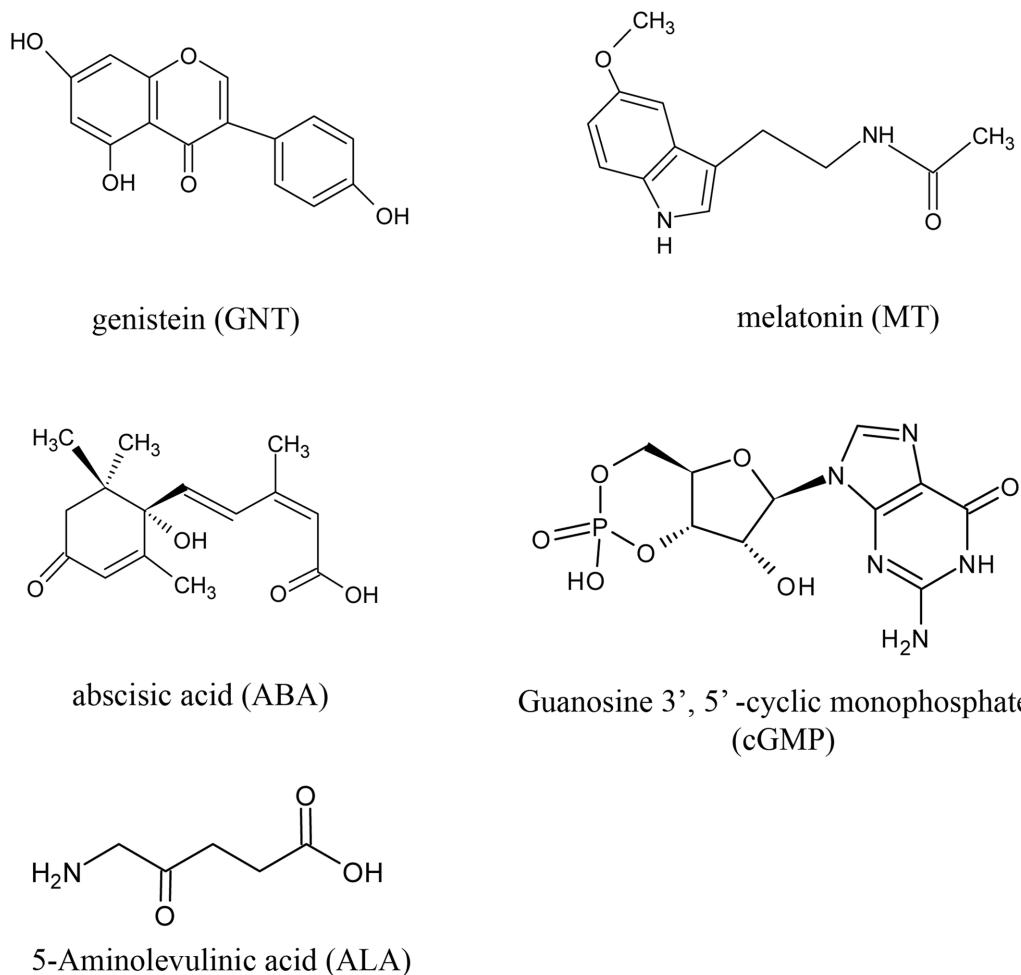


FIGURE 1 | The structure of the chemicals applied to red cabbage.

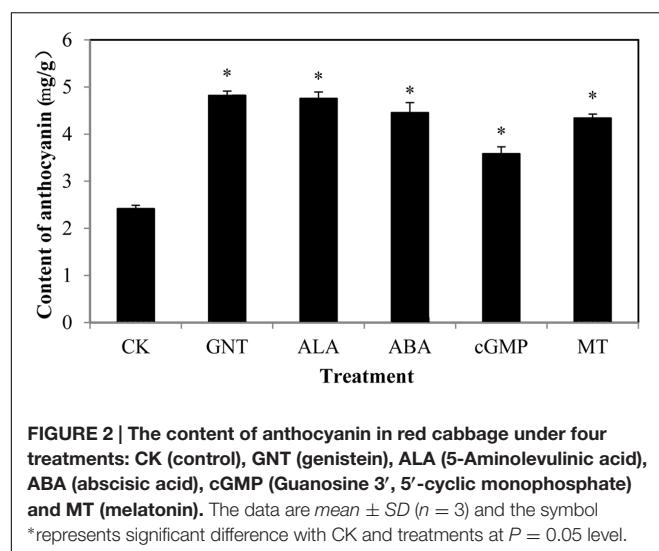
## Light-Dependent Promotive Effect of GNT on Anthocyanin Accumulation

To determine whether the promotive effect of GNT on anthocyanin accumulation is light-dependent, we considered light conditions in our experiments. The GNT solution used was the optimal concentration (30 mg/L) obtained, as shown in **Figure 3**. Under dark conditions, anthocyanin levels were similar in the GNT-treated and control groups (**Figure 4**). No significant difference was observed after GNT treatment. While anthocyanin accumulation levels were significantly increased under light conditions in both the GNT-treated groups and the control group. Under light conditions, GNT treatment significantly improved the anthocyanin level to twice that of the control (**Figure 4**). This result indicated that the promotive effect of GNT on anthocyanin accumulation is light-dependent. We also evaluated the chlorophyll content in GNT treatments under dark and light conditions. A difference in chlorophyll content was observed between light and dark conditions but not between the GNT-treated group and the control group (**Table 2**). This finding

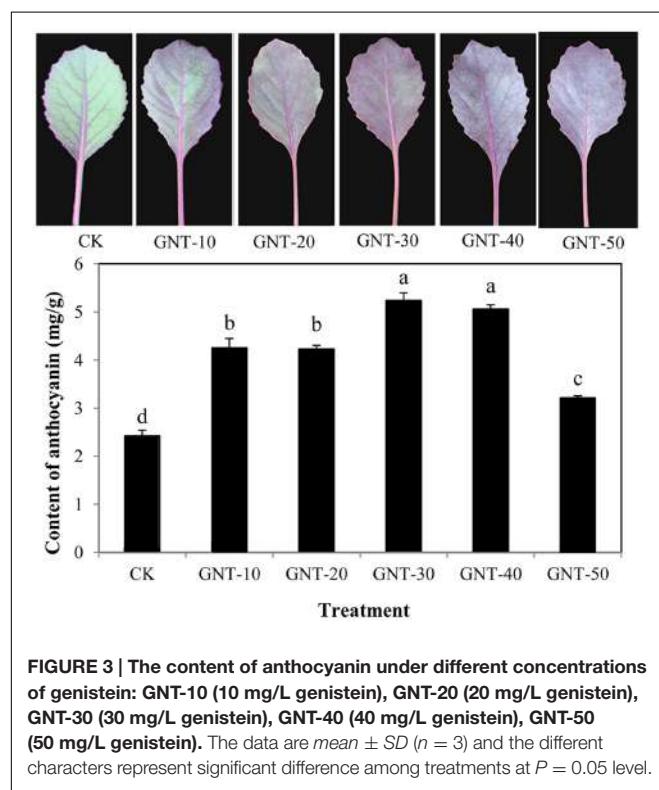
indicated that GNT treatment exerted no effect on chlorophyll synthesis.

## Positive Effect of GNT Treatment on the Expression of Genes Along the Anthocyanin Biosynthetic Pathway

To investigate whether the GNT induced anthocyanin accumulation is due to the upregulated gene expression, we measured the expression levels of transcripts that encode 7 anthocyanin biosynthetic genes (*BoPAL*, *BoCHS*, *BoCHI*, *BoF3'H*, *BoDFR*, *BoLDOX*, and *BoGST*) by Quantitative real-time PCR. The expression patterns of the seven biosynthetic genes under dark and light conditions in red cabbage are presented in **Figure 5**. Under light conditions, the 7 genes showed upregulated expression with GNT treatment. The genes *BoF3'H*, *BoDFR*, and *BoLDOX*—the key enzymes in the last step of anthocyanin biosynthesis—showed higher upregulation with GNT treatment (**Figure 5A**). Meanwhile, under dark conditions, several genes also exhibited upregulated expression after GNT treatment. These genes included *BoPAL*, *BoCHS*,



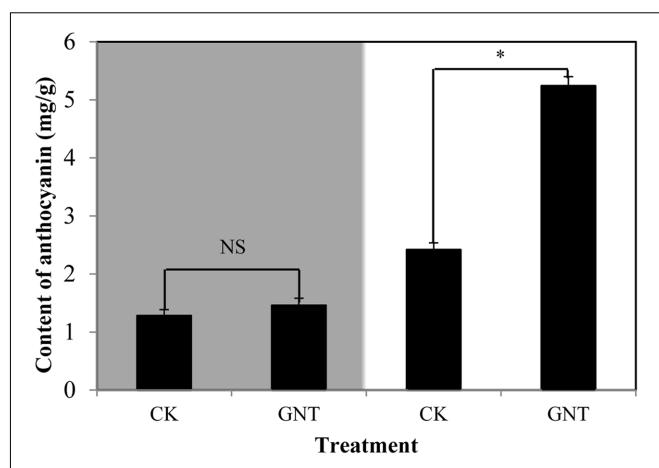
**FIGURE 2 |** The content of anthocyanin in red cabbage under four treatments: CK (control), GNT (genistein), ALA (5-Aminolevulinic acid), ABA (abscisic acid), cGMP (Guanosine 3', 5'-cyclic monophosphate) and MT (melatonin). The data are mean  $\pm$  SD ( $n = 3$ ) and the symbol \*represents significant difference with CK and treatments at  $P = 0.05$  level.



**FIGURE 3 |** The content of anthocyanin under different concentrations of genistein: GNT-10 (10 mg/L genistein), GNT-20 (20 mg/L genistein), GNT-30 (30 mg/L genistein), GNT-40 (40 mg/L genistein), GNT-50 (50 mg/L genistein). The data are mean  $\pm$  SD ( $n = 3$ ) and the different characters represent significant difference among treatments at  $P = 0.05$  level.

and *BoCHI*, which participate in the early steps of anthocyanin biosynthesis (Figure 5B). Under dark conditions, genes of the late-step enzymes, which play a more direct role in converting the intermediate products to anthocyanin, showed no significant change in expression with GNT treatment (Figure 5B). However, *BoDFR* is an exception.

We aimed to investigate whether transcripts corresponding to the anthocyanin regulatory genes accumulated in response to GNT both under dark and light conditions. We also intended to examine possible correlations between their expression



**FIGURE 4 |** Anthocyanin levels of red cabbage under GNT treatment in dark and light conditions. The colored section is dark condition and bright section is light condition. The data are mean  $\pm$  SD ( $n = 3$ ) and the symbol \*represents significant difference with CK and treatments at  $P = 0.05$  level while NS means not significant.

patterns and those of the structural genes. We evaluated several transcription factors that were known to regulate the structural genes in the anthocyanin biosynthetic pathway, including *BoMYB2*, *BoMYB4*, *BoTT2*, *BoTT8*, *BoEGL3*, *BoTTG1*, and *BoMYB12*. Anthocyanin biosynthetic genes are regulated by the interaction of the transcription factors MYB, bHLH, and WD40. The bHLH transcription factors participate in the regulation of anthocyanin biosynthesis, including TT8 and EGL3. TTG1 is a WD40 protein that correlates with anthocyanin biosynthesis. The remaining transcription factors that were evaluated were MYBs. All transcription factors showed low expression levels with GNT treatment under dark and light conditions (Figure 6). The largest upregulation in expression, which was only onefold higher than that of the control, was observed under light conditions in *BoMYB2* and *BoMYB12* (Figure 6A). The transcription factor *BoTT2* exhibited a slight downregulation. Under dark conditions, the transcription factors showed no change or downregulation with GNT treatment (Figure 6B). These results indicated that GNT may directly regulate the expression of structural genes in anthocyanin biosynthesis instead of by transcription factors.

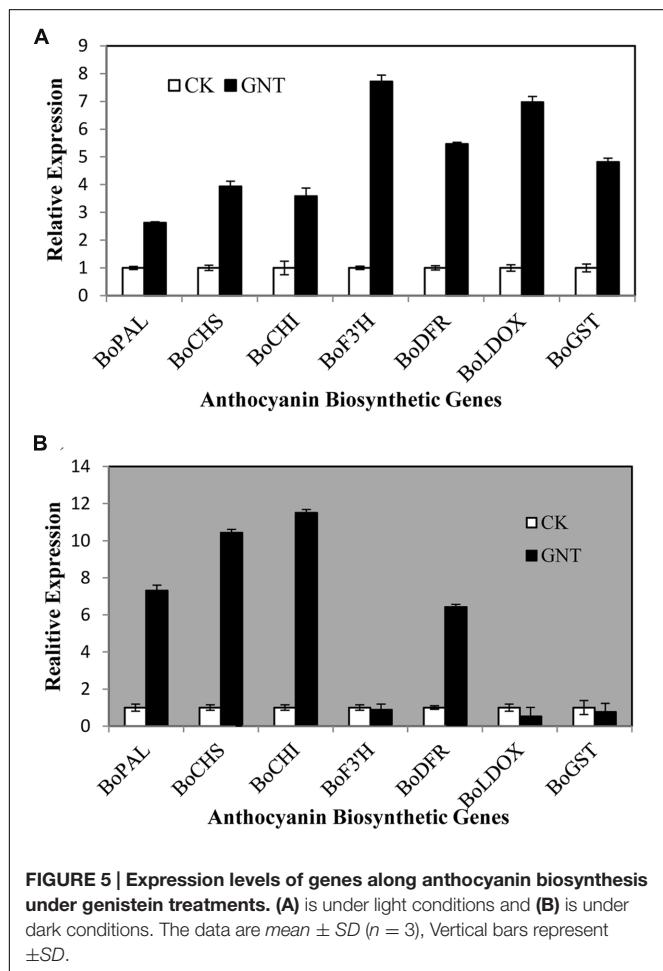
## Effects of GNT Treatment on the Antioxidant System in Red Cabbage

GNT treatment not only promoted anthocyanin accumulation but also improved antioxidant activity in red cabbage. Reactive oxygen species (ROS) cause lipid peroxidation, which is presented by MDA content. ROS can be used as an efficient indicator of the integrity of cell membranes in plants subjected to ROS stress. In the present study, the GNT-treated groups showed lower MDA content compared with the control group (Figure 7). This difference indicated that GNT treatment helped maintain the integrity of cell membranes in red cabbage seedlings. GNT treatment also increased the activities of classical antioxidant enzymes, such as SOD, POD, and CAT. The

**TABLE 2 | Chlorophyll content of GNT treatments under dark and light conditions: Chla (chlorophyll a), Chlb (chlorophyll b).**

Treatments	Chl a/(mg/g <sup>-1</sup> )	Chl b/(mg/g <sup>-1</sup> )	carotenoid(mg/g <sup>-1</sup> )
CK	0.971 ± 0.027a	0.386 ± 0.006a	0.333 ± 0.013a
GNT	0.901 ± 0.043a	0.343 ± 0.014b	0.312 ± 0.013a
CK	0.725 ± 0.059b	0.300 ± 0.019c	0.271 ± 0.021b
GNT	0.692 ± 0.012b	0.285 ± 0.010c	0.267 ± 0.007b

Different letters in the same column indicate a significant difference in particular series at  $P = 0.05$  according to ANOVA and Duncan's multiple range tests.

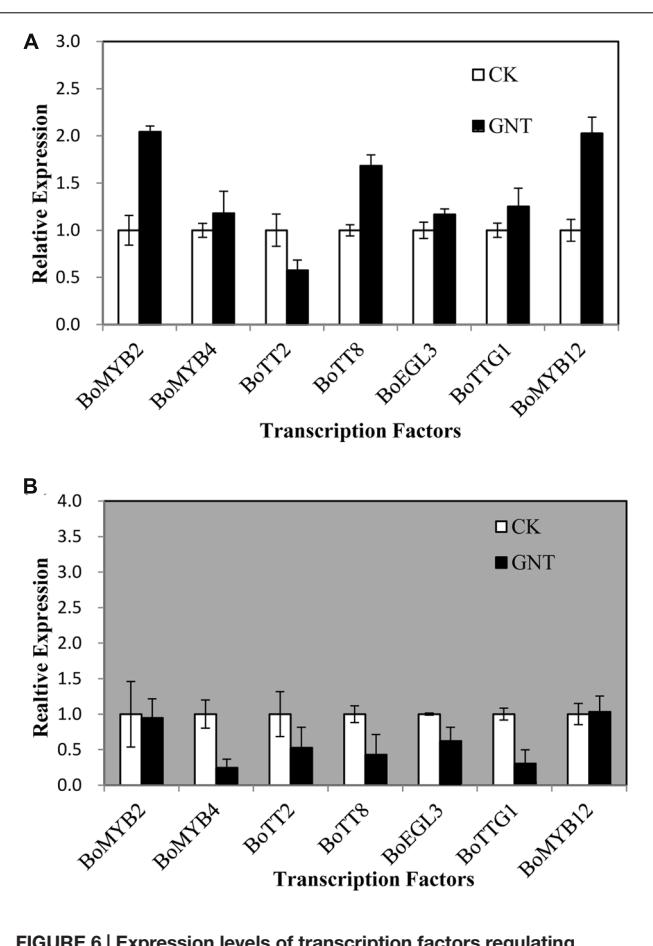


**FIGURE 5 | Expression levels of genes along anthocyanin biosynthesis under genistein treatments. (A)** is under light conditions and **(B)** is under dark conditions. The data are  $\text{mean} \pm \text{SD}$  ( $n = 3$ ), Vertical bars represent  $\pm \text{SD}$ .

activities of these enzymes were significantly upregulated by GNT treatment (Figure 7). The activities of these enzymes were highly collaborated to anthocyanin levels with GNT treatment. Anthocyanins were reported to possess antioxidant activity and upregulated antioxidant activity may be attributable to upregulated anthocyanin accumulation (Zhu, 2002).

