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Exogenous Melatonin Positively Regulates Rice Root Growth through Promoting the Antioxidant System and Mediating the Auxin Signaling under Root-Zone Hypoxia Stress

Juan Liu [1,2], Jiajia Wang [1], Tianhai Zhang [1], Meng Li [1], Huimin Yan [1], Qiuyuan Liu [1], Yunfei Wei [1], Xin Ji [1,*] and Quanzhi Zhao [2,*]

[1] College of Agronomy, Xinyang Agriculture and Forestry University, Xinyang 464000, China
[2] College of Agronomy, Henan Agricultural University, Zhengzhou 450046, China
* Correspondence: jixin@xyafu.edu.cn (X.J.); qzzhaoh@126.com (Q.Z.)

Abstract: Root growth and development is an important indicator of root-zone hypoxia tolerance in rice. Melatonin has been suggested to function as a crucial regulator in modulating root growth and improving plant abiotic stress resistance. To explore the role and potential mechanism of melatonin in regulating the root growth under root-zone hypoxia stress, rice seedlings were treated with hypoxia (oxygen level at 0.9–2.1 mg·L^{-1}), combined with or without a 20 μmol·L^{-1} melatonin pretreatment under a hydroponic condition. The results showed that the exogenous application of melatonin significantly alleviated the inhibition of the rice root growth that was induced by the hypoxia stress. The morphological–phenotypic analyses showed that after the melatonin pretreatment, the primary root length, lateral root length, and lateral root density increased by 11.6%, 8.2%, and 36.8%, respectively, under hypoxia stress. The physiological–biochemical analyses showed that the exogenous melatonin significantly increased the root activity and O_2 influx in the root meristem zone under hypoxia stress to 1.5 times that observed in the hypoxia stress group. The melatonin pretreatment significantly improved the activity of superoxide dismutase (SOD) and decreased the accumulation of superoxide anions ($O_2^{\bullet-}$) in the seedling roots, whereas it increased the content of hydrogen peroxide (H_2O_2) under hypoxia stress. The exogenous melatonin pretreatment significantly increased the content of indole-3-acetic acid (IAA) by 51.5% in the rice roots compared to the plants without melatonin pretreatment under hypoxia stress. Quantitative real-time PCR (qRT-PCR) analyses revealed that the melatonin pretreatment induced the expression of *OsPIN1a~1d*, *OsPIN8*, *OsPIN9*, *OsAUX1*, *OsARF19*, and *OsGH3-2* in the rice seedling roots under aerated conditions, whereas it only obviously upregulated the expression of *OsPIN1b*, *OsPIN2*, and *OsGH3-2* under hypoxia stress. These results indicate that melatonin positively regulates root growth and development under hypoxia stress, through improving the antioxidant system and directly or indirectly activating the auxin signaling pathway. This study demonstrates the important role of melatonin to modulate root growth under hypoxia stress, providing a new strategy for improving hypoxia tolerance.

Keywords: melatonin; hypoxia; root; reactive oxygen species; auxin

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1. Introduction

Waterlogging refers to excessive water in the root zone, resulting from poor soil drainage and extreme rainfall [1]. Globally, around 30% of cultivated land is affected by waterlogging, and the proportion is expected to increase due to rapid climate change [2]. Waterlogging has become the second most important abiotic stress factor, resulting in crop growth inhibition and crop yield loss [3]. Thus, it is essential to understand the mechanisms of crop waterlogging tolerance to maintain crop productivity.

Roots are the part of the plant most vulnerable to oxygen deficiency (hypoxia or anoxia) under waterlogging and flooding conditions [4], due to the slow diffusion of oxygen in the

waterlogged soil and the rapid consumption of oxygen by the plant roots [5,6]. Improving roots resistance to environmental stress is essential to ensure crop production. Plants adjust their roots' morphological and anatomical characteristics, such as adventitious roots, lateral roots, and root aerenchyma, to improve the oxygen supply in order to enhance the roots vitality under waterlogged conditions [7]. Previous studies have shown that many plant species reorganize root configuration characteristics by inducing adventitious root formation [8,9], inhibiting primary root growth [10], and promoting root bending [11] under waterlogged conditions. Hypoxia-tolerant rice genotypes exhibit a lower reduction in the root length, root biomass, and root activity and form more aerenchyma and adventitious roots under hypoxia stress [12,13]. Phytohormones play an important role in regulating plant growth and development under waterlogging stress. Waterlogging stress causes ethylene production, and ethylene induces root aerenchyma formation and promotes adventitious root development under hypoxic conditions [14]. It has been previously demonstrated that ethylene induces adventitious root formation by mediating the auxin transport, distribution, and accumulation in plant roots [9,15,16]. Hypoxic conditions cause root bending, mediated by the auxin signaling in the root tip [11].

