Exogenous Melatonin Enhances Rice Plant Resistance Against *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Rice bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most serious diseases of rice. In this study we found that exogenous melatonin can increase rice resistance to BB. Treatment of rice plants with exogenous melatonin (20 µg/ml) increased nitrate reductase, nitric oxide synthase, and peroxidase activity, enabling high intracellular concentrations of melatonin, nitric oxide, and H₂O₂. The expression of *NPR1*, a key regulator in the salicylic acid signaling pathway, was upregulated more than 10-fold when the plants were challenged with melatonin. Similarly, the messenger RNA level of *PDF1.2*, a jasmonic acid-induced defense marker, was 15 times higher in the treated plants

than in the control plants. Moreover, three pathogenesis-related proteins, *PR1b*, *PR8a*, and *PR9*, were upregulated 20-fold in the presence of melatonin. The application of melatonin (100 µg/ml) to soil-grown rice reduced the incidence of BB by 86.21%. Taken together, these results not only provide a better understanding of melatonin-mediated innate immunity to *X. oryzae* pv. *oryzae* in rice but also represent a promising cultivation strategy to protect rice against *X. oryzae* pv. *oryzae* infection.

Keywords: bacterial blight, melatonin, rice resistance, *Xanthomonas oryzae* pv. *oryzae*

Melatonin (*N*-acetyl-5-methoxytryptamine) is an evolutionarily conserved molecule that exists in the plant kingdom (Shi et al. 2015a), animal kingdom (Rocchitta et al. 2006), and microbes (Jiao et al. 2016). Melatonin was first identified in plants in 1995 (Dubbels et al. 1995; Hattori et al. 1995). Since then, many studies have revealed that melatonin regulates plant development through several physiological processes, including seed germination (Zhang et al. 2013), flowering (Byeon and Back 2014), leaf senescence (Liang et al. 2015), root development (Liang et al. 2017), and fruit ripening (Sun et al. 2015). Melatonin is a potent free radical scavenger and can protect plants from the damage caused by reactive oxygen species (ROS). In this field, melatonin was demonstrated to confer plant tolerance against numerous environmental stresses, including Cd (Gu et al. 2017), Cu (Cao et al. 2019), Al (Zhang et al. 2019), drought (Cui et al. 2017), salt (Li et al. 2017), cold (Lei et al. 2004), ultraviolet radiation (Afreen et al. 2006), and organic toxins (Reiter et al. 2008). Recently, melatonin was found to induce plant innate immunity against pathogen infection. In this sense, the application of exogenous melatonin led to improved resistance to *Marssonina* blotch in

apple leaves (Yin et al. 2013). It was found that exogenous melatonin enabled the apple trees to maintain high intracellular hydrogen peroxide (H₂O₂) concentrations and induced the overexpression of pathogenesis-related proteins (PRs). The application of exogenous melatonin also increased disease resistance against *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*DC3000) in *Arabidopsis thaliana* (Lee et al. 2014). ROS and nitric oxide (NO) bursts were observed in *A. thaliana* upon *Pst*DC3000 infection under exogenous melatonin application (Lee and Back 2017; Shi et al. 2015b). The expression of salicylic acid (SA)-related genes, *MAPK* cascade genes *MAPK3* and *MAPK6*, and defense-related genes *PR1* and *ISC1* was upregulated in *A. thaliana* in the presence of exogenous melatonin (Lee and Back 2017). Moreover, the content of melatonin and SA decreased in the serotonin *N*=acetyltransferase knockout mutant of *A. thaliana*, which exhibited high susceptibility to *Pst*DC3000 (Lee et al. 2015). Collectively, these data indicate that melatonin acts as an important signaling molecule and mediates plant immune response to pathogen infection.