## DISCUSSION

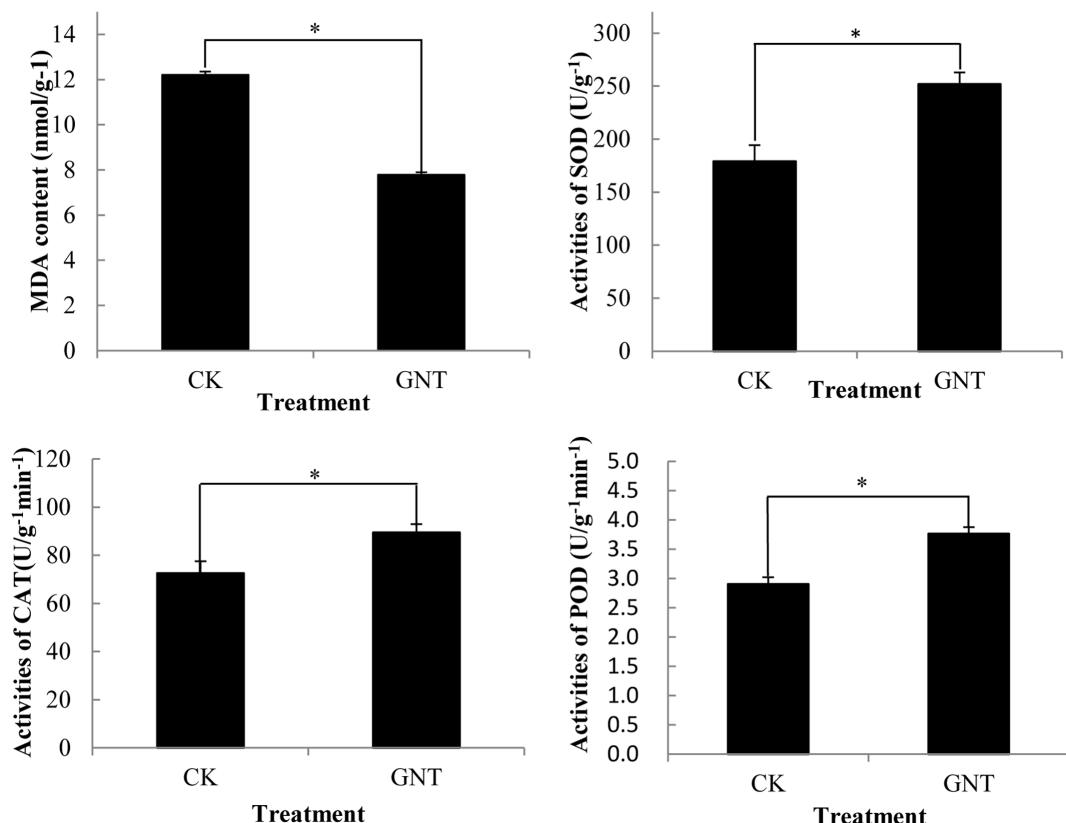
In the current study, GNT exerted a promotive effect on anthocyanin accumulation in red cabbage, similar to the previously reported chemicals, including ALA, ABA, cGMP, and



**FIGURE 6 | Expression levels of transcription factors regulating anthocyanin biosynthesis under genistein treatments. (A)** is under light conditions and **(B)** is under dark conditions. The data are  $\text{mean} \pm \text{SD}$  ( $n = 3$ ), Vertical bars represent  $\pm \text{SD}$ .

MT (Figure 2). No studies have reported on the effect exerted by GNT; thus, we evaluated the effect of GNT on anthocyanin accumulation in detail. We found that  $30 \text{ mg L}^{-1}$  GNT induced the highest anthocyanin level, which is 2.4-fold that of the CK group (Figure 3). Furthermore, the anthocyanin promotive effect is light-dependent. Under dark conditions, no significant change in anthocyanin level was observed (Figure 4).

Anthocyanin biosynthesis is regulated primarily at the transcriptional level. To explore the mechanisms of upregulated anthocyanin accumulation by GNT, we evaluated the expression of several genes and transcription factors in the anthocyanin biosynthetic pathway. Figure 8 presents the mechanisms identified. The middle section of the graph is the anthocyanin biosynthetic pathway. PAL is the first committed enzyme in the anthocyanin biosynthetic pathway, which leads to the production of numerous flavonoids (Springob et al., 2003). This enzyme catalyzes the formation of *trans*-cinnamic acid from phenylalanine. CHS catalyzes the formation of a triketide intermediate from 4-coumaroyl-CoA and three molecules of malonyl-CoA; subsequently, spontaneous cyclization of triketide intermediate results in the formation of naringenin chalcone.

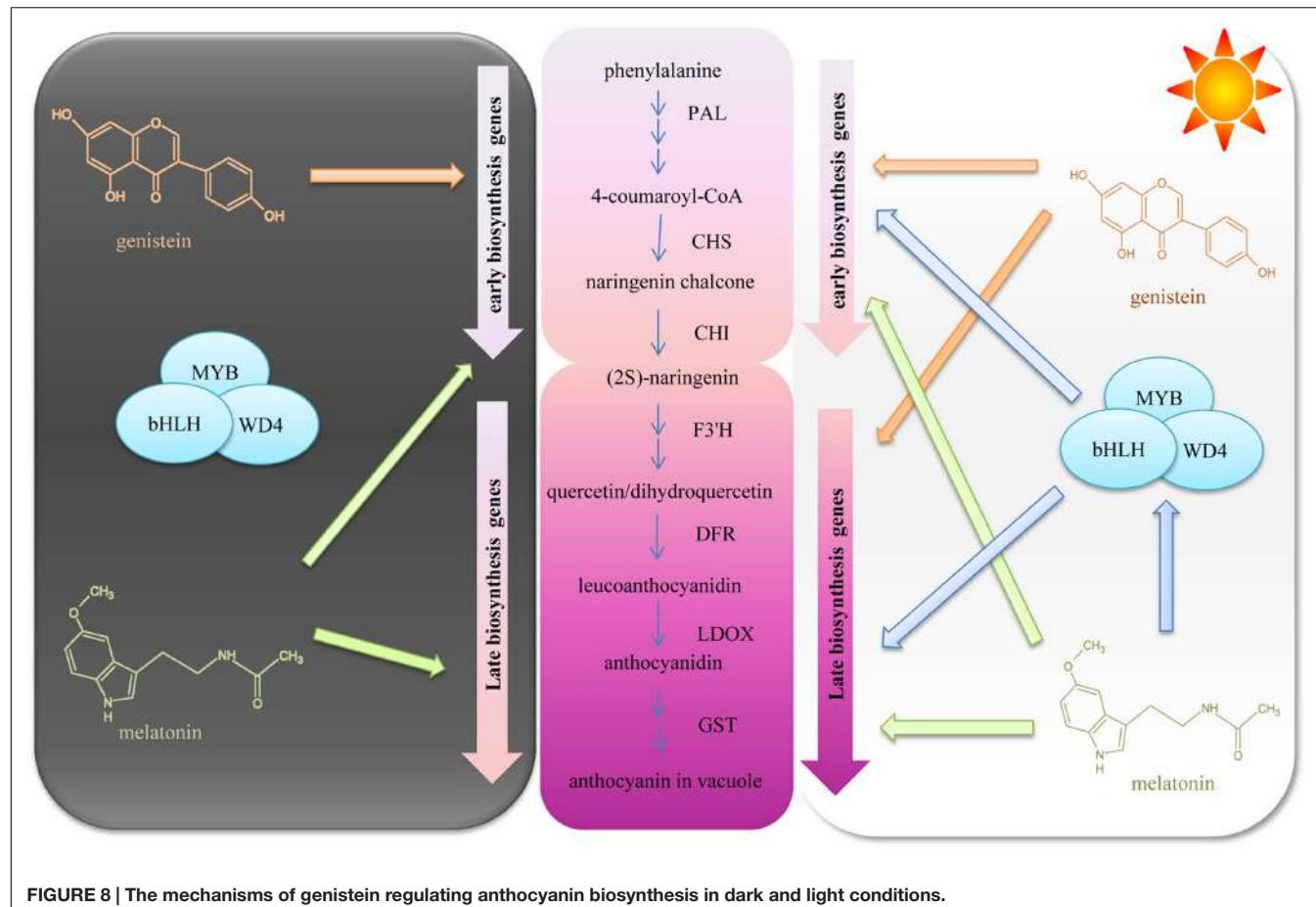


**FIGURE 7 |** Genistein treated cabbage showed lower MDA levels and higher antioxidant system enzyme activities. The data are mean  $\pm$  SD ( $n = 3$ ). Vertical bars represent  $\pm$  SD. The symbol \* represents significant difference with CK treatment at  $P = 0.05$  level.

CHI catalyzes the stereospecific cyclization of naringenin chalcone to (2S)-naringenin (Zhang et al., 2016). These genes participate in the early steps of anthocyanin biosynthesis. The products of these steps are also intermediate products of other products. We found that these genes can be regulated by GNT under both dark and light conditions, as shown in Figure 5. The compound synthetic pathways are complex, resembling a network. The product of every step is also an intermediate of other compounds. No significant change in anthocyanin levels were observed with GNT treatment under dark conditions (Figure 4), maybe some other products share the early steps were upregulated. GNT directly regulated the early biosynthetic genes under dark and light conditions without the participation of transcription factors (Figure 8). This process slightly differed from the mechanism of melatonin. Melatonin directly regulates the expression of early genes under both dark and light conditions. However, in the presence of light, melatonin can regulate the genes both directly and with transcription factors (Zhang et al., 2016). F3'H is a cytochrome P450 monooxygenase committed in the hydroxylation of the 3'-position of the B-ring of flavonoid (Holton and Cornish, 1995). This enzyme can accept either dihydrokaempferol or kaempferol as a substrate and convert them to dihydroquercetin and quercetin, respectively.

DFR catalyzes a reduction reaction of dihydroflavonol to leucoanthocyanidin. The enzyme LDOX catalyzes the formation of anthocyanidin from leucoanthocyanidin with 2-oxoglutarate and oxygen as co-substrates. GST protein is the flavonoid carrier, forming conjugates with anthocyanins, thereby preventing them from oxidation. It participates in the transport of anthocyanins from the cytosol to the vacuole (Zhang et al., 2016). These genes are late biosynthetic genes in the anthocyanin pathway. Under light conditions, GNT upregulation of these gene expression levels resulted in high anthocyanin biosynthesis (Figures 4 and 5A). However, under dark conditions, these late genes exhibited no response to GNT and produced no increase in anthocyanin accumulation (Figures 4 and 5B).

In all higher plants studied to date, the anthocyanin pigment pathway is regulated by a suite of transcription factors that include MYB, bHLH, and WD-repeat proteins (Broun, 2005; Gonzalez et al., 2008; Xu et al., 2015). Many signaling molecules affect anthocyanin synthesis by activating the transcription factors. ALA was reported to upregulate these transcription factors (Xie et al., 2013). ABA, jasmonate, or cytokinins induced anthocyanin biosynthesis; GA, ethylene, or brassinosteroids repressed anthocyanin accumulation, which were related to the activation and repression of these transcription factors (Loreti et al., 2008; Carvalho et al., 2010; Jeong et al., 2010). The



**FIGURE 8 |** The mechanisms of genistein regulating anthocyanin biosynthesis in dark and light conditions.

results of our study indicated that the transcription showed no response to GNT treatment. GNT upregulated the anthocyanin biosynthetic genes. This effect suggested that GNT can be a potential downstream factor along the anthocyanin biosynthetic pathway.

Light is one of the most important environmental factors regulating plant development and gene expression. Light exposure can increase the concentration of anthocyanins by activating many genes along the biosynthetic pathway. Under dark conditions, GNT could only regulate the early genes of anthocyanin biosynthesis (Figure 8); under light conditions, all genes along the biosynthetic pathway were activated and exerted a higher expression with GNT treatment. Melatonin regulated the expression levels of both early and late genes under dark conditions but only mildly. Under light conditions, the expression of these genes sharply increased with melatonin treatment (Zhang et al., 2016). Not only the structural genes but the transcription factors as well strongly responded to melatonin under light conditions.

## CONCLUSION

In this study, we found a novel anthocyanin biosynthesis promoter: GNT. It is a plant-derived molecule that plays a

role in maintaining health. We also found that GNT affects anthocyanin biosynthesis in a light-dependent manner. Anthocyanin biosynthetic genes showed an upregulated expression under light conditions. Under dark conditions, only the early biosynthetic genes responded to GNT. GNT directly regulated the structural genes—i.e., without the participation of transcription factors—unlike other signaling molecules. Anthocyanin is shown to be an effective antioxidant. Our study found that GNT treatment also improved the antioxidant activity of red cabbage.

## AUTHOR CONTRIBUTIONS

NZ, YQ, and Y-DG designed research; NZ, YQ, H-JZ, XW, HL, and YS performed the experiments; NZ, YQ, H-JZ analyzed the data; NZ and YQ wrote the manuscript. NZ and Y-DG revised the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Melatonin Improved Anthocyanin Accumulation by Regulating Gene Expressions and Resulted in High Reactive Oxygen Species Scavenging Capacity in Cabbage

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In this work, we found, that exogenous melatonin pretreatment improved anthocyanin accumulation (1- to 2-fold) in cabbage. To verify the relationship with melatonin and anthocyanin, an *Arabidopsis* mutant, snat, which expresses a defective form of the melatonin biosynthesis enzyme SNAT (Serotonin N-acetyl transferase), was employed. Under cold conditions, the foliage of wild-type *Arabidopsis* exhibited a deeper red color than the snat mutant. This finding further proved, that exogenous melatonin treatment was able to affect anthocyanin accumulation. To gain a better understanding of how exogenous melatonin upregulates anthocyanin, we measured gene expression in cabbage samples treated with melatonin and untreated controls. We found that the transcript levels of anthocyanin biosynthetic genes were upregulated by melatonin treatment. Moreover, melatonin treatment increased the expression levels of the transcription factors MYB, bHLH, and WD40, which constitute the transcriptional activation complex responsible for coordinative regulation of anthocyanin biosynthetic genes. We found, that free radical generation was downregulated, whereas the osmotic adjustment and antioxidant capacities were upregulated in exogenous melatonin-treated cabbage plants. We concluded, that melatonin increases anthocyanin production and benefits cabbage growth.

**Keywords:** anthocyanin, cabbage, gene expression, melatonin, reactive oxygen species

## INTRODUCTION

The pigmentation of most plants is controlled by the relative concentrations of anthocyanin, chlorophyll, and carotenoid pigments. Anthocyanins are the most important group of water-soluble pigments in plants and contribute to the blue, red, or purple colors of leaves, flowers, or fruits. Anthocyanins belong to a group of plant natural products with antioxidant activity and play important roles in protecting humans from diseases. The consumption of vegetables and fruits with these colors has become popular among health-conscious consumers due to their high levels of anthocyanins.

Anthocyanins belong to a class of flavonoids synthesized via the phenylpropanoid pathway. They are synthesized, along with flavonols, from phenylalanine and malonyl-CoA (Winkel-Shirley, 2002). The biosynthesis of anthocyanin pigments and the gene networks, that regulate synthesis have been extensively studied (Grotewold, 2006; Allan et al., 2008). Two classes of genes are involved in anthocyanin biosynthesis, structural genes encoding the enzymes, that directly participate in the formation of anthocyanins and other flavonoids and regulatory genes that control the transcription of structural genes. The structural genes, including phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), and UDP -glucose: flavonoid 3-O-glucosyltransferase (UFGT), and glutathione S-transferase (GST), encode the corresponding enzymes responsible for the biochemical reactions of anthocyanin synthesis. The second group consists of transcription factors that regulate the structural gene expression in anthocyanin biosynthesis. These transcription factors principally belong to two classes, MYB and bHLH, and are thought to co-operatively regulate the anthocyanin biosynthetic genes with a WD40 protein (Gonzalez et al., 2008; Feller et al., 2011). The transcription factors may form complexes to regulate the expression of the biosynthetic genes, e.g., MYB-bHLH-WDR (MBW) transcription factor complex (Zhang et al., 2003; Xu et al., 2014). Many MYB transcription factors, such as MYB12, have been shown to coordinately regulate the early flavonoid biosynthetic genes CHS, CHI, and F3H (Mehrtens et al., 2005), MYB111 (Stracke et al., 2007). MYB-recognition elements have been identified in the promoters of three genes in *Arabidopsis* and are important for light responsiveness (Hartmann et al., 1998, 2005). The MYB transcription factors PAP1 and PAP2 together with three potential bHLH partners [transparent testa8 (TT8), glabra (GL3) and enhanced glabra (EGL3)], and the WD40 protein transparent testa glabra 1 (TTG1) participate in the regulation of the late anthocyanin biosynthesis genes DFR, LDOX, and UFGT (Baudry et al., 2004). *Arabidopsis* plants, that overexpressed PAP1 or PAP2 showed intense purple pigmentation in many vegetative organs throughout development. A detailed analysis of PAP1 over-expressing plants showed that some anthocyanin biosynthetic genes are highly expressed, and the accumulation of anthocyanins is strongly enhanced (Borevitz et al., 2000; Tohge et al., 2005). MYBL2 expression is controlled by negative regulatory feedback that involves an atypical bHLH BES1, which plays a role in the brassinosteroid (BR)-regulated anthocyanin accumulation pathway (Ye et al., 2012). GL3, EGL3, and TT8 have partially redundant roles in the regulation of anthocyanin accumulation. Specifically, EGL3 appears to play a dominant role, as demonstrated by analyses of single, double, and triple mutants (Zhang et al., 2003). These factors can form a ternary complex, that regulates anthocyanin biosynthesis in *Arabidopsis* seeds (Baudry et al., 2004). A yeast two hybrid analysis revealed that EGL3, GL3, and TT8 interact with TTG1 as well as PAP1 and PAP2. Furthermore, transient expression assays of the DFR promoter have shown that different combinations of EGL3

and GL3 with PAP1 and PAP2 result in very strong activation (Zimmermann et al., 2004).