Melatonin (*N*-acetyl-5-methoxytryptamine, Mel) is a pleiotropic molecule that was initially identified in plants in 1995 [17,18]. Melatonin, as a master regulator, regulates plant development and stress responses [19]. A range of studies have shown that melatonin, as a broad-spectrum antioxidant, can enhance the tolerance of plants under various environmental stresses, such as cold [20], drought [21], salt [22], alkaline [23], and heavy metal stress [24]. However, few studies have been conducted to elucidate the role of melatonin-mediated plant root-zone hypoxia stress tolerance, especially in rice. It has been shown that melatonin pretreatment improves waterlogging tolerance in alfalfa by reprogramming polyamine and ethylene metabolism [25]. In apple, exogenous melatonin application improved the aerobic respiration, chlorophyll content, and photosynthetic rate, which were suppressed by waterlogging stress [26]. Additionally, melatonin treatment can alleviate waterlogging-induced oxidative damage [27]. Melatonin treatment protected apple plants from waterlogging stress, mainly by improving the antioxidant enzyme activity and decreasing the reactive oxygen species (ROS) accumulation [26]. A recent study on peach revealed that melatonin enhanced the antioxidant enzyme activity and reduced the ROS concentrations in both roots and leaves to alleviate oxidative damage under waterlogging stress [28].

Additionally, due to the chemical similarity and the common biosynthetic route of melatonin and indole-3-acetic acid (IAA), melatonin has been reported to exhibit some similar functions to auxin in many plant species, such as Arabidopsis, sweet cherry, barely, oat, tomato, soybean, wheat, and rice [29–31]. Melatonin treatment facilitated lateral root development and growth in tomato and cucumber [32,33]. Rice seedlings overexpressing sheep serotonin *N*-acetyltransferase (NAT), which produced more melatonin than the wildtype plants, showed enhanced seminal root growth [34]. Melatonin has been reported to regulate the root architecture by modulating the auxin response in rice [35]. Exogenous melatonin promotes adventitious root development by regulating auxin transport and signal transduction [36]. Although many studies have revealed the interaction of melatonin and auxin in regard to plant root growth, the interactions of melatonin and auxin to promote plant adaption to biotic and abiotic stresses have rarely been reported [37]. Exogenous application of melatonin increased the endogenous IAA content in maize under semiarid conditions, thus improving maize survival and yields under stress [38]. In a previous study, hypoxia stress altered the auxin response and transport, and auxin may be involved in modulating the root architecture characteristics in rice under hypoxia conditions [12]. Thus, can exogenous melatonin regulate the growth and development of rice roots under hypoxia stress? Is this achieved by mediating the auxin signaling or other signaling pathways? Furthermore, the underlying mechanisms of melatonin in mediating rice roots response to hypoxia stress are unclear.

Therefore, we investigated the effects of melatonin on the root morphological parameters, root vitality, antioxidant activity, and auxin signaling in rice roots under hypoxia stress. This study aimed to elucidate the potential mechanisms of melatonin regulating rice root growth under hypoxia stress. These results will contribute to further elucidating the role and potential mechanism of melatonin in improving root growth under root-zone hypoxia stress and provide a new strategy to enhance hypoxia tolerance.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The rice (*Oryza sativa* L.) variety IRAT109, an upland tropical japonica cultivar, was used in this study. The rice seeds were surface-sterilized with 10% H_2O_2 for 10 min and rinsed thoroughly with distilled water. After soaking in darkness and at 30 °C for 48 h, the germinated seeds were placed into a bottomless 96-well plate, floating on a water solution for 4 days. Then, Yoshida nutrient solutions, ranging from one quarter and one half to full strength, were applied for 6 days, followed by the full-strength nutrient solution. The seedlings were grown in a growth incubator under 28 °C/25 °C (day/night) and a 14 h light/10 h dark photoperiod. The humidity was maintained at 70% throughout the study.

Ten-day-old seedlings were pretreated with 20 μmol·L^{-1} melatonin (Mel) for 24 h. Then, they were transferred to the Yoshida nutrient solution with different dissolved oxygen concentrations for 3 or 5 days. In the aerated (Aer) treatment, fresh air was applied to the nutrient solution to maintain the dissolved oxygen concentration at 4.5–6.0 mg·L^{-1}. The hypoxic (Hyp) treatment was achieved by passing nitrogen gas into the solution with 0.1% agar to maintain the dissolved oxygen concentration at 0.9–2.1 mg·L^{-1}. Moreover, the preparation method for the 0.1% agar referred to a previously described method [39]. The dissolved oxygen concentration was continuously monitored with a Fiber-Optic Oxygen Meter (Firesting O_2: PyroScience GmbH, Aachen, Germany).

2.2. Determination of the Root Morphology Parameters and Root Activity

Ten uniform seedlings were selected to measure the longest root length with a ruler. The number of adventitious roots and lateral roots on the seminal root were counted manually. The morphology of the lateral roots was observed with a scanning microscope. The length of the lateral roots was measured with ImageJ software, and the root vitality was determined by the improved triphenyl tetrazolium chloride (TTC) method. The protocol was conducted according to a previous study [40].

2.3. Determination of the Net Oxygen Flux on the Root Tip Surfaces

The net oxygen flux on the root tip surfaces was determined using the noninvasive microtest technique (NMT, MT100 series; YoungerUSA, LLC, Amherst, MA, USA; Xuyue (Beijing) Sci. & Tech. Co., Ltd., Beijing, China). An oxygen microelectrode (tip diameter, 2–3 μm, XY-DJ-501; YoungerUSA, Amherst, MA, USA) and a reference electrode (YG003-Y10; YoungerUSA, Amherst, MA, USA) were used to complete the circuit at a −750 mV polarization voltage [12]. The oxygen microelectrode was moved repeatedly from one point to another, perpendicular to the root surface (amplitude: 30 μm, 0.3–0.5 Hz), to obtain the current difference. The oxygen fluxes were calculated according to Fick's law of diffusion: $J_0 = -D\,(dc/dx)$.

2.4. Determination of the Antioxidant Enzyme Activity, Superoxide Radical Anions ($O_2^{\bullet -}$), and Hydrogen Peroxide (H_2O_2)

To assess the activity of superoxide dismutase (SOD) and catalase (CAT), the frozen roots (0.2 g) were homogenized with ice-cold extraction buffer (50 mmol·L^{-1} potassium phosphate buffer, pH 7.8), containing 1 mmol·L^{-1} EDTA and 2% (*w*/*v*) polyvinylpolypyrrolidone. The homogenate was transferred and centrifuged at 12,000× *g* for 15 min at 4 °C, and the supernatant was used for the following enzyme assays. The activity of the SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue

tetrazolium (NBT), according to a previous method [41]. The activity of the CAT was determined as the decline in the absorbance at 240 nm due to the decrease in H_2O_2 extinction [42]. Moreover, the $O_2^{\bullet -}$ and H_2O_2 content in the rice roots was quantified with $O_2^{\bullet -}$ and H_2O_2 content determination kits, according to the manufacturer instructions (Comin Biotechnology, Suzhou, China).

2.5. Quantification of the IAA Content

Root tissues were harvested from the different melatonin and hypoxia treatments, rinsed with distilled water, and then frozen in liquid nitrogen immediately. The endogenous free IAA levels of rice roots were determined according to the method described previously [36]. Briefly, 0.1 g of each root sample was homogenized, and the free IAA was extracted using an aqueous solution of methanol. The extracts were purified through a C18 column on a solid-phase extractor, and the eluate was collected. The IAA was eluted with 80% MeOH. The eluent was evaporated to dryness under a stream of nitrogen. The IAA levels were quantified by high-performance liquid chromatography using an HPLC system (Waters, E2695, Milford, MA, USA) equipped with a compass C18 column (250 mm × 4.6 mm, 5 μm) and a fluorescence detector (Waters, 2475, Milford, MA, USA). The excited and the emitted wavelength was 275 nm and 345 nm, with a flow rate of 1.0 mL·min^{-1}.

2.6. RNA Extraction and Quantitative Real-Time PCR Analysis

After hypoxia treatment for 3 d, the root tissues were sampled, frozen in liquid nitrogen immediately, and stored in a −80 °C refrigerator. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed into cDNA using a HiFi-MMLV cDNA Kit (Cwbiotech, Beijing, China). Quantitative real-time PCR (qRT-PCR) was performed using iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on the CFX connect real-time PCR detection system (Bio-Rad, CFX96, Hercules, CA, USA). The gene expression was normalized using the rice *β-actin* gene as an internal control. The primers used for qRT-PCR are listed in Supplementary Table S1. For each gene, qRT-PCR was performed on three biological replicates.

2.7. Statistical Analysis

Statistical analysis was performed using SPSS software (21.0, SPSS, Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used to determine the significant differences between the mock and melatonin treatments under aerated or hypoxia conditions at $p < 0.05$. All values were reported as means ± SE (standard error). The graphs were made using Sigma plot software (12.5, Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Exogenous Melatonin Alleviates Hypoxia-Induced Root Growth Inhibition in Rice

To investigate the effect of melatonin on the root growth under hypoxia stress, we determined the maximum root length and the adventitious root number. The hypoxia significantly inhibited the root lengths of the rice seedlings (Figure 1A,B). Compared to the aerated condition, the maximum root length decreased by 17.9% under hypoxia stress (Figure 1B). However, a remarkable alleviating effect was observed in the melatonin-pretreated plants under hypoxia stress. The pretreatment with melatonin improved the maximum root length by 11.6% compared with the non-melatonin pretreatment under hypoxia conditions.