Many environmental factors affect the biosynthesis of anthocyanin in plants. The effects of light on anthocyanin synthesis have been extensively studied in many plants. Anthocyanin can be induced by broad wavelengths of light, including the UV, visible, and far-red regions (Chalker-Scott, 1999). By absorbing high-energy quanta, anthocyanic cell vacuoles protect chloroplasts from both the photoinhibitory and phototoxic effects of strong light (Gould, 2004). Foliar anthocyanins also act as sunscreens against potentially damaging UV-B radiation (Gould, 2004). In many plants, the accumulation of anthocyanin is also promoted by low temperatures (Leyva et al., 1995), a phenomenon, that is strikingly apparent in autumn leaves and alpine plants. Osmotic stress, such as saline excess, water deficiency, and flooding stress, can all induce anthocyanin accumulation (Chalker-Scott, 1999; Hughes et al., 2013). These environmental or developmental regulations mostly rely on the coordinated expression of anthocyanin biosynthesis genes. Hormone signals have also been shown to be involved in anthocyanin regulations [e.g., induction by abscisic acid (ABA), jasmonate (JA), or cytokinins, and repression by gibberellic acid (GA), ethylene, or BRs; (Peng et al., 2011; Shi and Xie, 2014; Xu et al., 2015)].

Anthocyanins, other flavonoids, and phenolic acids belong to a group of plant natural products with antioxidant activity and play important roles in plant protection from stress. Active oxygen produced under stress has generally been accepted as a detrimental factor that causes the gradual peroxidation of lipid structures (Baryla et al., 2000), antioxidant enzyme inactivation (Teisseire and Guy, 2000), and oxidative DNA damage (Kasprzak, 2002). Many experiments have proven, that anthocyanins can inhibit the formation of free radicals and reduce the level of reactive oxygen species (ROS). For example, anthocyanins inhibit hydroxyl radical generation by chelating ferrous ions and effectively scavenging the super-oxide and hydrogen peroxide generated by mechanical injury, sudden temperature changes, or exposure to strong light (Gould et al., 2002). H<sub>2</sub>O<sub>2</sub> rapidly diffuses through membranes, which may allow vacuolar anthocyanins to scavenge ROS (Hatier and Gould, 2009). In addition to their function as scavengers of free radicals, flavonoids, and phenolic acids also act as chelating metals, that generate ROS via the Fenton reaction. The presence of the OH group at the 3-position of the flavonoid skeleton is the main structural feature responsible for chelating metal ions, such as iron, copper, zinc, and aluminum (Kidd et al., 2001; Verdan et al., 2011). UV-B radiation modifies the production of anthocyanins (Mazza et al., 2000; Bassman, 2004). The UV-absorbing characteristics of anthocyanins have long been considered evidence for the role of UV protection. Anthocyanins offer multifaceted, versatile, and effective protection to plants under stress: they are the Swiss army knife of the plant kingdom (Gould, 2004).

Melatonin is endogenously produced in all plant species and has recently been identified as a free radical scavenger (Galano et al., 2011; Reiter et al., 2015). Solid evidence implicates melatonin as a growth promoter and rooting agent

(Hernández-Ruiz et al., 2004, 2005; Arnao and Hernández-Ruiz, 2007; Hernández-Ruiz and Arnao, 2008; Sarrou et al., 2014; Zhang N. et al., 2014). In addition to its roles in plant development, melatonin plays an important role in plant stress defense (Arnao and Hernández-Ruiz, 2009, 2013, 2014, 2015; Zhang et al., 2015). In our previous study, we found, that melatonin alleviated drought- and saline-induced germination inhibition (Zhang et al., 2013; Zhang H. J. et al., 2014). We also found, that melatonin played an important role in fruit ripening and quality in tomato (Sun et al., 2015). Serotonin N-acetyltransferase (SNAT) is the penultimate enzyme in the melatonin biosynthesis pathway in plants. snat is a SNAT gene-inactivation mutant line generated by T-DNA insertion. The melatonin level in the snat knockout mutant lines was 50% less than that in wild-type *Arabidopsis* plants (Lee et al., 2015).

In this study, we examined the biochemical and transcriptional changes responsible for the increased anthocyanin accumulation of cabbage sprouts treated with melatonin. We showed, that melatonin treatment upregulated the expressions of both structural genes in the anthocyanin biosynthesis pathway and their transcription factors. Melatonin application resulted in a significant increase in anthocyanin accumulation in cabbage sprouts, and these samples consequently exhibited downregulated ROS levels and upregulated antioxidant activities and markers of osmotic adjustment.

## MATERIALS AND METHODS

### Plant Materials

Seeds of white cabbage and red cabbage were sterilized in 5% sodium hypochlorite solution for 10 min, rinsed in distilled water five times, and air-dried prior to melatonin treatment. Sterilized seeds were hydro primed using distilled water and melatonin solution at concentration of 10, 50, 500, and 1000  $\mu\text{mol/L}$  for 12 h. After air-dried to the initial water content, seeds were spread on the Petri dish with three layer of moisture filter paper (Whatman International Ltd, Maidstone, UK) to germinate. Seedlings were cultured under 14-h-light/10-h-dark cycle (23–25°C/15–18°C day/night) environment-controlled cabinets. The 7-day-old seedlings were collected without colorless root to measure the anthocyanin content. For gene expression experiments, seedlings were first cultured in constant dark conditions for 3 days and then light conditions for another 3 days after germination. The samples for Q-PCR were collected every day. Each treatment consisted of 20 dishes with 100 seedlings per dish. All experiments were conducted in triplicate.

Mutant and wild-type *Arabidopsis thaliana* lines were of the Columbia (Col-0) ecotype. T-DNA insertion SNAT-deficient mutants designated *snat* (SALK\_032239) was obtained from the Arabidopsis Biological Resource Center (<http://abrc.osu.edu/>). *Arabidopsis* plants were grown in 7 × 7 cm plots in a controlled environment growth room at 23°C under a 16 h light/8 h dark photoperiod. After 4 weeks' culturing, *Arabidopsis* plants were transferred to a growth chamber with temperature of 5°C, an d

photoperiod of 16 h light/8 h dark. This 5°C treatment remained for 14 days.

### Reagents

All chemicals used in experiments were of analytical grade. Melatonin (N-acetyl-5-methoxytryptamine) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were purchased from Sinopharm Chemical Reagent (Beijing Co., Ltd, China).

### Total Anthocyanin Content

Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra (Pazmino-Duran et al., 2001). Plant material was ground to powder under liquid nitrogen and then was dissolved in a 0.025 M potassium chloride buffer, pH = 1.0, and 0.4 M sodium acetate buffer, pH = 4.5. The absorbance of each solution was measured at 510 and 700 nm, against a blank cell filled with distilled water.

The absorbance (A) of the diluted sample was calculated by the following formula:

$$A = (A_{510} - A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5} \quad (1)$$

The monomeric anthocyanin pigment concentration in the original sample was expressed in equivalence of cyanidin-3-glucoside that is the main anthocyanin in cabbage, and calculated by the following formula:

$$\text{Monomeric anthocyanin pigment(mg/L)} = (A^* \text{MW} * 1000) / (E^* 1) \quad (2)$$

Where MW is the molecular weight of cyanidin-3-glucoside in 449.2; and E is the molar absorptivity, which equal to 26,900 for cyanidin-3-glucoside.

### Evaluation of ROS and Antioxidant Enzymes

The content of ROS and the activities of antioxidant enzymes were measured following the method described in our previous work (Zhang et al., 2013). The ROS in this research included  $\text{H}_2\text{O}_2$ ,  $\text{OH}^-$ , and  $\text{O}_2^-$ . The antioxidant enzymes in this research included superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase.

### Lipid Peroxidation Measurements

The level of lipid peroxidation of each sample was measured as malondialdehyde (MDA) content determined by reaction with 2-thiobarbituric acid (TBA) reactive substances as described in our previous paper (Zhang et al., 2013). A 0.5 g leaf sample was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10,000 g for 5 min. Four milliliters of 20% TCA containing 0.5% TBA were added to 1 ml of the supernatant then incubated in boiling water for 20 min. The reaction was stopped by placing the reaction tubes on ice. MDA absorption was measured spectrophotometrically at 450, 532, and 600 nm. The concentration of lipid peroxides was thus quantized in terms of the MDA concentration and expressed as nmol/g.

## Proline Determination

The sprouts (200 mg) were homogenized with 4 ml of 3% sulphosalicylic acid using a mortar and pestle. The homogenate was centrifuged at 5000 × g for 10 min at 4°C. To 1 ml of the supernatant, 1 ml of glacial acetic acid, and 1 ml of acid ninhydrin was added and boiled for 1 h. After cooling 4 ml of toluene was added and shaken for 30 s. After separating the layers, the upper (toluene) was collected and absorbance was measured at 520 nm on a spectrophotometer (UV2800A; UNICO, Shanghai, China). Proline content was determined using a calibration curve prepared with known concentrations. All tests were repeated at least three times, and the results are presented as means ± SD.

## PAL Activity Assay

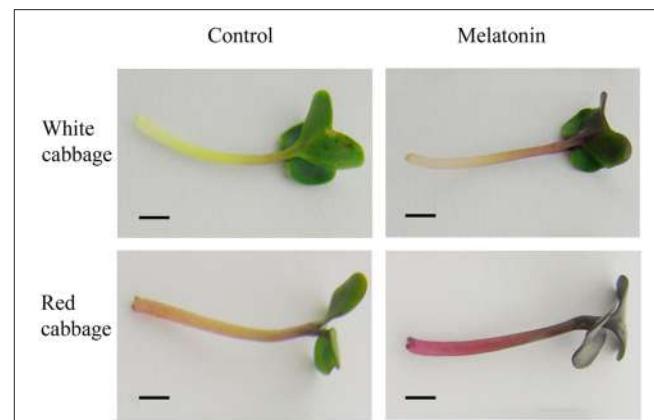
Phenylalanine ammonialyase activity was determined according to Flores et al. (2005) with slight modifications. Total protein was extracted from 500 mg (FW) sprouts in 1 ml extraction buffer (50 mM borate buffer, pH 8.8 containing 5 mM β-mercapto ethanol and 2% w/v polyvinyl pyrrolidone from Sigma, USA). The reaction mixture contained 0.25 ml of enzyme extract, 0.75 ml extraction buffer, 0.35 ml double-distilled water, and 0.15 ml 100 mM L-phenylalanine (Sigma). The reaction mixture was incubated for 30 min at 40°C, and terminated by adding 50 µl of 2.5 M HCl. PAL activity was determined by measuring the concentration of E-cinnamic acid produced at A290 (Flores et al., 2005). Protein concentration was determined using Bradford reagent (Bio-Rad, USA). PAL activity was expressed as micromole E-cinnamic acid in 1 min per gram protein.

## Real-Time Quantitative PCR (qPCR) Analysis

Total RNA was extracted from the samples using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). The primers of selected genes were designed using Primer-priemer 5 software (PREMIER Biosoft, Palo Alto, CA, USA) and synthesized by Sangon. The primer pairs are summarized in **Table S1**. The cDNA was synthesized from 1 µg of total RNA using PrimeScript RT reagent Kit (Takara, Da Lian, China) in 20 µl of reaction mixture. The amount of the amplified DNA was monitored by fluorescence at the end of each cycle using 7500 Real-Time PCR System (Applied Biosystems). Each plate was repeated three times in independent runs for all reference and selected genes. Gene expression was evaluated by the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

## Melatonin Extraction and Analysis

Melatonin was extracted from cabbage seeds and seedlings according to the method described in our previous work (Zhang H. J. et al., 2014). Approximately 1 g frozen samples were ground into powder with liquid nitrogen and homogenized with 10 mL methanol. After centrifugation at 11,417 g at 4°C for 15 min, the supernatants were collected and dried using nitrogen gas. The extracts were then dissolved in 5% methanol and purified using a C18 solid phase extraction (SPE) cartridge (Waters, Milford, MA, USA). The cartridge was next washed with 10 mL 5% methanol, and melatonin was eluted finally at a natural flow rate with 2 mL 80% methanol. The extract was subsequently filtered



**FIGURE 1 | Melatonin treatment influenced the color depth in white cabbage and red cabbage.** The concentration of melatonin pretreatment is 1000 µmol/L in white cabbage and 100 µmol/L in red cabbage.

through a 0.22 µm PTFE syringe filter before UHPLC-ESI-MS/ MS analysis. Melatonin determination and quantification was analyzed using a UHPLC-ESI-MS/MS (UHPLC-1290 Series and a 6460 QqQ-MS/MS; Agilent Technologies, Waldbronn, Germany) with an Agilent SB-C18 column (4.6 9 50 mm; 1.81 m; Agilent Technologies, Santa Clara, CA, USA).

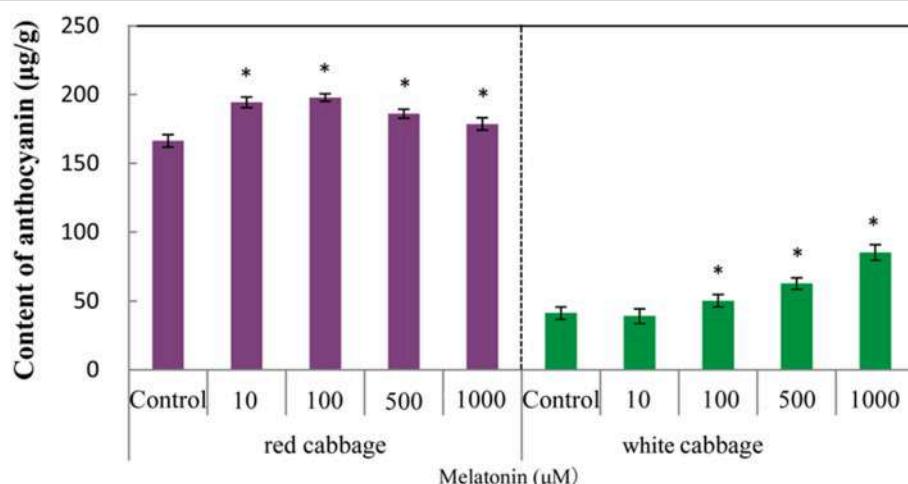
## RESULTS

### Melatonin Treatment Increased Anthocyanin Concentration in Cabbage Seedlings

We analyzed the anthocyanin content in white and red cabbage sprouts pretreated with melatonin and untreated controls. **Figure 1** shows, that melatonin-treated cabbage sprouts exhibited darker hypocotyls and cotyledons (**Figure 1**). The total anthocyanin contents of the two cabbage cultivars are presented in **Figure 2**. To assess the effect of melatonin on the anthocyanin accumulation in cabbage sprouts, the sprouts were pre-treated with four concentrations of melatonin (10, 100, 500, and 1000 µM/L). Melatonin promotes anthocyanin accumulation in both red and white cabbage. In red cabbage, the four concentrations of melatonin all significantly increased anthocyanin content of sprouts. Specifically, the anthocyanin concentration was highest in red cabbage sprouts treated with 100 µM/L melatonin (198 µg/g FW, 18.7% higher than, that in the control). All concentrations of melatonin pretreatment except 10 µmol/L, which was the lowest concentration, significantly increased the anthocyanin content in white cabbage. The most effective concentration was 1000 µmol/L, which doubled the anthocyanin content in white cabbage compared with the control. The results indicated that melatonin improved the anthocyanin content of cabbage.

### Melatonin-Deficient *Arabidopsis* Mutant Accumulated Low Levels of Anthocyanin

To further confirm the relationship between melatonin and anthocyanin synthesis, we studied a T-DNA insertion



**FIGURE 2 | The anthocyanin levels in white cabbage and red cabbage.** Vertical bars at each column represent standard deviation of three replications.

\*Significant difference between this column and control at  $P < 0.05$ .



**FIGURE 3 | Anthocyanin accumulation in wild type *Arabidopsis* and melatonin defect mutant.**

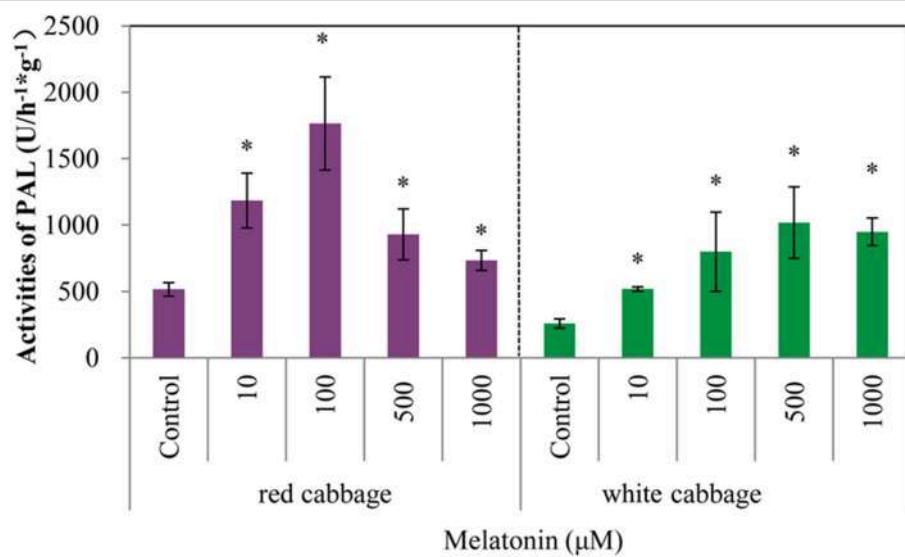
SNAT-deficient mutant that accumulates low levels of melatonin. SNAT is the penultimate enzyme in the melatonin biosynthesis pathway in plants. The melatonin level in the snat knockout mutant lines was 50% less than, that in wild-type *Arabidopsis* Col-0 plants (Lee et al., 2015). Wild-type and mutant seedlings exhibited color differences after 10 days of cold treatment (Figure 3). The seedlings of the wild-type Columbia ecotype darkened after cold treatment, whereas the melatonin-deficient mutant seedlings were not as dark as the wild-type seedlings. Because the snat mutant accumulates low levels of melatonin and correspondingly accumulates less anthocyanin than the wild type, we speculated that melatonin positively affects anthocyanin accumulation.