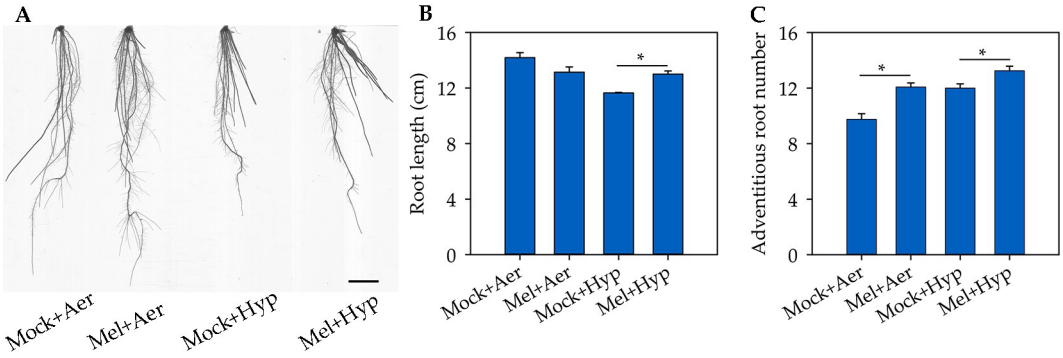

Figure 1. The effects of exogenous melatonin on the root morphology (**A**), maximum root length (**B**), and adventitious root number (**C**) under aerated (Aer) and hypoxia (Hyp) conditions. Scale bar = 2 cm. * indicates a significant difference between the melatonin (Mel) and non-melatonin (Mock) pretreatments under Aer and Hyp conditions at $p < 0.05$.

The hypoxia stress caused a significant increase in the adventitious root number compared to the aerated condition (Figure 1C). Pretreatment with melatonin also induced a significant increase in the adventitious root number by 23.9% compared with the non-melatonin pretreatment under the aerated condition. Under hypoxia conditions, the pretreatment with melatonin further increased the number of adventitious roots by 9.2% compared with the non-melatonin pretreatment.

3.2. Melatonin Promoted Lateral Root Formation under Hypoxia Stress

As shown in Figure 2A, the hypoxia treatment and the melatonin pretreatment affected the growth and development of the lateral roots. Compared to the aerated condition, the hypoxia stress increased the lateral root (LR) density (Figure 2B). Under the hypoxia stress, the melatonin pretreatment further induced an increase in the LR density (Figure 2B). Interestingly, both the hypoxia stress and the melatonin pretreatment inhibited the LR elongation (Figure 2C). The melatonin pretreatment significantly decreased the LR length by 10.4% compared with the non-melatonin pretreatment under the aerated condition. The hypoxia stress decreased the LR length by 61.9% compared to the aerated condition. However, the melatonin pretreatment significantly alleviated the LR length inhibited by the hypoxia stress and improved the LR length by 36.8% compared to the non-melatonin pretreatment under hypoxia stress.

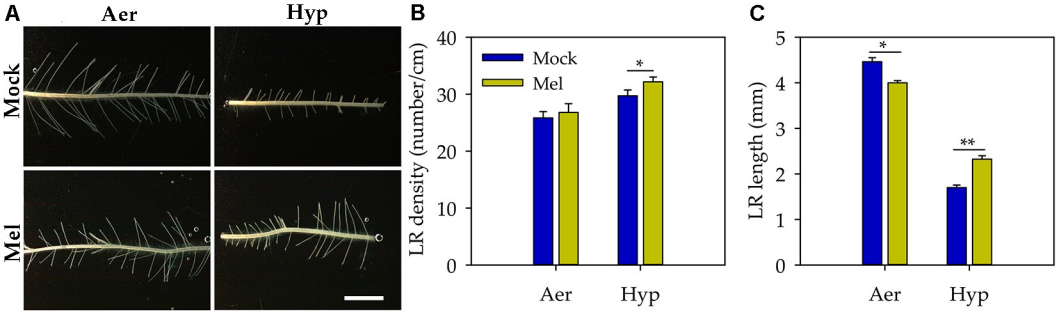

Figure 2. The effects of exogenous melatonin on the morphology of the lateral root (**A**), the lateral root density (**B**), and the lateral root length (**C**) in the rice seedling roots under Aer and Hyp conditions. Scale bar = 3 mm. * and ** indicate significant differences between the Mel and Mock pretreatments under Aer and Hyp conditions at $p < 0.05$ and $p < 0.01$, respectively.

3.3. Exogenous Melatonin Improved the Root Activity and Oxygen Influx in the Root Tips under Hypoxia Stress

The root activity was determined using the measurement of respiratory activity with TTC (Figure 3A). The hypoxia stress significantly inhibited the root activity by 34.5% compared to the aerated condition. The pretreatment with melatonin did not significantly change the root activity under the aerated condition. However, the melatonin pretreatment significantly improved the root activity, which increased 1.4-fold over the non-melatonin pretreatment under hypoxia stress.

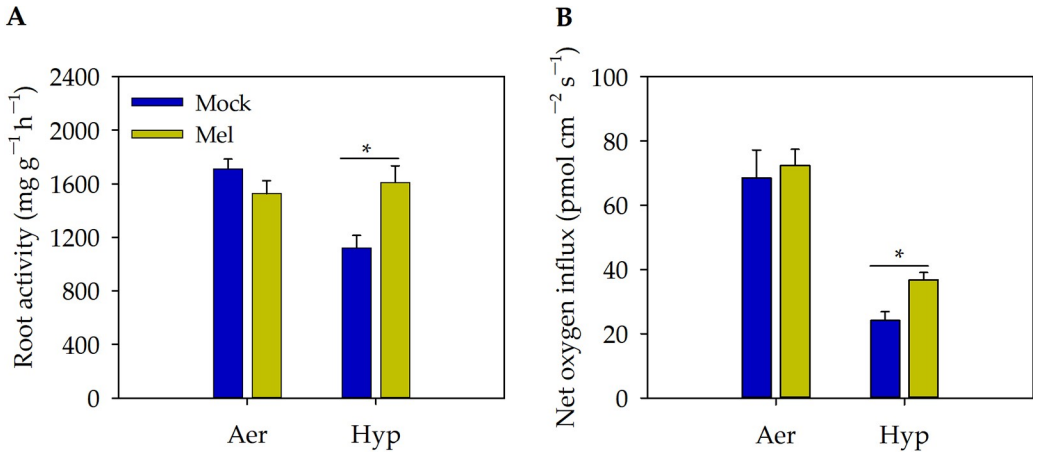

Figure 3. Changes in the root activity (**A**) and the net oxygen influx from the root meristematic zone (**B**) as affected by the application of exogenous melatonin under Aer and Hyp conditions. * indicates a significant difference between the Mel and Mock pretreatments under the Hyp condition at $p < 0.05$.

The oxygen flux is affected by the rhizosphere dissolved oxygen status. Our previous study showed that the meristem zone in the root tip was sensitive to hypoxia stress. Accordingly, we analyzed the effect of melatonin on the oxygen flux in the meristem zone under hypoxia stress (Figure 3B). The change trend of the hypoxia stress and the melatonin pretreatment regarding the oxygen influx was similar to that of the root activity. The hypoxia stress significantly suppressed the oxygen influx in the meristem zone. The oxygen influx was 68.55 pmol·cm^{-2}·s^{-1} under the aerated condition without the melatonin pretreatment but only 24.13 pmol·cm^{-2}·s^{-1} under the hypoxia condition without the melatonin pretreatment (Figure 3B). However, the oxygen influx significantly improved in the hypoxia-stressed seedlings after the melatonin pretreatment, which increased by 52.5% compared with the non-melatonin pretreatment under hypoxia stress.

3.4. Exogenous Melatonin May Positively Modulate Root Growth by Improving Redox Homeostasis

For rice seedlings under the aerated condition, melatonin pretreatment had no significant effect on the $O_2^{\bullet -}$ and H_2O_2 content (Figure 4A,B). The hypoxia stress significantly induced the accumulation of the $O_2^{\bullet -}$ content, which increased 1.3-fold as compared to the aerated condition. Moreover, the melatonin pretreatment significantly alleviated the increase in the hypoxia-induced $O_2^{\bullet -}$ content, reducing the $O_2^{\bullet -}$ content by 24.3% compared to the non-melatonin pretreatment under hypoxia stress (Figure 4A). The pretreatment with melatonin significantly enhanced the increase in the H_2O_2 content under hypoxia stress, increasing the H_2O_2 content by 35.2% compared with hypoxia stress alone (Figure 4B).