### Melatonin Treatment Upregulated the Activity of Phenylalanine Ammonia-Lyase

Phenylalanine ammonia-lyase initiates anthocyanin biosynthesis (Feng et al., 2008). The anthocyanin contents of white cabbage and red cabbage generally correlated with the PAL activities (Figure 4). Melatonin treatment significantly increased the activity of PAL in a concentration-independent manner. The maximum PAL activity in melatonin-treated samples was 4-fold higher than the level of the control. The PAL activity in red cabbage was higher than, that in white cabbage, which is consistent with the anthocyanin biosynthesis in these species. Figures 2, 4 show a positive linear relationship between the PAL activity and the amount of accumulated anthocyanin. Therefore, although PAL participates in the early steps of the synthesis of numerous phenolic and flavonoid compounds, its activity significantly affects anthocyanin synthesis.

### Effect of Melatonin Treatment on the Expression Levels of Genes along the Anthocyanin Biosynthetic Pathway

To assess whether the increase in the anthocyanin concentration following melatonin treatment is due to an increase in its



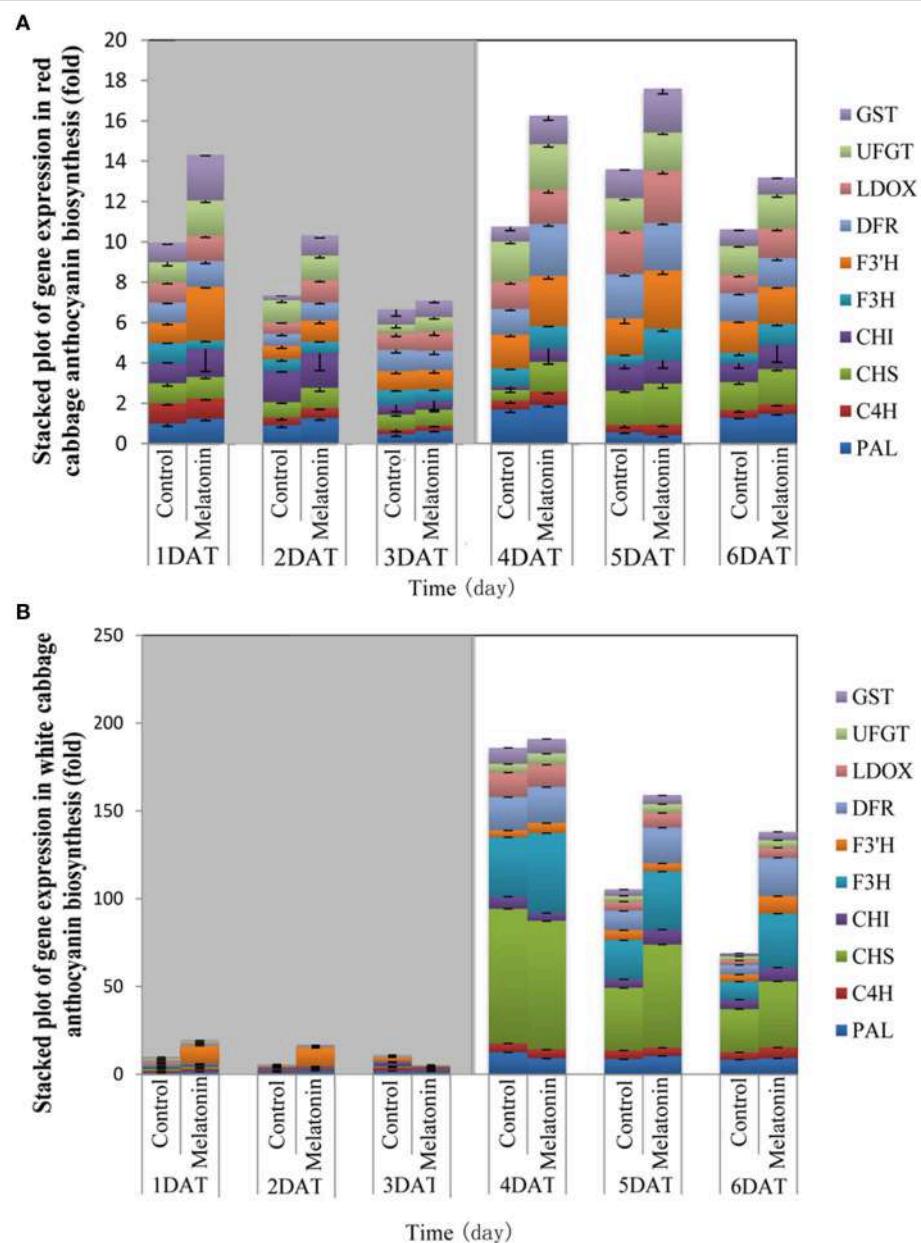
**FIGURE 4 | Melatonin treatment affected PAL activities in white cabbage and red cabbage.** Vertical bars at each column represent standard deviation of three replications. \*Significant difference between this column and control at  $P < 0.05$ .

biosynthesis, the expression levels of transcripts that encode ten anthocyanin biosynthesis genes (*PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *LDOX*, *UGT*, and *GST*) were measured using quantitative real-time PCR.

The expression patterns of the ten biosynthesis genes in white and red cabbage under dark and light conditions are shown in **Figure 5**. Under dark conditions, the expression levels of anthocyanin biosynthesis genes were low and increased when exposed to light. This phenomenon was more marked in white cabbage than in red cabbage (**Figure 5**). The expression levels of anthocyanin biosynthesis genes were upregulated by melatonin treatment under both dark and light conditions. The expression levels of these ten genes were low when seedlings were maintained in the dark. Melatonin treatment tended to increase the expression levels of these biosynthesis genes in both white and red cabbage, despite their low expression levels in the dark. When light was added to the culture environment, the expression levels of genes significantly increased. In white cabbage, the expression levels of genes increased 100-fold when stimulated by light (**Figure 5B**). This change indicated that the expression of anthocyanin biosynthesis genes is triggered by light in white cabbage, whereas these genes maintain a relative high expression in red cabbage under both light and dark conditions.

*PAL* is the first committed enzyme in the anthocyanin biosynthesis pathway, that leads to the production of many flavonoids. This enzyme catalyzes the formation of trans-cinnamic acid from phenylalanine. The melatonin-treated samples expressed higher levels of *PAL* at four of six tested time points (**Figures 6, 7**). This finding indicated, that melatonin modulates the expression of genes starting during the early steps in anthocyanin biosynthesis. *C4H* is a cytochrome P450 monooxygenase that catalyzes the hydroxylation of trans-cinnamic acid at the C-4 position to yield 4-coumaric acid. In

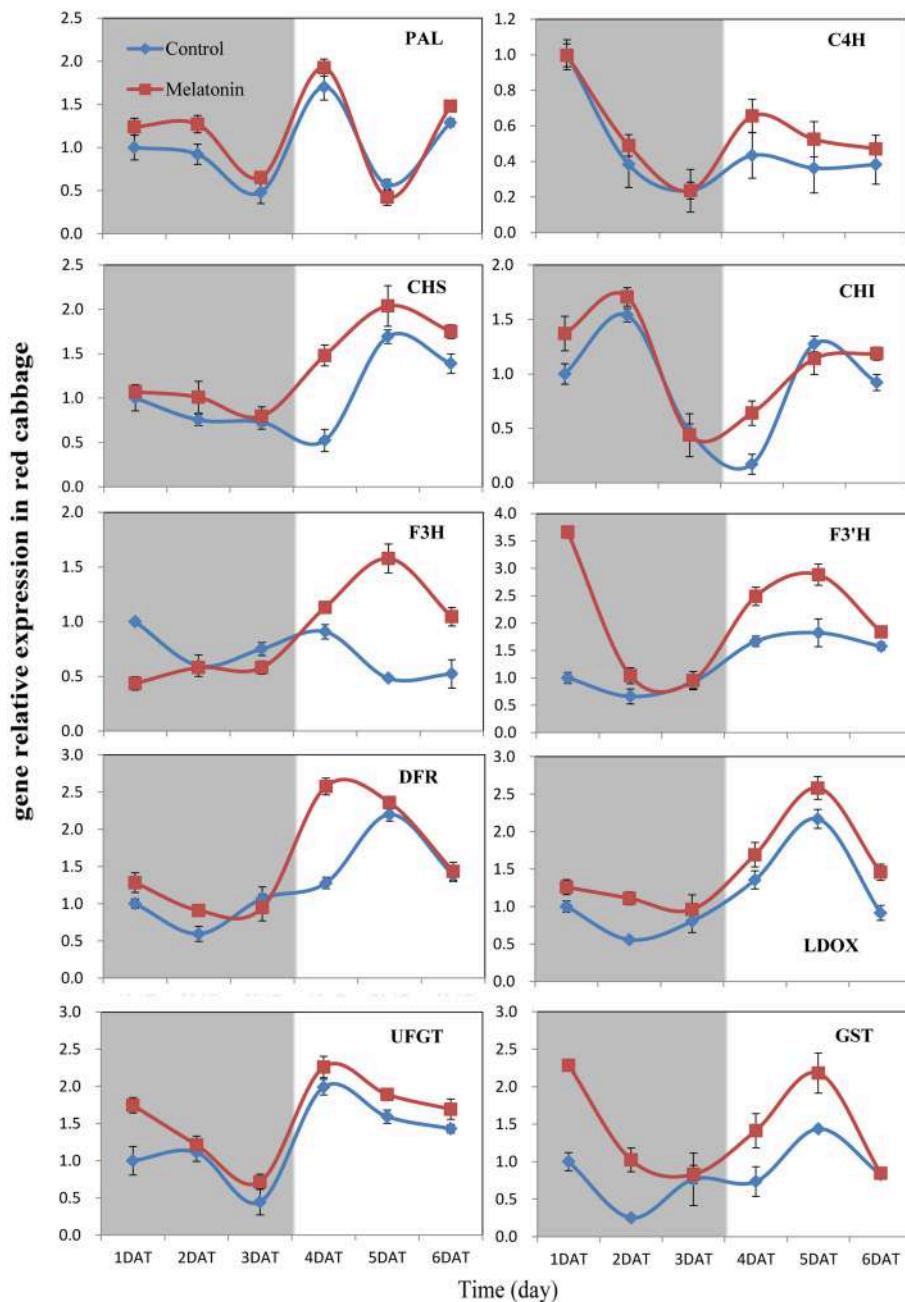
the dark, the expression levels of *C4H* of control and melatonin treated samples were similar. When light was introduced to the culture environment, the expression levels of *C4H* in melatonin-treated samples were much higher than that in the control (**Figures 6, 7**). *CHS* catalyzes the formation of a triketide intermediate from p-coumaroyl-CoA and three molecules of malonyl-CoA; the subsequent spontaneous cyclization of the triketide intermediate then results in the formation of naringenin chalcone. When exposed to light, melatonin also strongly stimulated the expression of *CHS* in both white and red cabbage (**Figures 6, 7**). *CHI* catalyzes the stereospecific cyclization of naringenin chalcone to (2S)-naringenin. The expression of the *CHI* gene differed between white cabbage and red cabbage. In white cabbage, the expression levels of *CHI* were low in the dark in both control and melatonin-treated samples. After light treatment, the expression levels of *CHI* significantly increased. Conversely, the expression levels of *CHI* were initially high in red cabbage, and melatonin-treated samples expressed high levels of *CHI* both under dark and light conditions (**Figures 6, 7**). This difference may be responsible for the fact, that red cabbage synthesized more anthocyanin than white cabbage. *F3H* catalyzes the oxygenation at the 3-position of flavanone [(2S)-naringenin] to form dihydroflavonol (dihydrokaempferol), and this reaction concomitantly produces CO<sub>2</sub> and succinate from oxygen and 2-oxoglutarate as co-substrates. The expression patterns of *F3H* were similar in white and red cabbage: melatonin-treated samples expressed higher levels of *F3H* than control samples (**Figures 6, 7**). *F3'H* is a cytochrome P450 monooxygenase committed in the hydroxylation of the 3'-position of the flavonoid B-ring. This enzyme accepts either dihydrokaempferol or kaempferol as a substrate and converts it to dihydroquercetin or quercetin, respectively. Melatonin appeared to particularly promote the



**FIGURE 5 | The expression patterns of anthocyanin synthesis genes in red cabbage (A) and white cabbage (B) in dark and light conditions.** Vertical bars at each column represent standard deviation of three replications.

expression of *F3'H*. Melatonin-treated samples expressed high levels of this gene during initial seedling growth, i.e., 1 day after germination (Figures 6, 7). As the plant continued to grow, the expression levels of this gene tended to be consistent among the two treatment groups. Generally, melatonin significantly upregulated the expression of *F3'H*. DFR catalyzes the reduction reaction of dihydroflavonol to leucoanthocyanidin. The expression of *DFR* showed similar response to melatonin with *F3H* (Figures 6, 7). The enzyme LDOX catalyzes the formation of anthocyanidin from leucoanthocyanidin, producing 2-oxoglutarate and oxygen as co-substrates. In

red cabbage, the expression of *LDOX* remained higher in melatonin-treated samples than in control samples, both in dark and light conditions (Figure 6). Conversely, this gene only slightly responded to melatonin in white cabbage under light conditions (Figure 7). UFGT catalyzes the glycosylation of flavonoid skeletons and utilizes UDP-sugars as sugar donors. The expression patterns of this gene in white cabbage and red cabbage were similar to those of *LDOX* (Figures 6, 7). Anthocyanins are unstable at pH > 4.0. GST protein is the flavonoid carrier and forms conjugates with anthocyanins, preventing them from oxidizing. It is involved in the transport of

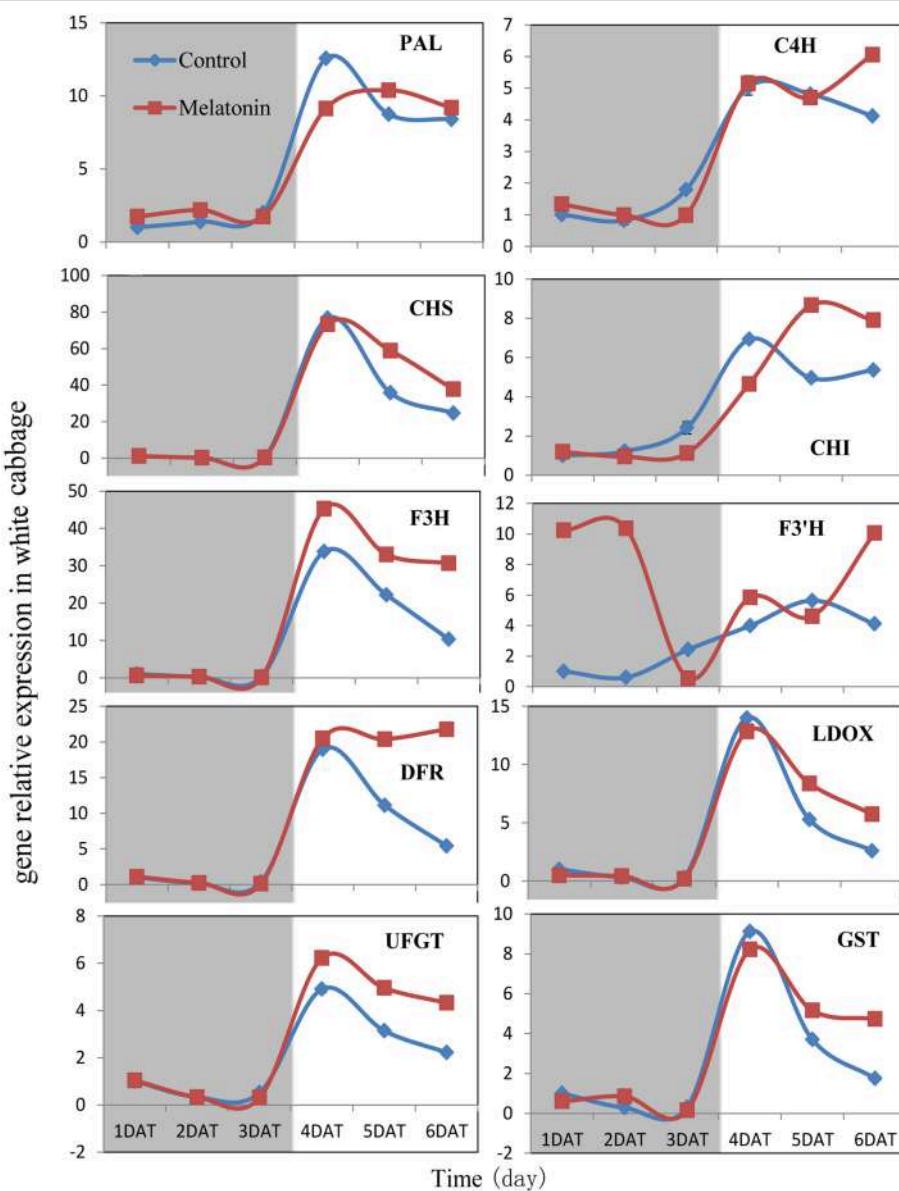


**FIGURE 6 | Expressions of anthocyanin synthesis genes in red cabbage under dark and light conditions.** The concentration of melatonin pretreatment in red cabbage is 100  $\mu\text{mol/L}$ . Vertical bars at each column represent standard deviation of three replications.

anthocyanins from the cytosol to the vacuole. Melatonin-treated samples showed improved expression levels of *GST* compared with control samples, especially in red cabbage (Figure 6). In summary, we found that melatonin treatment upregulated the expressions of structural genes in the anthocyanin biosynthesis pathway. Furthermore, this finding is consistent with the increase in anthocyanin levels in cabbage in response to melatonin.