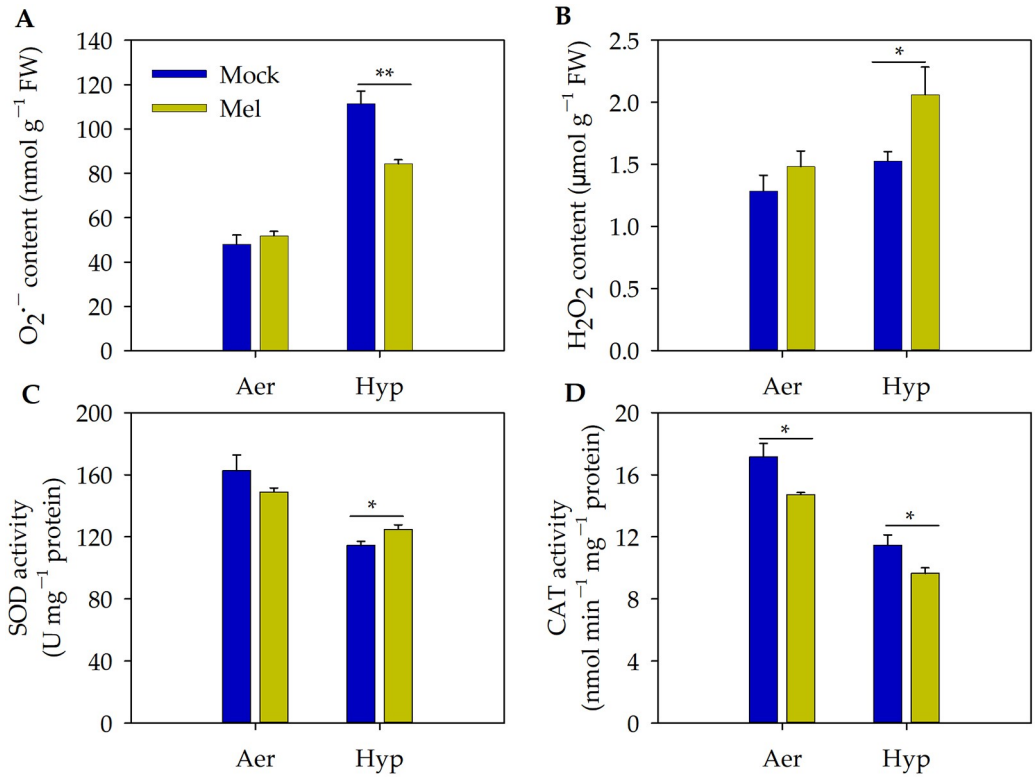

Figure 4. The effects of exogenous melatonin on the contents of superoxide anion ($O_2^{\bullet-}$; (**A**)) and hydrogen peroxide (H_2O_2; (**B**)) and the activity of superoxide dismutase (SOD; (**C**)) and catalase (CAT; (**D**)) in the rice seedling roots under Aer and Hyp conditions. * and ** indicate significant differences between the Mel and Mock pretreatments under Aer and Hyp conditions at $p < 0.05$ and $p < 0.01$, respectively.

To further explore the effect of the melatonin on the redox homeostasis under hypoxia stress, the changes in some antioxidant enzymes activities were determined. Under the aerated condition, the activity of the SOD and CAT was significantly reduced in the melatonin-pretreated seedlings (Figure 4C,D). Moreover, hypoxia stress significantly reduced the activity of the SOD and CAT by 29.6% and 33.2% as compared to the aerated condition. The pretreatment with melatonin significantly improved the activity of SOD by 9.1% under hypoxia stress as compared to hypoxia stress alone (Figure 4C). However, melatonin pretreatment further reduced the activity of CAT by 15.8% under hypoxia stress when compared with the non-melatonin pretreatment (Figure 4D).

3.5. Auxin May Act as a Downstream Signal of Melatonin to Regulate the Root Length under Hypoxia Stress

To explore the changes in the phytohormones under melatonin pretreatment and hypoxia stress, we evaluated the IAA content. The results showed that under the aerated condition, the melatonin pretreatment significantly improved the IAA content by 2.4 times compared with the non-melatonin pretreatment (Figure 5). The hypoxia stress significantly induced an increase in the IAA content, which increased by 1.3 times compared with the aerated condition. The melatonin pretreatment further improved the IAA content by 51.5% compared with the non-melatonin pretreatment under hypoxia conditions.

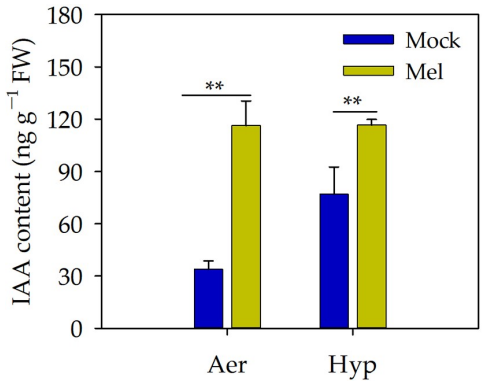

Figure 5. The effect of the exogenous melatonin on the IAA content in the adventitious roots of the rice seedlings under Aer and Hyp conditions. ** indicates a significant difference between the Mel and Mock pretreatments under Aer and Hyp conditions at $p < 0.01$.

We further investigated the expression levels of some auxin-related genes in the roots exposed to the melatonin pretreatment and hypoxia stress (Figure 6). The pretreatment with melatonin had no obvious effect on the expression of *OsPIN2* but significantly induced the expression of *OsPIN1a~1d*, *OsPIN8*, *OsPIN9*, *OsAUX1*, and *OsARF19* under the aerated condition (Figure 6). The hypoxia stress dramatically increased the expression levels of *OsPIN1d* and *OsPIN9*, which increased by 2.1- and 13.5-fold compared to the aerated condition. However, the melatonin pretreatment did not significantly increase the expression levels of *OsPIN1d* and *OsPIN9* compared with the non-melatonin pretreatment under the hypoxia condition. The melatonin pretreatment significantly increased the expression level of *OsPIN1b* and *OsPIN2* under hypoxia stress. The hypoxia stress and melatonin pretreatment had no significant effect on the expression level of *OsIAA13*. Interestingly, the pretreatment with melatonin remarkably increased the expression level of *OsGH3-2* compared with the non-melatonin pretreatment, which increased by 12.2- and 2.9-fold, respectively, under the aerated and hypoxia conditions.