## Induction of Regulatory Gene Expression in Response to Melatonin under Dark and Light Conditions

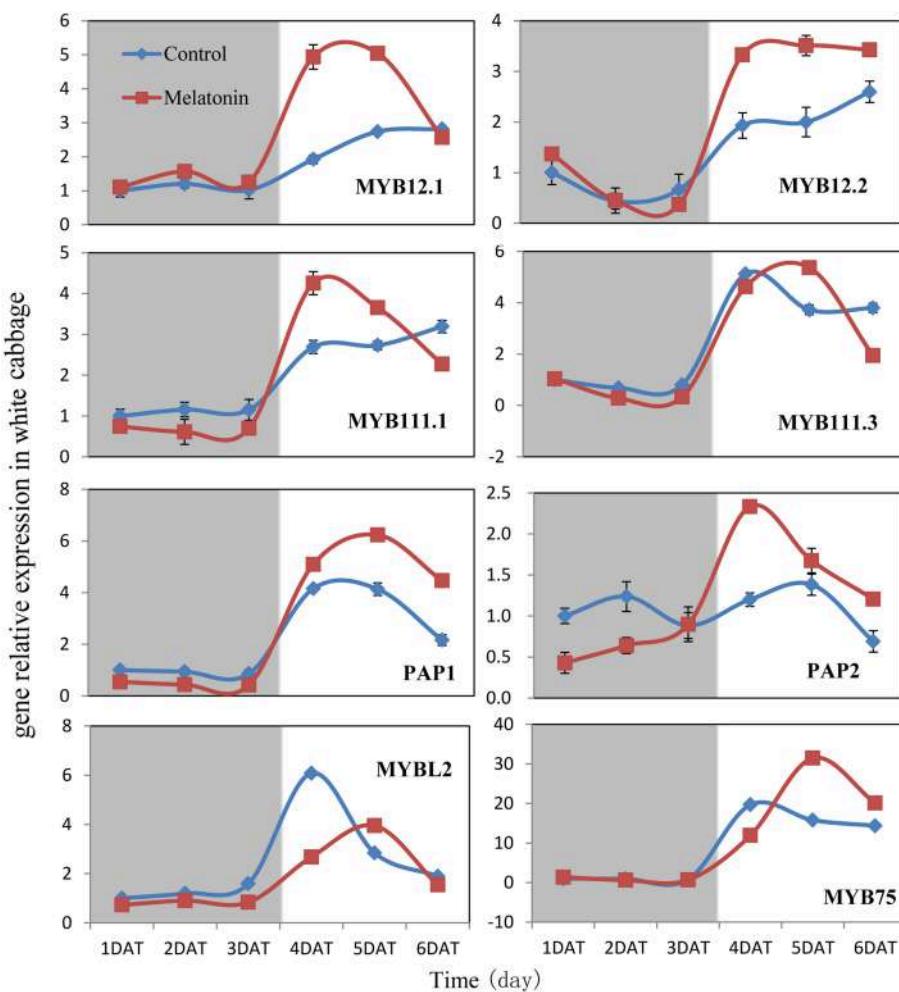
We aimed to investigate the accumulation of transcripts corresponding to the anthocyanin regulatory genes in response to melatonin in dark-grown and light-grown seedlings and to examine possible correlations between their expression patterns and those of the structural genes. Transcripts corresponding to



**FIGURE 7 | Expressions of anthocyanin synthesis genes in white cabbage under dark and light conditions.** The concentration of melatonin pretreatment in white cabbage is 1000  $\mu\text{mol/L}$ . Vertical bars at each column represent standard deviation of three replications.

the 8 MYB transcription factor genes analyzed were expressed at a very low level in dark-grown samples (Figure 8), and the expression levels of control and melatonin-treated samples did not significantly differ. After light treatment, we observed a strong increase in the transcript levels of all MYB transcription factors. Melatonin-treated samples expressed higher levels than control samples (Figure 8). *MYBL2* is an exception to this pattern because it is a negative regulator of anthocyanin biosynthesis genes. This finding was consistent with changes in the expression of structural genes. Anthocyanin biosynthesis genes are regulated by the interaction of the transcription factors MYB, bHLH, and WD40. The bHLH transcription factors participate in anthocyanin biosynthesis regulation, including *TT8*, *EGL3*, and

*GL3*. *TTG1* is a WD40 protein that correlates with anthocyanin biosynthesis. In the dark, the expression levels of these genes were similar between the control and melatonin-treated samples for all points except for the first, at which melatonin-treated samples expressed lower levels than the control. The expression of *TT8* significantly increased after illumination (Figure 9) and peaked to a level that was six-fold higher than that of the dark-grown plant 1 day after light treatment. Moreover, the maximum expression of *TT8* in melatonin-treated samples was 8-fold higher than that of the dark-grown sample at the same time point. Generally, the expressions of *EGL*, *GL3*, and *TTG1* only slightly fluctuated, but melatonin-treated samples continued to express higher levels of these genes than the control (Figure 9).



**FIGURE 8 | Expressions of MYB type transcription factors participate in anthocyanin synthesis in white cabbage.** The concentration of melatonin pretreatment is 1000  $\mu\text{mol/L}$ . Vertical bars at each column represent standard deviation of three replications.

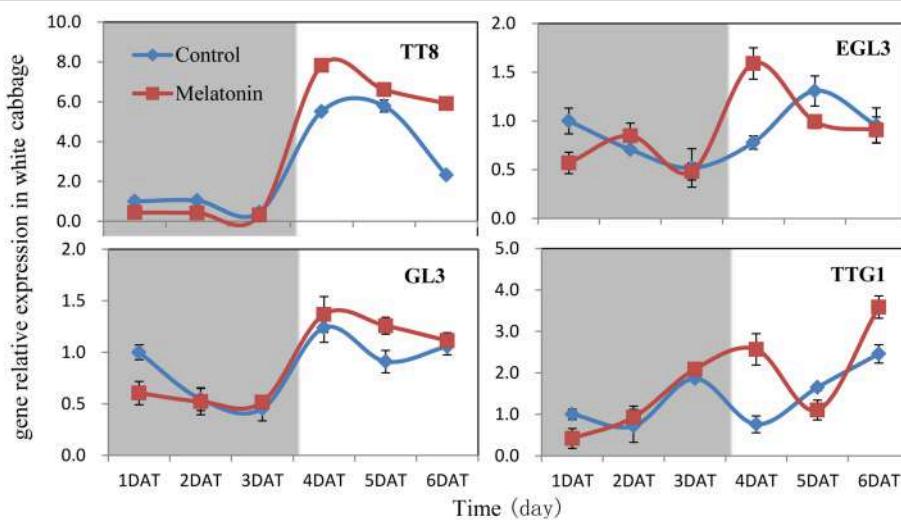
## Effect of Melatonin on TBARS, ROS Generation, Proline Levels, and Antioxidant Enzymes Activities in Cabbage Sprouts

Melatonin treatment not only promoted anthocyanin accumulation in cabbage but also improved the capacity to resist adverse conditions. **Figure 10** shows, that the ROS levels, including the level of  $\text{H}_2\text{O}_2$  and the production rates of  $\text{OH}^-$  and  $\text{O}_2^-$ , were all decreased in melatonin-treated samples. This decrease was associated with the rapid drop in the TBARS levels in these samples (**Figure 10**). Adverse environments induce oxidative stress and lipid peroxidation, and changes in the TBARS level have been suggested as indicators of the integrity of cell membranes in plants subjected to stress. Melatonin and anthocyanin can both scavenge excess ROS. Proline overproduction is a well-known response to adverse conditions in plants, and this amino acid is non-toxic, even at high concentrations. Soluble protein has a similar effect. Melatonin treatment increased the endogenous concentration of soluble protein and proline in cabbage compared with the

control (**Figure 10**). These compounds protect plants from stress in various ways, including contributions to cellular osmotic adjustment, the protection of membrane integrity, and the stabilization of enzymes/proteins. Given, that certain agents were observed to alleviate stress and exert beneficial effects, the study of individual antioxidant enzymes was deemed necessary. Melatonin treatment increased the SOD activities by 9%, the CAT activity by 10%, the POD activity by 75%, and the APX activity by 53% (**Figure 11**). These enzymes are all important antioxidants, that contribute to detoxification of ROS. Melatonin treatment significantly promoted the antioxidant enzymes activities, especially those of peroxidase and ascorbate peroxidase. This finding is consistent with the results of our previous study (Zhang N. et al., 2014).

## DISCUSSION

Anthocyanin biosynthesis is regulated primarily at the transcriptional level. The biosynthesis of anthocyanins is derived

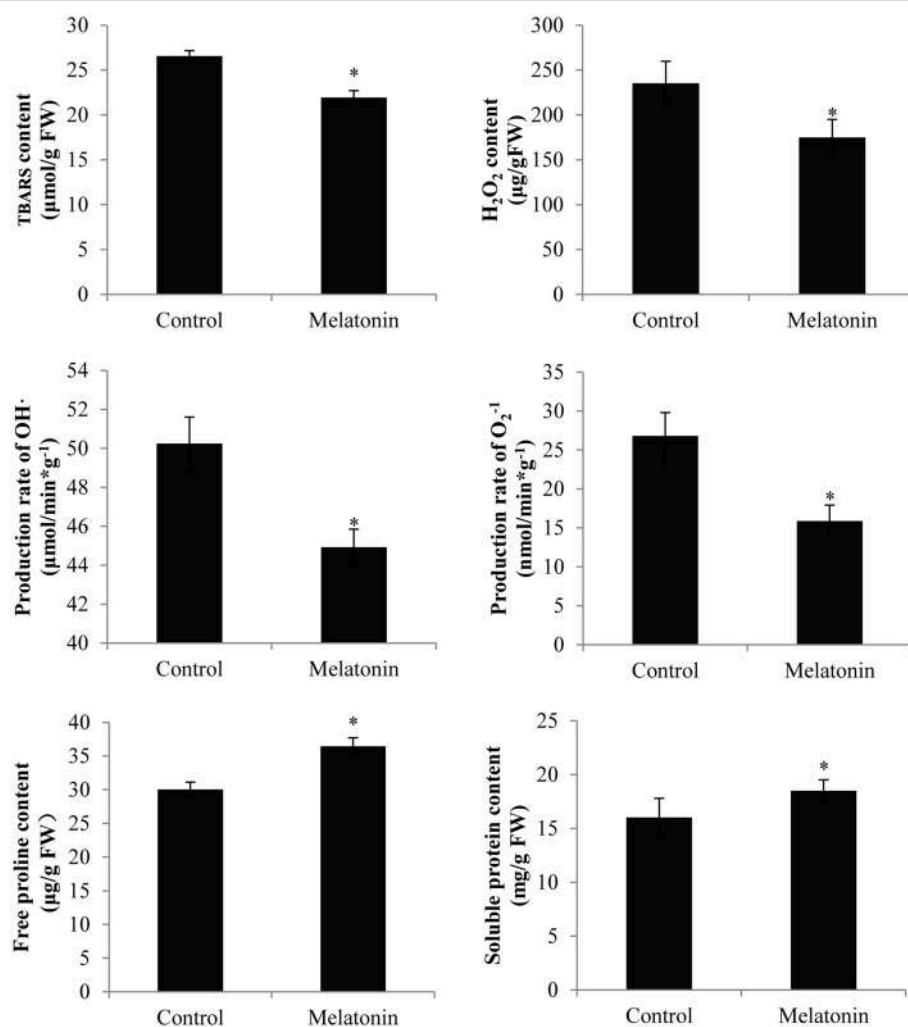


**FIGURE 9 | Expressions of bHLH (TT8, EGL3, GL3) and WD40 type (TTG1) transcription factors participate in anthocyanin synthesis in white cabbage.**  
The concentration of melatonin pretreatment is 1000  $\mu\text{mol/L}$ . Vertical bars at each column represent standard deviation of three replications.

from a branch of the flavonoid pathway. PAL and C4H are two key catalytic enzymes upstream of the flavonoid biosynthesis pathway that catalyze phenylalanine to 4-coumaroyl-CoA. They participate in many common metabolic pathways. Therefore, the expression levels of the two structural genes were not as important as those of genes downstream of the synthesis of anthocyanin. CHS facilitates a committed step by condensing one molecule of p-coumaroyl-CoA with three molecules of malonyl-CoA to produce tetrahydroxychalcone. The expression of CHS in white cabbage increased 80 times in response to light compared with dark conditions (Figure 7). A detailed functional analysis of the CHS gene promoter revealed a light-responsive unit carrying a G-box and a MYB recognition element (Hartmann et al., 1998). The G-box can bind bHLH factors. In our result, CHS gene exhibited a relatively strong response to a light signal. This result may be related to the light-responsive unit in the CHS promoter, which may also be responsible for the upregulation of CHS gene expression in melatonin-treated samples. The F3H promoter contains two putative MYB-recognition elements and three ACGT-containing elements, and these motifs are necessary to confer light responsiveness (Zhu et al., 2015). The expression of F3H in white cabbage also strongly responded to light signals, increasing by approximately 50-fold compared with dark conditions (Figure 7). Similarly, other upregulated genes included DFR (23-fold higher), LDOX (14-fold higher), CHI (9-fold higher), GST (9-fold higher), and UFGT (7-fold higher; Figure 7). Interestingly, all highly light-responsive genes were significantly upregulated in white cabbage in response to light but were minimally affected in red cabbage (2.5-fold maximum change; Figures 5–7). In red cabbage, these genes were expressed at high levels in the dark (Figure 5A). We speculated, that the motifs in the promoters of these genes may differ between white cabbage and red cabbage. However, more experimental evidence is needed to confirm this speculation. These genes were upregulated in response to melatonin, irrespective of light

treatment. Moreover, anthocyanin accumulation in response to melatonin differed between white and red cabbage (Figures 1, 2). Red cabbage showed a significant response to a very low concentration of melatonin (10  $\mu\text{mol/L}$ ), whereas white cabbage responded to higher concentrations of melatonin (Figure 2). However, the anthocyanin content exhibited larger fluctuations, which indicated that red cabbage is more sensitive to melatonin, whereas white cabbage had higher potential in anthocyanin synthesis.

Transcription factors are crucial in the regulation of anthocyanin biosynthesis. Deficiencies in related transcription factors specifically inhibit the expression of anthocyanin biosynthesis genes, but their ectopic expression can activate the entire anthocyanin pathway (Xu et al., 2015). The over-expression of PAP1, which was the first isolated MYB member of this complex in Arabidopsis, results in purple-colored leaves that contain increased levels of anthocyanin and quercetin glycosides (Tohge et al., 2005). MYB and bHLH TFs are found in all eukaryotes and are among the largest families of plant TFs (Feller et al., 2011). We analyzed the expression of related MYB transcription factors, including MYB12, MYB111, PAP1, PAP2, MYB75, and the negative regulator MYBL2, in white cabbage. We found, that melatonin treatment upregulated the positive regulators in most tested samples and downregulated the negative regulator MYBL2 (Figure 8). The bHLH proteins, such as TT8, GL3, and EGL3, participate in anthocyanin biosynthesis and interact with various MYBs. bHLHs contain an N-terminal arginine residue, which is conserved among the bHLH TFs. It can interact with the R3 repeat in MYBs. MYBs and the bHLH proteins, together with TTG1, can form a ternary protein complex named MBW. TTG1 is crucial for the activity of this complex in plants because it regulates both the specific activity (i.e., interactions with other proteins or DNA) and the quantity (e.g., stability and localization) of the MBW complex (Baudry et al., 2004). In our study, melatonin treatment

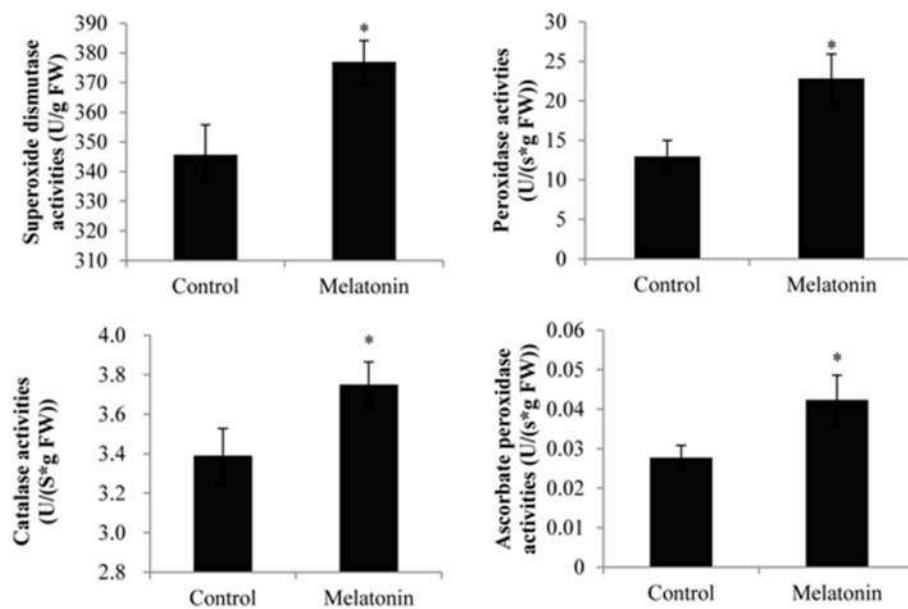


**FIGURE 10 | Melatonin treated cabbage seedlings showed lower radical levels and high proline and soluble protein content.** The concentration of melatonin pretreatment is 1000  $\mu\text{mol/L}$ . Vertical bars at each column represent standard deviation of three replications. \*Significant difference between this column and control at  $P < 0.05$ .

upregulated the expression of MBW-related transcription factors (**Figure 8**). Many reports have concluded, that melatonin affects various biological processes by regulating the expressions of many transcription factors. For example, melatonin treatment upregulated the expression of class A1 heat-shock factors and consequently improved the thermo-tolerance of *Arabidopsis* (Shi et al., 2015b). Melatonin also alleviated cold stress by mediating several cold-related genes, including C-REPEAT-BINDING FACTORs (CBFs)/Drought Response Element Binding factors (DREBs), COR15a, and three transcription factors [CAMTA1, ZINC FINGER OF ARABIDOPSIS THALIANA 10 (ZAT10), and ZAT12; (Bajwa et al., 2014; Shi and Chan, 2014)].