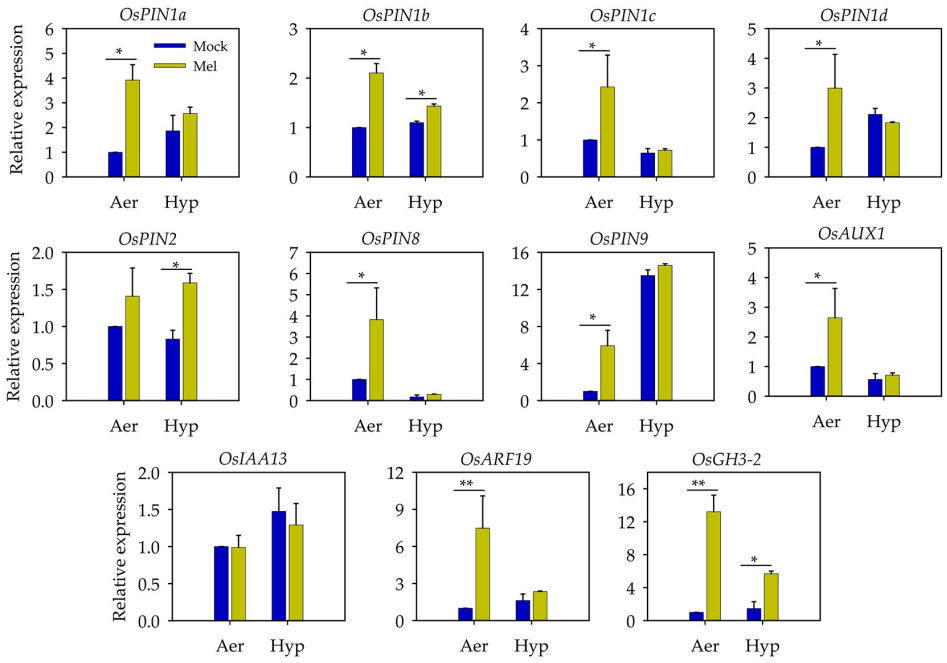

Figure 6. The effect of the exogenous melatonin pretreatment on the expression of the genes involved in auxin transport, response, and synthesis in the adventitious roots of the rice seedlings under hypoxia stress. * and ** indicate significant differences between the Mel and Mock pretreatments under Aer and Hyp conditions at $p < 0.05$ and $p < 0.01$, respectively.

4. Discussion

Hypoxia stress suppresses the growth and development of rice roots [12]. A number of studies have revealed that melatonin acts not only as a biostimulator, alleviating the damage caused by various stresses, but also as a plant growth regulator that regulates plant growth and development [43]. Melatonin has been shown to play a role in root growth and development, including primary root growth and lateral root formation [32,35]. Exogenously applied melatonin significantly suppressed embryonic root elongation in rice [35]. Here, melatonin pretreatment and hypoxia stress obviously inhibited primary root elongation, but this inhibition of the root growth was greatly alleviated by melatonin under hypoxia stress. Melatonin significantly promoted the lateral root formation and development in rice and cucumber [33,35]. In *Arabidopsis*, melatonin increased the number of lateral roots [44]. In this study, we found that melatonin pretreatment significantly alleviated the lateral root length inhibited by hypoxia stress and caused a further increase in the lateral root density under hypoxia stress. It has been demonstrated that exogenous melatonin improved drought tolerance and promoted lateral root generation in cucumber under drought stress [45]. Taken together, these results suggested that melatonin might function as a positive regulator of rice lateral root growth and development under hypoxia stress.

Root respiration intensity, as the core element of the root metabolism, plays an important role in the nutrient absorption, root regeneration, and plant growth and development [46]. Generally, the change trend of the root activity is consistent with the root respiratory intensity. Long-term hypoxia stress suppresses the physiological activity of plant roots [47]. Here, hypoxia stress caused a dramatic decrease in the root activity, whereas the melatonin pretreatment significantly improved the root activity under hypoxia stress. This was consistent with a previous report that exogenous melatonin enhanced root activity in cucumber under drought stress [45]. In another previous study, hypoxia stress inhibited the oxygen influx in the root tips of plants [48], especially in the root meristem zone of rice [12]. In this study, the oxygen influx in the root meristem zone was similar to the change trend of the root activity in rice. The hypoxia stress decreased the oxygen influx in the root meristem zone, whereas the exogenous melatonin pretreatment alleviated the inhibitory effects. A previous study demonstrated that a hypoxia-sensitive genotype exhibited a more severe 'energy crisis' than a hypoxia-tolerant genotype [12]. All these results suggest that exogenous melatonin can improve the root respiration intensity to enhance roots physiological activity under hypoxia stress.