Many signaling molecules can affect anthocyanin synthesis. Hormones can also affect the synthesis of anthocyanin. ABA is involved in the regulation of ripening related to anthocyanin biosynthesis in non-climacteric fruits. Silencing an ABA pathway gene, *FaNCED1*, resulted in decreased levels of ABA, and non-colored strawberry fruits. Furthermore, the

silencing of a putative ABA receptor also inhibited the ripening and anthocyanin accumulation of strawberries (Jia et al., 2011). Ethylene inhibited sugar- and photosynthesis-induced anthocyanin accumulation in *Arabidopsis* by suppressing the expression of the MBW complex. Ethylene can also inhibit anthocyanin accumulation induced by sucrose and light by suppressing the expression of *TT8*, *GL3*, and *PAP1* while concomitantly stimulating the expression of the negative regulator *MYBL2* (Jeong et al., 2010). Cytokinins have been found to enhance light- and sugar-induced anthocyanin biosynthesis in *Arabidopsis* (Das et al., 2012). The endogenous application of auxin has been found to retard anthocyanin accumulation in grapevine (Jeong et al., 2004; Wheeler et al., 2009). Moreover, cell culture studies showed, that different auxins regulate anthocyanin biosynthesis, mainly by inducing the expression of *TT8*, *GL3*, and *PAP1* (Liu et al., 2014). Jasmonates have been found to affect color formation in apples and grapevine, possibly by interacting with ethylene biosynthesis (Fan et al.,



**FIGURE 11 | Melatonin treated cabbage seedlings showed high antioxidant enzyme activities.** The concentration of melatonin pretreatment is 1000  $\mu\text{mol/L}$ . Vertical bars at each column represent standard deviation of three replications. \*Significant difference between this column and control at  $P < 0.05$ .

1998; Rudell et al., 2005). BRs negatively regulate JA-induced anthocyanin accumulation by inhibiting MBW complexes (Peng et al., 2011). Furthermore, gibberellins can retard the ripening-related accumulation of anthocyanin in fruits (Awad and De Jager, 2002). The DELLA and JASMONATE-ZIM DOMAIN (JAZ) proteins act as key crosstalk components of the GA and JA signaling cascades, respectively, and interact with different partners of the MBW complex (Qi et al., 2014). Sucrose can also affect anthocyanin accumulation. Specifically, it synergistically interacts with phosphate and ABA and with JA, cytokinins, and other hormones involved in the responses to various biotic and abiotic stresses. Moreover, it antagonistically interacts with GA, ethylene, and BRs (Li et al., 2014; Shi and Xie, 2014). In our study, the expression levels of almost all genes, that participate in anthocyanin biosynthesis and regulation fluctuated in response to melatonin treatment (Figures 6–9). However, melatonin did not target one gene or a group of genes to regulate anthocyanin accumulation; instead, melatonin exerted a comprehensive effect.

Environmental factors can also affect the synthesis of anthocyanin. Light exposure can increase the concentration of anthocyanins. In our study, a light signal was sufficient to elicit a large increase in the transcription of the transcriptional activation complex and consequently, the biosynthetic pathway. In addition to light intensity, the light quality also affects the biosynthesis of anthocyanins, especially UV light. However, other specific light qualities (e.g., blue light) have also been associated with the regulation of anthocyanin biosynthesis (Ubi et al., 2006; Li et al., 2013). Low temperatures are well known to induce anthocyanin biosynthesis in plants, especially in light conditions (Catalá et al., 2011). Excess nitrogen application has been found to decrease the anthocyanin content and

the contents of other phenolic compounds (Stefanelli et al., 2010). In *Arabidopsis*, higher nitrogen concentrations reportedly decrease in the expression levels of PAP1 and TT8, which are part of the TTG1-GL3/TT8-PAP1 (WD40-bHLH-MYB) anthocyanin biosynthesis regulatory complex, and increase in the expression levels of three lateral organ-boundary domain genes (LBD37, LBD38, and LBD39), which act as negative regulators of anthocyanin biosynthesis (Zhou et al., 2012). Nitrogen deficiency (low N/C balance) also triggers anthocyanin accumulation in seedlings and rosette leaves, especially at low temperatures, by inducing PAP1, PAP2, and GL3 expression (Lillo et al., 2008; Nemie-Feyissa et al., 2014; Shi and Xie, 2014).

Hu et al. found, that black corn exhibits higher antioxidant activity than yellow and white corn varieties (Hu and Xu, 2011). Moreover, Harakotra et al. found, that the purplish black genotype (KKU-WX111031) consistently exhibited the highest levels of anthocyanins and antioxidant activity among the tested genotypes (Harakotra et al., 2014). Studies of purple potatoes identified that anthocyanins significantly contribute to antioxidant activity (Hu et al., 2016). The health benefits of anthocyanins in purple vegetables have been attributed to their high antioxidant activities.

Anthocyanins appear to protect against both abiotic and biotic stressors. They may reduce the propensity for photoinhibition or mitigate the effects of drought, salinity, heavy metal, or oxidative stress (Chalker-Scott, 1999; Gould, 2004; Agati and Tattini, 2010; Falcone Ferreyra et al., 2012). Different environmental stressors can lead to the generation of ROS in various cellular compartments, e.g., mitochondria and chloroplasts. Melatonin and anthocyanin both exhibit antioxidant capacity. Specifically, melatonin treatment not only upregulated the levels of anthocyanin but also downregulated the ROS levels

(**Figures 2, 10**). Moreover, melatonin treatment increased the activities of antioxidant enzymes (**Figure 11**), which resulted in a higher ROS-scavenging potential. Proline and soluble protein play important roles in cabbage plants exposed to osmotic stress. These osmoregulation substances were also upregulated in our study (**Figure 10**). These changes may all improve the ability of cabbage seedlings to resist adverse conditions.

Melatonin has been proven to increase lycopene levels during the post-harvest life of tomato fruits (Sun et al., 2015). Specifically, the expression levels of the SPSY1 and SICRTISO genes, which are involved in lycopene biosynthesis, were upregulated in melatonin-treated tomatoes. We also detected the anthocyanin content in melatonin-treated samples (data not shown). We found, that melatonin not only regulated the color of tomatoes by affecting lycopene levels but also affected the anthocyanin content. This phenomenon observed in tomatoes is consistent with, that observed in cabbage. We previously found plant materials can absorb that melatonin. Melatonin pretreatment can significantly increase the levels of melatonin in treated samples, such as tomato fruits and cucumber seeds, whereas the levels of melatonin decreased during ripening and germination (Zhang H. J. et al., 2014; Sun et al., 2015). In this experiment, we also measured the melatonin levels in white cabbage seeds after pre-sowing with melatonin (1000 μmol/L) and seedlings cultured for 1 week after germination (**Figure S1**). We found, that cabbage seeds absorbed large amounts of melatonin from the soaking solutions. As the plants grew, the melatonin levels decreased, which indicated, that melatonin participates in growth. This finding is consistent with the results of our previous work. Exogenous melatonin application is an effective method to improve the melatonin levels and adversity resistance of plants. Specifically, exogenous melatonin applied to Bermuda grass conferred improved salt, drought, and cold stress resistances (Shi et al., 2015a). Arabidopsis grown in the melatonin-supplemented culture media showed higher survival rates in response to heat shock (Shi et al., 2015b), and applying the optimal dose of melatonin effectively ameliorated Cd-induced phytotoxicity in tomato by affecting Cd transport (Harson et al., 2015). Moreover, melatonin has been proved to scavenge ROS and improve antioxidant activities (Zhang et al., 2013; Zhang H. J. et al., 2014; Li et al., 2015; Shi et al., 2015c), which also corroborates our results.

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## CONCLUSION

Melatonin treatment prior to sowing resulted in a darker red color in cabbage seedlings, and this color is due to anthocyanin accumulation. Melatonin treatment improved the anthocyanin accumulation in white and red cabbage. The expression levels of anthocyanin biosynthesis pathway genes, i.e., PAL, C4H, CHS, CHI, F3H, F3'H, DFR, LDOX, UFGT, and GST, were upregulated in melatonin-treated samples, especially in the presence of light. The upregulated expression of structural genes coincided with a coordinated increase in the transcript levels of MYB transcription factors, bHLH transcription factors, and a WD40 gene. Moreover, the amount of total anthocyanins in cabbage was found to be positively correlated with the total antioxidant power and the content of some osmoregulation substances, suggesting, that anthocyanins may improve the stress resistance of cabbage during growth.

## AUTHOR CONTRIBUTIONS

NZ, QS, and YG designed research; NZ, QS, HL, XL, YC, and HZ performed the experiments; NZ, SL, LZ, YQ, and SR analyzed the data; BZ and YG revised the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00197>

**Table S1 | The genes and primers used for Quantitative PCR.**

**Figure S1 | Identification of melatonin by UPLC- ESIMS/MS.** (A) UPLC spectrum of melatonin standard. (B) Melatonin levels of cabbage samples presoaked with melatonin solutions (1000 μmol /L) and water control. (1DAT: 1 day after presoaked with melatonin; 7DAT: 7-day-old seedlings). Vertical bars at each column represent standard deviation of three replications.

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# Melatonin Improves Waterlogging Tolerance of *Malus baccata* (Linn.) Borkh. Seedlings by Maintaining Aerobic Respiration, Photosynthesis and ROS Migration

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Waterlogging, one of the notorious abiotic stressors, retards the growth of apple plants and reduces their production. Thus, it is an urgent agenda for scientists to identify the suitable remedies for this problem. In the current study, we found that melatonin significantly improved the tolerance of apple seedlings against waterlogging stress. This was indicated by the reduced chlorosis and wilting of the seedlings after melatonin applications either by leaf spray or root irrigation. The mechanisms involve in that melatonin functions to maintain aerobic respiration, preserves photosynthesis and reduces oxidative damage of the plants which are under waterlogging stress. Melatonin application also enhances the gene expression of its synthetic enzymes (MbT5H1, MbAANAT3, MbASMT9) and increases melatonin production. This is the first report of a positive feedback that exogenous melatonin application promotes the melatonin synthesis in plants. A post-transcriptional regulation apparently participated in this regulation. When exogenous melatonin meets the requirement of the plants it is found that the protein synthesis of MbASMT9 was suppressed. Taken together, the results showed that melatonin was an effective molecule to protect plant, particularly apple plant, against waterlogging stress.

**Keywords:** melatonin, waterlogging, ROS, oxidative stress, aerobic respiration, photosynthesis

## INTRODUCTION

Waterlogging, is a major agricultural constraint that limits crop growth and reduces their yield (Xu et al., 2013). It is frequently encountered during the raining seasons in many areas worldwide. The excessive waterlogging causes root damage, impairs the water uptake and, finally leads to chlorosis and wilting of the plants (Arbona et al., 2008). It was estimated that waterlogging stress resulted in nearly 40–80% of the crop yield loss in the area greater than 17 million km<sup>2</sup> (Voesenek and Sasidharan, 2013; Shabala et al., 2014).

The reactive oxygen species (ROS) is believed to play a critical role in the response of plant to waterlogging stress. At the early stage during waterlogging, the elevated ROS molecules functions as an important second messenger in signaling for response. Following the prolonged waterlogging, the increased anaerobic respiration of root and the responsive stomata closure in leaves induce a burst of excessive ROS production. If the excessive ROS is not migrated properly, it will cause plant oxidative damage and finally, it leads to roots rotting and leaves wilting (Hossain et al., 2009).

Plants have already developed a series of antioxidant mechanisms to defend themselves against oxidative stress. These include small molecule antioxidants (SMA) and antioxidant enzymes (Apel and Hirt, 2004; Mittler et al., 2004). SMA includes ascorbic acid, carotenoids, tocopherol, glutathione, polyphenol, etc. They can scavenge ROS with different chemical reactions (Smirnoff, 2000; Kuzniak and Skłodowska, 2001; Foyer and Noctor, 2005). The antioxidant enzymes are mainly those of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), etc. Both SMA and antioxidant enzymes work coordinately to keep the oxidative stress in check (Hossain et al., 2009). In addition to the SMA mentioned above, melatonin (*N*-acetyl-5-methoxytryptamine) is a potent free radical scavenger and an antioxidant (Tan et al., 1993, 2012). Melatonin was identified in plants in Dubbels et al. (1995) and Hattori et al. (1995). Since then it has been reported to exist in many plants and plant products (Ramakrishna et al., 2012; Yip et al., 2013; Shi et al., 2015b; Zhao et al., 2015a; Li C. et al., 2016; Ma et al., 2016; Xu et al., 2016). Different from other antioxidants, it is an amphiphilic molecule which makes it distribute in all cellular compartments including cytosol, membrane, mitochondria and chloroplasts (López et al., 2009; Byeon et al., 2013; Back et al., 2016). Also, melatonin as well as its metabolites can eliminate different kinds of ROS including superoxide anion ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\bullet OH$ ), singlet oxygen ( $^1O_2$ ), peroxynitrite anion ( $ONOO^-$ ) and nitric oxide (NO) (Tan et al., 2013). Melatonin has been reported to protect plants against a variety of abiotic and biotic stresses (Zuo et al., 2014; Liang et al., 2015; Liu et al., 2015; Shi et al., 2015a; Wang et al., 2015; Zhao et al., 2015b; Li C. et al., 2016; Xu et al., 2016). The transgenic *Arabidopsis* plants, ecotypically expressing melatonin synthetic gene *MzASMT1*, had higher endogenous melatonin production and significantly lower ROS than that of their wild types. The melatonin enriched transgenic *Arabidopsis* had a greater tolerance to drought stress than the wild types (Zuo et al., 2014). The exogenous melatonin also improved tolerance of tomato plants against alkaline stress by migrating  $O_2^{\bullet-}$  and  $H_2O_2$  (Liu et al., 2015). Melatonin application to apple leaves alleviated the drought-induced inhibition of photosynthesis (Wang et al., 2012). However, the effects of the exogenous melatonin application on apple plants which are under the sustained waterlogging condition have not been reported yet.

Reduced apple yield caused by waterlogging stress is a worldwide problem to be solved. Apple is a perennial woody plant, thus, the damage from waterlogging stress not only reduces apple yield of the current year, but also suppresses the tree vigor which also leads to the yield loss in the following years (Qu et al., 1999). *Malus baccata* (Linn.) Borkh. is used as a rootstock, which frequently suffers from the waterlogging stress (Wang et al., 2013). Therefore improvement of its waterlogging resistance by melatonin application will provide a potential cultivation method for apple production.

In current study, we investigated the protective effects of melatonin on *M. baccata* seedlings, which were subjected to the waterlogging stress. Furthermore, the potential mechanisms of these protections were also explored and discussed.

## MATERIALS AND METHODS

### The Cultivation of Plant Material

Seeds of apple (*M. baccata*) were sown in soppy vermiculite. Two weeks later, the seedlings were watered with half-strength Hoagland's nutrient solution (Li M.Q. et al., 2016). When the seedlings developed to have four leaves, they were watered with complete nutrient solution. The plants were kept in green house with the temperature at a constant  $22 \pm 2^\circ\text{C}$  and a 16/8 h light/dark cycle. The light intensity was approximately  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

### Waterlogging Stress/Melatonin Treatment and Sample Collection

After the *M. baccata* seedlings developed to have four leaves, a total of 96 seedlings were transplanted into the glass container with sterilized matrix soil. They were divided into eight groups. Plants in group I were watered with the 200 mL normal nutrient solution. Totally 25 mL normal nutrient solution was added every 3 days as control. The waterlogging stress was conducted in the remaining seven groups by keeping the soil being covered with 300 mL the nutrient solution and added 25 mL normal nutrient solution every 3 days.

The seedlings from group II was waterlogging stressed without supply of exogenous melatonin. The waterlogging stressed seedlings from group III to VIII were treated with different concentration of melatonin by spraying or irrigation. Melatonin was dissolved in 100% ethanol at a concentration of 10 mM and stored at  $-20^\circ\text{C}$  as a stock solution. When use, melatonin was then diluted into 50, 100, and 200  $\mu\text{M}$ , respectively with deionized water. These different concentrations of melatonin were sprayed to the leaves of seedlings every other day in group III, IV, and V, respectively. Two pieces of hardboard were used to avoid sprayed melatonin dropping into the soil. In group VI, VII, and VIII, melatonin was directly supplemented to the nutrient solution at the concentrations of 200, 400, and 600  $\mu\text{M}$ , respectively. Groups I and II was also applied with equal volume of ethanol. The experiments were independently repeated three times. Photos were taken before and after 9 days of waterlogging stress/melatonin treatments.

The leaves and roots were collected from the all groups of seedlings, respectively, before and after 9 days of waterlogging stress/melatonin treatment.

### Melatonin Measurement

The leaves collected from the seedlings were immediately frozen at  $-80^\circ\text{C}$  for future melatonin detection. Around 1 g of frozen leaves of *M. baccata* was ground to a fine powder in liquid nitrogen. The powder was mixed with 10 mL methanol and ultrasonicated (80 Hz) for 35 min at  $45^\circ\text{C}$ . The sample preparation and HPLC detection of melatonin were performed as described by Zhao et al. (2013). Each experiment was independently repeated three times.

### RNA Extraction and RT-PCR Analysis

Total RNA was isolated from the leaves of seedlings of the eight groups, respectively, before and after 9 days of waterlogging

stress/melatonin treatment, using the EASY spin Plant RNA Rapid Extraction Kit (Biomed, Beijing, China). The first-strand cDNA was synthesized following the protocol of Kit (Promega, Madison, WI, USA). The cDNAs were used as template for RT-PCR. The specific primers were designed according to the sequence of melatonin synthesized enzyme genes *MbASMT9* (KJ156531), *MbAANAT3* (KJ156532), and *MbT5H1*, respectively, by Primer 5 software and checked by BLAST search in the apple genome<sup>1</sup> (*MbASMT9* Forward Primer 5'-TGATCTGCCCATGTCGT-3', Reverse Primer 5'-CTTTGTGGCGAGGGAAAC-3'; *MbAANAT3* Forward Primer 5'-CGCTCCCTAACTACCAACCA-3', Reverse Primer 5'-ACAAATCCCTTCCCTACCAAG-3'; *MbT5H1* Forward Primer 5'-ATCCGTAAGATTGTATACTTGAGCT-3', Reverse Primer 5'-TCACCGACCAAGATAATAGCCT-3').

RT-PCRs were performed use of 20  $\mu$ L reaction mixtures containing 20 ng of first-strand cDNA, 2  $\times$  PCR Mix 5  $\mu$ L (CWBIOD, Beijing, China), 0.5  $\mu$ M of each of the forward and reverse primers and appropriate amounts of ddH<sub>2</sub>O. The *Actin* gene was used as the internal standard, and the PCR program for *Actin* was as following: 94°C for 5 min; 28 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 10 min, amplified with primers (Forward Primer 5'-CAATGCCTGCCATGTATG-3', Reverse Primer 5'-CCAGCAGCTCCATTCCAAT-3'). The PCR products were analyzed in 1% TAE-agarose gel stained by goodview. The quantification of amplified *Actin*, *MbT5H1*, *MbAANAT3*, and *MbASMT9* fragment was done by the ImageJ software<sup>2</sup>, the ratios of *MbT5H1*, *MbAANAT3*, *MbASMT9* and *Actin* were calculated.

## The Cloning of *MbASMT9* Gene and the Expression of *MbASMT9* Protein in *Escherichia coli*

The coding frame of *MbASMT9* was amplified and ligated into pMD 19-T Simple, which was then digested with BamHI/EcoRI and inserted into the pGEX-6p-1. The protein expression of *MbASMT9* was analyzed according to Li et al. (2015). The purity of the GST-MbASMT9 protein was confirmed by SDS-PAGE.

## Protein Extraction and Western Blot Analysis

The total protein was isolated from leaves of seedlings according to Wang et al. (2006). Western blot was applied with antibody of *MbASMT9* (rabbit, 1:3000). The recombinant GST-MbASMT9 was used as an antigen to raise polyclonal GST-MbASMT9 antibodies in rabbit. The preparation of *MbASMT9* antibody was carried out in accordance with relevant guidelines and regulations. The experimental protocols of the *MbASMT9* antibody preparation were reviewed and approved by Beijing Municipal Science and Technology Commission. The chemiluminescent signals were detected using an ECL detection kit (Amersham-Pharmacia, USA). The loading control of the Western blot was stained by Coomassie Brilliant Blue.

## Detection of Antioxidant Enzyme Activities

A total of 0.3 g leaves or 0.3 g roots of *M. baccata* seedlings were ground with 8 mL chilled 50 mM phosphate buffer (pH 7.8), then they were transferred into 10 mL tubes and centrifuged at 4°C for 15 min at 10,000 g. The supernatants were diluted with phosphate buffer to 10 mL and used for enzyme activity detection. The enzyme activity of SOD was detected according to the method of Stewart and Bewley (1980). CAT activity was measured as the absorbance at 240 nm wave length according to the method of Ma et al. (2016). POD (Peroxidase) activity was measured by the changes in absorbance at 470 nm due to guaiacol oxidation according to the method of Shi et al. (2015b). Each experiment was independently repeated at least three times.

## Measurements of Enzyme Activities of Alcohol Dehydrogenase (ADH) and Succinate Dehydrogenase (SDH)

A total of 0.3 g roots for each group were collected to detect the enzyme activity of ADH and SDH before and after 9 days of waterlogging stress/melatonin treatment to identify the alterations of anaerobic and aerobic respiration. ADH activity was measured as reported by Yamashita et al. (2015). SDH activity was detected according to Landi et al. (2009). Each experiment was independently repeated at least three times.

## Detections of ROS Level and Malondialdehyde (MDA) Content

The ROS level in roots was detected according to the method modified from Zuo et al. (2014). The intact roots were collected from each group and the fresh roots were immediately used for ROS detection before and after 9 days of waterlogging stress/melatonin treatment. Simply, the entire roots were incubated with the 5-(and 6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCFDA) solution for 20 min and washed with distilled H<sub>2</sub>O to remove excess CM-H<sub>2</sub>DCFDA. The fluorescence images were obtained with a Leica stereoscope (Leica, Wetzlar, Germany) ( $\times 10$ ).

In addition to roots, the ROS level in leaves was also detected. The leaves were collected from the each group, respectively before and after 9 days of waterlogging stress/melatonin treatments and used for detection of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> immediately. For O<sub>2</sub><sup>•-</sup> measurement, the leaf histochemical staining was vacuum infiltrated with 0.1 mg·mL<sup>-1</sup> nitroblue tetrazolium in 25 mM K-HEPES buffer (pH 7.9) for 40 min. Then the samples were kept at 25°C in dark for an additional 4 h. For the H<sub>2</sub>O<sub>2</sub> detection the leaves were vacuum infiltrated with 0.1 mg·mL<sup>-1</sup> DAB in 50 mM Tris-acetate (pH 3.8) and were incubated at 25°C in dark for 24 h. Then leaves for either O<sub>2</sub><sup>•-</sup> or H<sub>2</sub>O<sub>2</sub> detection were washed in 80% ethanol every 10 min at 80°C until the leaves lost green color completely (Gong et al., 2014). The MDA detection in the leaf samples were performed according to Zhao et al. (2013). Each experiment was independently repeated at least three times.

<sup>1</sup><https://www.rosaceae.org/species/malus/all>

<sup>2</sup><http://en.wikipedia.org/wiki/ImageJ>

## Analyses of Chlorophyll (Chl) Content and Photosynthetic Rate (Pn) in Leaves of Seedlings

The extraction of Chl was conducted according to Porra et al. (1989). A total of 0.2 g leaves were homogenized in 2–3 mL 80% acetone. After filtration with four layers of gauze (1 mm × 1 mm), the homogenate was diluted with 80% acetone to 25 mL. The absorbance of the extract was measured at 645 and 663 nm. Chl concentration was calculated from the following equations: Chl a =  $12.72 \times OD_{663} - 2.59 \times OD_{645}$ ; Chl b =  $22.88 \times OD_{645} - 4.67 \times OD_{663}$ ; chl = chl a + chl b (Arnao and Hernández-Ruiz, 2009).

The photosynthetic rate was measured by LI-6400XT (LI-COR, Lincoln, NE, USA) according to the producer's protocol. The light intensity was at  $800 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The humidity was about 50% and the temperature was 23°C. Each experiment was independently repeated three times.

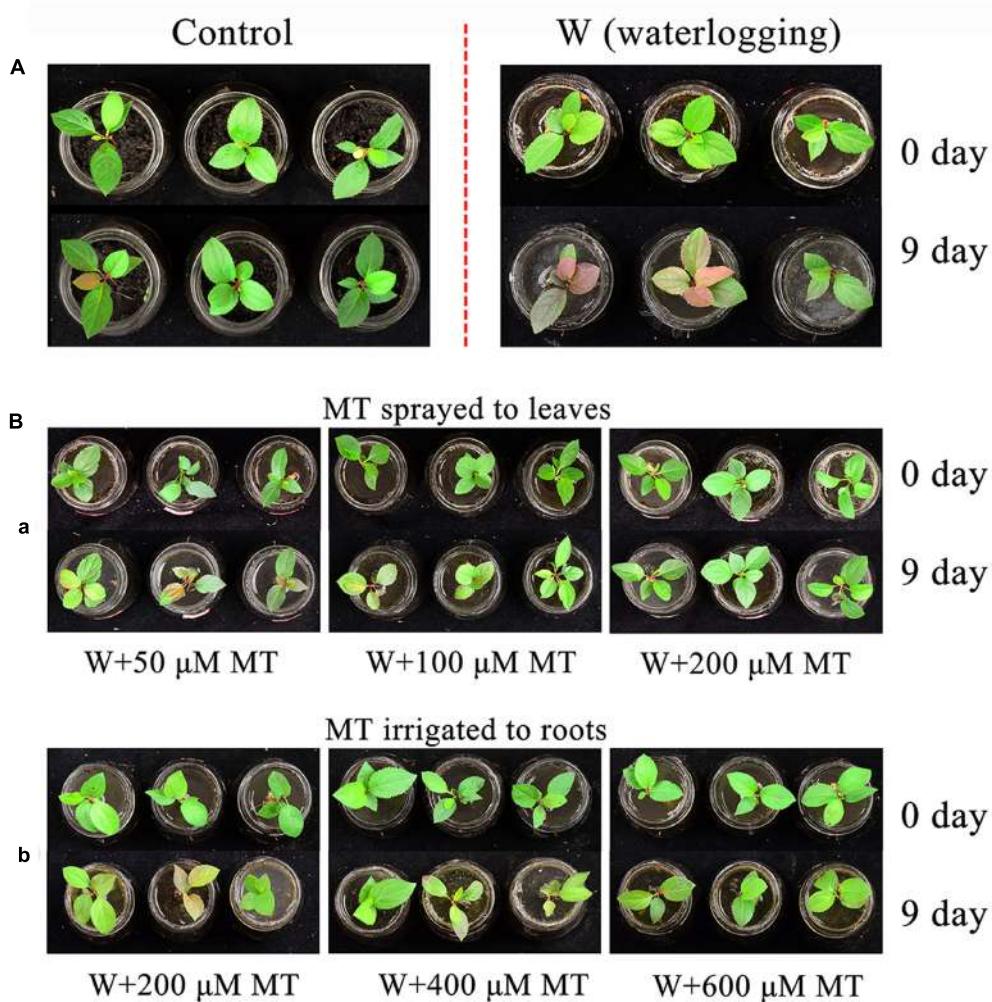
## Statistical Analysis

The data are expressed as means ± SD. One-way ANOVA was used for the normality evaluation followed by a Tukey–Kramer multiple comparison test. The statistical significant difference was set up when  $P < 0.05$ . Statistical evaluations were carried out using SPSS software (IBM, Armonk, NY, USA).

## RESULTS

### Melatonin Applications Improved the Tolerance of *M. baccata* Seedlings against Waterlogging Stress

The results showed that waterlogging stress significantly impaired the *M. baccata* seedling growth. Those plants which were under waterlogging stress for 9 days wilted severely compared to the normal controls (Figure 1A). When melatonin was sprayed to



**FIGURE 1 |** The effects of melatonin on the *M. baccata* seedlings which were suffered from waterlogging stress. **(A)** Before and after waterlogging stress. **(B):** (a) The phenotype of *M. baccata* seedlings with melatonin leaf spray at different concentrations before and after waterlogging stress. (b) The phenotype of *M. baccata* seedlings with melatonin irrigation at different concentrations before and after waterlogging stress.

the seedlings at the concentrations of 50, 100, and 200  $\mu\text{M}$ , respectively, it was apparent that melatonin spraying improved tolerance of seedlings against waterlogging stress. The protective effects of melatonin indicated a dose-responsive manner. The seedlings treated with 200  $\mu\text{M}$  of melatonin, even under the waterlogging stress, showed a similar phenotype as the normal controls. The similar results were also observed in the seedlings those melatonin was irrigated (**Figure 1B**). When melatonin was irrigated to the seedlings at the concentrations of 200, 400, and 600  $\mu\text{M}$ , respectively, it also increased waterlogging resistance of seedlings as a dose-dependent manner. The irrigation with 600  $\mu\text{M}$  of melatonin, gained a similar phenotype as spraying with 200  $\mu\text{M}$  melatonin. The percentage of leaf chlorosis was also analyzed. It was clearly to see that the percentage of leaf chlorosis was significantly increased after waterlogging treatment. After melatonin was applied to the seedlings under waterlogging, the percentage of leaf chlorosis were significantly decreased (Supplementary Figure 1).

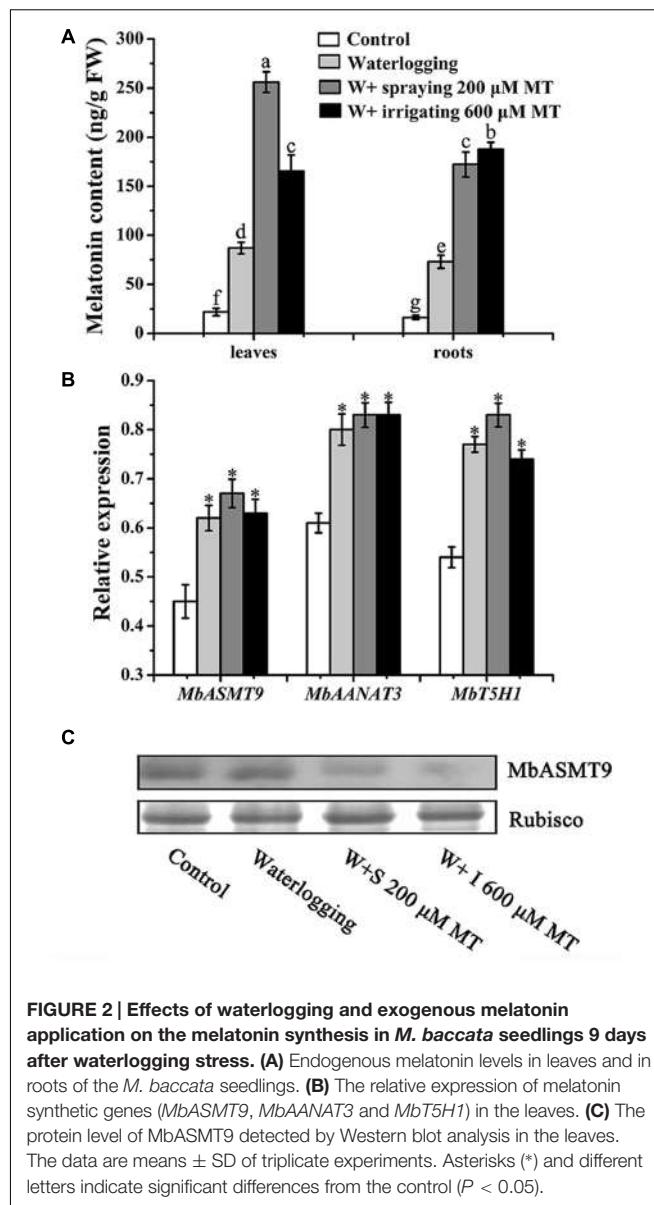
### The Effects of Waterlogging Stress and Melatonin Application on *De novo* Melatonin Synthesis in Seedlings

To find the potential effects of waterlogging stress and melatonin application on the endogenous melatonin synthesis, the melatonin level and its synthetic gene expression were detected. The results showed that waterlogging stress significantly elevated plants melatonin levels in leaves (87 vs. 21 ng/g FW of control) and in roots (73 vs. 16 ng/g FW of control) (**Figure 2A**).

When exogenous melatonin was applied to the stressed seedlings, their endogenous melatonin was further increased compared to the plants under waterlogging stress alone (**Figure 2A**). For example, the endogenous leaf melatonin level in melatonin (200  $\mu\text{M}$ ) sprayed plants was 256 ng/g FW, which was 2.9 times higher than that in leaves of waterlogged seedling alone (87 ng/g FW). The similar results were observed in roots and also in melatonin irrigated seedlings. In accordance with the elevated melatonin production, the gene expressions of melatonin synthetic enzymes including ASMT (acetylserotonin O-methyltransferase), AANAT (aralkylamine N-acetyltransferase) and T5H (tryptamine 5-hydroxylase), were slightly upregulated under the waterlogging stress and melatonin treatment compared to the controls (**Figure 2B**). The upregulated gene expression of ASMT9, which is the supposed melatonin synthetic rate-limiting enzyme, failed to result in the increase in its protein level. In contrast, its protein level was declined when compared to the controls (**Figure 2C**). Obviously, the post-transcriptional regulation occurred for ASMT9.