Multiple abiotic stresses, including waterlogging, flooding, and salt, induce ROS overproduction, such as $O_2^{\bullet -}$, H_2O_2, and hydroxyl radicals, causing oxidative damage [49,50]. To protect plant cells from the excessive accumulation of ROS and oxidative damage, plants have evolved antioxidant defense machinery, including ROS-scavenging enzymes, such as SOD, CAT, and ascorbate peroxidase [51]. Melatonin has been reported as a broad-spectrum antioxidant to directly scavenge most ROS and reactive nitrogen species [52], but it also improves the activity of various antioxidant enzymes [53]. A range of studies have revealed that melatonin improves abiotic stress tolerance by enhancing oxidation resistance [54]. In this study, exogenous melatonin significantly increased the SOD activity but decreased the CAT activity in the seedling roots under hypoxia stress. SOD can catalyze the disproportionation and dismutation of $O_2^{\bullet -}$ into H_2O_2, thereby scavenging $O_2^{\bullet -}$, while CAT is mainly responsible for the decomposition of H_2O_2 in plants. Previous studies indicated that exogenous melatonin decreased the overproduction of $O_2^{\bullet -}$ and H_2O_2 under waterlogging stress [26]. Exogenous melatonin improved the waterlogging tolerance in wheat through enhancing the antioxidant enzymatic activity and reducing the malondialdehyde (MDA) content and $O_2^{\bullet -}$ production rate [55]. In this study, melatonin significantly reduced the overaccumulation of $O_2^{\bullet -}$ but, interestingly, further increased the content of H_2O_2 in the rice roots under hypoxia stress. Previous studies have suggested that ROS, as a downstream signaling, is involved in melatonin-induced stress tolerance [56,57]. H_2O_2 has also been reported as a central redox signaling mechanism to regulate oxidative stress [58]. H_2O_2 acts downstream of melatonin to mediate the lateral root growth and development [32].

These results indicate that melatonin decreased the overaccumulation of $O_2^{\bullet -}$ in rice roots by improving the SOD activity, thus increasing the roots physiological activity and the hypoxia tolerance in rice. Moreover, H_2O_2 might serve as a signaling molecule, involved in melatonin-modulated rice root growth and development under hypoxia stress. However, this requires further study.

Furthermore, H_2O_2 has been considered as a vital signaling molecule regulating plant root growth and development [59,60]. Previous studies have suggested that H_2O_2 mediates root formation and growth via the regulation of auxin signaling in rice under abiotic stress [61]. Melatonin also mediates auxin transport and signaling to control the plant root architecture [35,36,62]. Our results suggested that exogenous melatonin pretreatment significantly increased the content of the IAA in the roots under hypoxia stress. Melatonin can increase the expression of auxin signaling and the efflux genes to modulate the root architecture [36]. In the present study, exogenous melatonin significantly induced the expression of *OsPIN1a~1d*, *OsPIN8*, *OsPIN9*, *OsAUX1*, *OsARF19*, and *OsGH3-2* under the aerated condition, whereas the melatonin pretreatment upregulated the expression of *OsPIN1b*, *OsPIN2*, and *OsGH3-2* under hypoxia stress. *OsGH3-2*, encoding an enzyme catalyzing IAA conjugation to amino acids, modulates the endogenous free IAA levels and positively regulates cold tolerance [63], and it might mediate lateral root development [64]. A recent study has revealed that the overexpression of *OsGH3-8* increases auxin metabolism, decreases the endogenous levels of free IAA, and improves rice anaerobic germination tolerance [65]. *OsPIN2*, encoding a member of the auxin efflux carrier proteins, modulates rice root elongation growth and lateral root formation by regulating auxin distribution [66]. Thus, melatonin is an important mediator of the root architecture. It likely alters the auxin metabolism and transport and might function by activating auxin signaling. However, the specific molecular mechanism of melatonin-mediated auxin transport and signaling in terms of regulating the root architecture and the response to hypoxia stress needs to be further investigated.

5. Conclusions

In summary, our results indicated that exogenous melatonin effectively alleviated the inhibition of primary roots and lateral roots and enhanced the increase in the lateral root density under hypoxia stress. Meanwhile, the melatonin pretreatment improved the rice roots activity and the oxygen influxes in the root tips under hypoxia stress. These changes may be mediated by two potential mechanisms: (1) the melatonin may promote SOD activity in the rice roots and decreased the overaccumulation of $O_2^{\bullet -}$ to mitigate the oxidative damage and thereby improve the hypoxia tolerance; and (2) auxin, as a potential downstream signal of melatonin, may be involved in regulating the root architecture characteristics in rice under hypoxia stress. This study highlights the role of melatonin for melatonin-enhanced hypoxia tolerance in rice and provides new evidence for melatonin-modulated rice root growth through promoting the antioxidant system and mediating the auxin signaling under root-zone hypoxia stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020386/s1, Table S1: List of the specific primers used for the qRT-PCR.

Author Contributions: X.J. and J.W. designed the experiments; T.Z., M.L. and H.Y. performed the experiments; Q.L. and Y.W. analyzed the data; J.L. and X.J. wrote the original draft; J.L. and Q.Z. revised and edited this manuscript. All authors have read and agreed to the published version of the manuscript.

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