### The Effects of Melatonin on Antioxidant Enzymes

The activities of the antioxidant enzymes including SOD, POD, CAT were measured in four groups of *M. baccata* seedlings. These groups included the normal control group, waterlogging stress alone, waterlogging stress treated with melatonin (200  $\mu\text{M}$ ) spray and waterlogging stress treated with

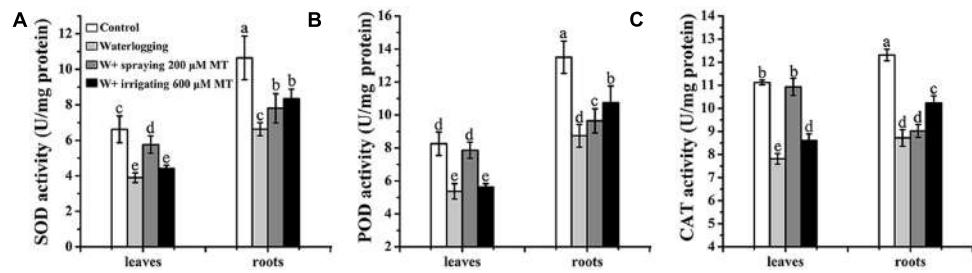


**FIGURE 2 |** Effects of waterlogging and exogenous melatonin application on the melatonin synthesis in *M. baccata* seedlings 9 days after waterlogging stress. **(A)** Endogenous melatonin levels in leaves and in roots of the *M. baccata* seedlings. **(B)** The relative expression of melatonin synthetic genes (*MbASMT9*, *MbAANAT3* and *MbT5H1*) in the leaves. **(C)** The protein level of *MbASMT9* detected by Western blot analysis in the leaves. The data are means  $\pm$  SD of triplicate experiments. Asterisks (\*) and different letters indicate significant differences from the control ( $P < 0.05$ ).

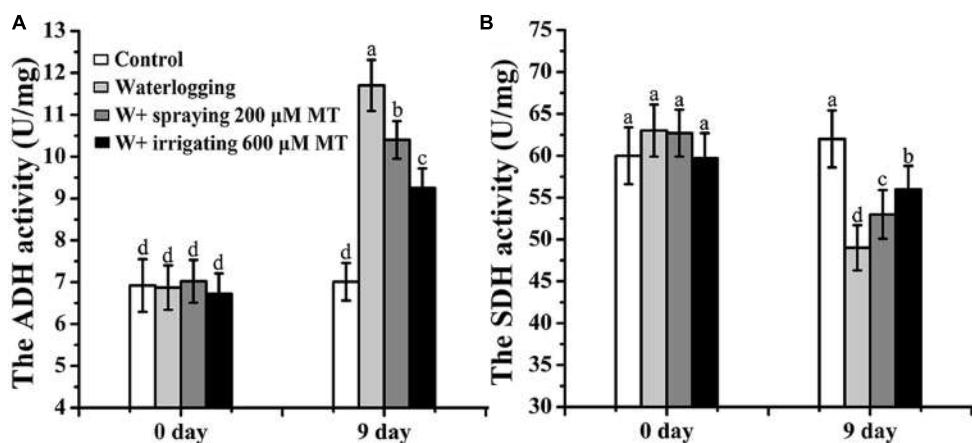
melatonin (600  $\mu\text{M}$ ) irrigation. Waterlogging stress significantly suppressed all antioxidant enzymes tested in both levels and roots of the seedlings. However, melatonin treatment could recover the activities of these antioxidant enzymes suppressed by waterlogging stress in a great degree. For example, melatonin (200  $\mu\text{M}$ ) spray recovered the activity of CAT in leaves to the level comparable to the control seedlings (**Figure 3**).

### Effects of Melatonin on Aerobic and Anaerobic Respiration of *M. baccata* Seedlings under Waterlogging Stress

The activities of ADH and SDH indicate the anaerobic and aerobic respirations, respectively. It was found that the ADH activity (11.71 U/mg), the index of anaerobic respiration, was significantly higher in the roots of waterlogging stressed seedlings



**FIGURE 3 | Effects of melatonin on activities of antioxidant enzymes. (A)** Superoxide dismutase (SOD). **(B)** Peroxidase (POD). **(C)** Catalase (CAT) in both leaves and roots. The waterlogging stress was lasted 9 days. The data are means  $\pm$  SD of triplicate experiments. Different letters indicate significant differences from the control ( $P < 0.05$ ).



**FIGURE 4 | Effects of melatonin on aerobic and anaerobic respirations of *M. baccata* seedlings under waterlogging stress. (A)** Anaerobic respiration which is indicated by ADH activity. **(B)** Aerobic respiration which is indicated by SDH activity. The waterlogging stress was last for 9 days. The data are means  $\pm$  SD of triplicate experiments. Different letters indicate significant differences from the control ( $P < 0.05$ ).

than that of normal control (7.01 U/mg). Both melatonin spray and irrigation reduced the anaerobic respiration indicated by decreased ADH activity (Figure 4A). In contrast, waterlogging stress suppressed the aerobic respiration indicated by the significantly reduced SDH activity compared to the control seedlings. This tendency was reversed by melatonin treatment in a great degree (Figure 4B).

### The Effect of Melatonin on $O_2^{\bullet-}$ and $H_2O_2$ Production and the Oxidative Damage Induced by Waterlogging Stress

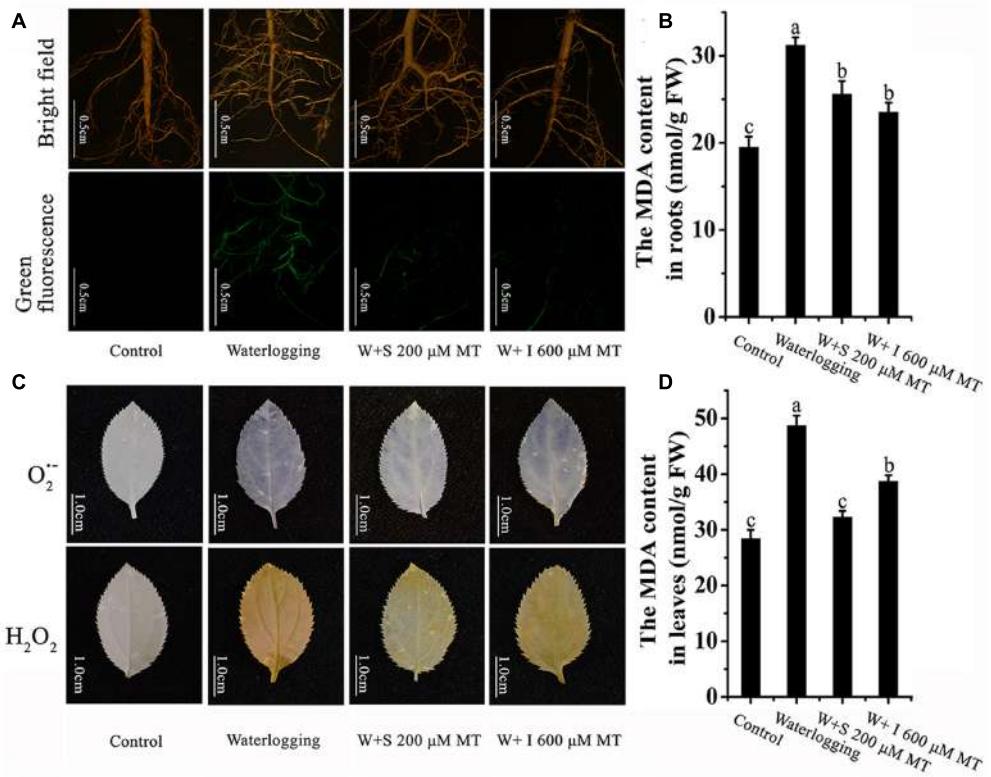
Waterlogging stress significantly elevated the  $O_2^{\bullet-}$  and  $H_2O_2$  production both in leaves and in roots of the seedlings. These were indicated by the increased fluorescent staining intensities on them. Melatonin treatment significantly reduced  $O_2^{\bullet-}$  and  $H_2O_2$  productions, no matter melatonin was sprayed to the leaves or irrigated to the roots of the seedlings (Figures 5A,C). Accordantly, waterlogging stress caused oxidative damage in both leaves and roots of the seedlings. This was indicated by the increased content of MDA. It was expected that melatonin treatment significantly reduced the MDA levels in leaves and also in roots (Figures 5B,D).

### The Effect of Melatonin on the Chlorophyll Content and Photosynthetic Rate

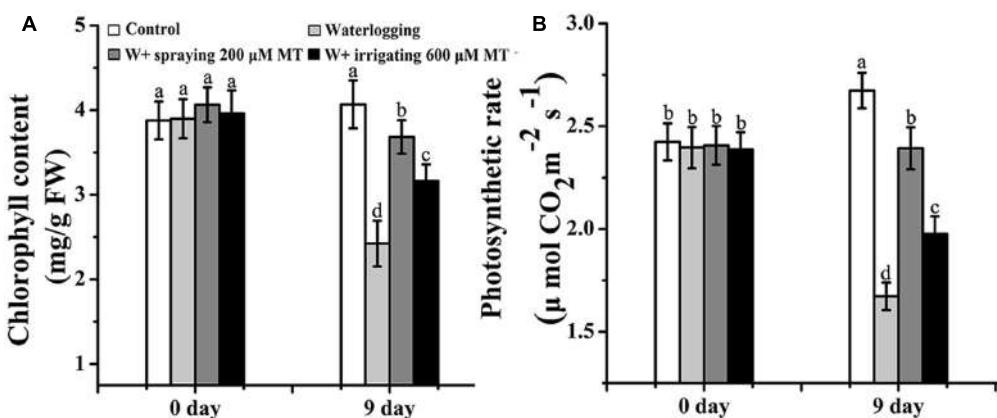
Waterlogging stress dramatically reduced the Chl content in the leaves of seedlings. After 9 days of waterlogging stress, the Chl content of these plants (2.4 mg/g FW) was only roughly half of the value of control seedlings (4.1 mg/g FW). The photosynthetic rate of the waterlogging stressed plants was  $1.67 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The value was significantly lower than that of control seedlings ( $2.67 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Melatonin treatments (spray or irrigation) improved both Chl content and photosynthetic rate which were suppressed by the waterlogging stress (Figures 6A,B).

### DISCUSSION

Waterlogging, caused by poor drainage, flooding, and long periods of rainfall, usually occurs in summer and autumn, which hampers apple tree growth and results in yield loss (Yu et al., 2015). Because of the global warming effect, it is predicted that this climate change will bring more rainfall yearly and thus



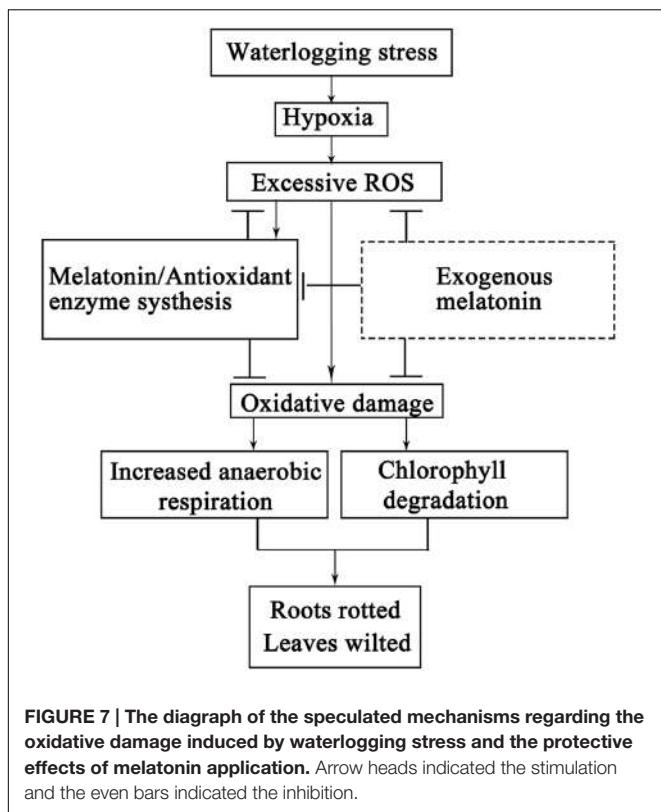
**FIGURE 5 | Effects of melatonin on ROS levels and oxidative damage in *M. baccata* seedlings which were under waterlogging stress. (A)** ROS level in roots. **(B)** MDA production in roots. **(C)** The levels of superoxide anion and hydrogen peroxide in leaves. **(D)** MDA production in leaves. The waterlogging stress lasted for 9 days. Scale bars in **(A)** represent 0.5 cm. Scale bars in **(C)** represent 1cm. The data are means  $\pm$  SD of triplicate experiments. Different letters indicate significant differences from the control ( $P < 0.05$ ).



**FIGURE 6 | Effects of melatonin on the chlorophyll content and photosynthetic rate of the *M. baccata* seedlings before and after waterlogging stress. (A)** Chlorophyll content. **(B)** Photosynthetic rate. The data are means  $\pm$  SD of triplicate experiments. Different letters indicate significant differences from the control ( $P < 0.05$ ).

the waterlogging will be frequently encountered by the crops worldwide. This will bring severe problem for these crops which are intolerant to the waterlogging stress including the apple trees. To solve this problem, it has become an urgent agenda for scientist.

In the current study, a unique molecule, melatonin, was tested with this purpose. Melatonin was reported to enhance plant tolerance against various abiotic stressors including cold, hot, drought, salinity, and chemical pollutants (Bajwa et al., 2014; Wei et al., 2014; Liang et al., 2015; Liu et al., 2015; Shi et al., 2015b;



Li X. et al., 2016; Xu et al., 2016). But the effect of melatonin on waterlogging resistance is still to be investigated.

Long lasting waterlogging caused apple seedling damage. Under waterlogging, the seedlings changed their metabolism from aerobic to anaerobic respiration (Fukao and Bailey-Serres, 2004). During this transition, a burst of ROS was generated and this resulted in oxidative damage of the seedlings (Hossain et al., 2009; Xu et al., 2013; Takahashi et al., 2015). This was indicated by the phenotype alterations of the seedlings such as chlorosis and wilting (Figure 1). However, all these changes were partially or completely prevented by melatonin application either by spraying to the leaves or irrigating to the roots. The primary mechanism of melatonin to improve the tolerance of apple seedlings against waterlogging stress may relate its potent free radical scavenging and antioxidant capacity (Tan et al., 1993, 2012). Melatonin not only directly scavenges the ROS but also upregulates the activities of a variety of antioxidant enzymes (Wang et al., 2012). This was in accordance with our observations in the current study (Figures 3, 5).

In the current study, both waterlogging stress and melatonin application significantly increased melatonin level of the plants. It is well known that various stressors induce melatonin production in plants as well as in animals (Byeon et al., 2012; Li C. et al., 2016; Xu et al., 2016). This is considered as self-defense of organisms against external insults (Tan et al., 2015). Our observation provided new evidence to support this consideration. Waterlogging induced the gene expression of the melatonin synthetases and increased melatonin

production in the apple seedlings. This positive response mechanism at the RNA level, for melatonin production in apple plants, probably also, exists in other plants. When exogenous melatonin was applied under waterlogging stress, the stress-induced expression of the melatonin synthetases still maintained their high expression. But obviously, the slight expression change between melatonin + waterlogging treatment and only waterlogging treatment can't explain the significant melatonin increase after exogenous melatonin applied under waterlogging. It seemed that melatonin absorbed from outside mainly resulted in melatonin increase. In addition, for the MbASMT9, a melatonin synthetic rate-limiting enzyme, which was located in chloroplasts (Kang et al., 2013; Zheng et al., 2017), its upregulated RNA expression did not result in elevated protein level. In contrast, its protein level reduced after melatonin application. This probably was the first report to document a post-transcriptional regulation of melatonin synthesis in plants. There are accumulated post-transcriptional regulation reported, including RNA processing, transport and degradation, translation control to fine-tune biological processes for plants in response to the environmental changes (Palatnik et al., 2003, 2007; Cowley et al., 2012; Saze et al., 2013). Obviously, herein, the post-transcriptional regulation conserves resources and energy of the plants to avoid extra melatonin production when exogenous applied melatonin meets their requirement. This observation provided valuable information as to use of exogenous melatonin to improve plant tolerance to against stressors.

There are accumulated reports described the waterlogging caused transition from aerobic to anaerobic respiration in root, which is also confirmed by us (Figure 4). Our results also uncovered the role of melatonin to maintain aerobic respiration under waterlogging stress, by efficient suppression of the ROS burst and subsequent mitochondria degradation. The chlorosis is a typical sign unavoidably happening after severe waterlogging. Due to the reason that waterlogging leads to water shortage and subsequent quick stomata closure, the high concentration of  $O_2$  can't be released out and photosynthetic electron transportation is blocked in chloroplasts. Therefore, the ion leakage from the electron transport chain would induce in over-produced  $O_2^-$  and  $H_2O_2$ , which destroy chlorophyll and lead to the disintegration of chloroplasts (Musgrave, 1994; Smethurst and Shabala, 2003). The protective effects of melatonin on Chl decay, photosynthetic capacity and stomata configurations have been reported previously in other stressors such as in drought and hot (Wang et al., 2012; Xu et al., 2016). Actually, chloroplasts, as one of the most suffered organelles from ROS, were proved to be the major site for melatonin production. A large amount of melatonin is needed to maintain its structure and function. Therefore absorbed and *in vivo* synthesized melatonin can function together to migrate waterlogging induced ROS and help to survive the stress. Here we reported that melatonin application preserved the Chl content and maintained the photosynthetic rate in seedlings suffered from waterlogging stress. The high content of Chl and efficient photosynthesis are required for high yield of apple production (Li et al., 2000). It is our

speculation that melatonin application in the field will increase the tolerance of apple tree and reduces apple yield loss against waterlogging stress. The speculated mechanisms are summarized in **Figure 7**.

## AUTHOR CONTRIBUTIONS

Designed the studies: JK, Undertook the experimental work: XZ, JZ, NW, LW, and DS. Contributed to figures and manuscript preparation: XZ, D-XT, and JK. 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: <http://journal.frontiersin.org/article/10.3389/fpls.2017.00483/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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