Rice bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most widespread and destructive diseases in rice-growing regions (Wing et al. 2018). BB was first reported by Japanese farmers in 1884 and has become a serious agricultural threat because of resistance mutations and new crop management practices (Adhikari et al. 1999; Chen et al. 2011; Tagami 1962). BB has been reported to cause yield losses up to 50% in uncontrolled spreads (Srinivasan and Gnanamanickam 2005). The most economical and effective method for managing BB consists of using plant resistant varieties. To date, at least 40 BB resistance (R) genes that confer host resistance against *X. oryzae* pv. *oryzae* have been identified, and 10 (R) genes have been cloned (Baliyan et al. 2018; Kim et al. 2015). Although BB has been “quiet” for 30 years, it has gradually emerged in fields planted with newly released rice varieties, particularly in China. Because of the high degree of pathogenic variation in *X. oryzae* pv. *oryzae*, many resistant varieties have succumbed to the disease (Singh et al. 2018). This trend highlights the need to continue discovering new strategies for controlling BB in rice.

Melatonin was reported to improve plant resistance by inducing the production of NO and H₂O₂, activation of SA and MAPK

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Funding: This research was supported by grants from the National Natural Science Foundation of China (31571974), the Special Fund for Agroscentific Research in the Public Interest (201303015), the National Key R&D Program of China (2017YFD0200900) and the Natural Science Foundation of Jiangsu Province of China (BK20170606), the State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products (2010DS700124-KF2007).

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary materials are published online.

The author(s) declare no conflict of interest.

Accepted for publication 22 January 2020.

signaling pathways, and expression of plant defense-related genes in *Arabidopsis* (Lee and Back 2017; Lee et al. 2014; Shi et al. 2015a; Yin et al. 2013). However, studies about the role of melatonin in rice are limited. In this work, we have examined the effects of melatonin-mediated resistance to *X. oryzae* pv. *oryzae* infection in rice and investigated how melatonin acts as a protector against this pathogen.

Materials and Methods

Plant and bacterial strains. Mature rice seeds (*Oryza sativa* subsp. *japonica* var. Nipponbare) were germinated and grown in a growth chamber alternating 28°C/12-h light and 30°C/12-h dark cycles with a photon flux density of 200 $\mu\text{mol}/\text{m}^2/\text{s}$. The rice seedlings were cultured in International Rice Research Institute nutrient solution (N, 40; P, 10; K, 40; Ca, 40; Mg, 40; Mn, 0.5; S, 40; Mo, 0.05; B, 0.2; Zn, 0.01; Cu, 0.01; Fe, 2.0; and Si, 50 ppm) for 5 weeks. The *X. oryzae* pv. *oryzae* strain PX099 was streaked on nutrient agar (containing 3 g of beef extract, 1 g of yeast extract, 5 g of polypeptone, 10 g of sucrose, and 15 g of agar per liter of distilled water) and incubated at 28°C for 2 days (X. Chen et al. 2018). Statistical analyses were performed in SPSS version 20.0. The variables were analyzed via Student's *t* test and tested for significance at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$ levels.

Bacterial pathogen inoculation. The virulent pathogen *X. oryzae* pv. *oryzae* strain PX099 (race P6) was used for the innate immunity assay. The pathogen was grown in nutrient broth (3 g/liter of beef extract, 1 g/liter of yeast extract, 5 g/liter of polypeptone, and 10 g/liter of sucrose) with shaking at 28°C and 180 rpm for 16 to 24 h. Then, the bacterial suspension was centrifuged at 6,000 rpm for 10 min, the upper phase was discarded, and the cells were suspended in water to an optical density at 600 nm (OD_{600}) of 1.0. The bacterial inoculation was performed in a greenhouse via the leaf clipping method, under the same conditions previously reported by Kauffman (1973). The 5-week rice seedlings were transferred into new pots containing 800 ml of aqueous solution. Thirty leaves were inoculated with *X. oryzae* pv. *oryzae*, and, at the same time, the roots of the plants were treated with melatonin (20 $\mu\text{g}/\text{ml}$, 0.02% MeOH, purity $\geq 98\%$). For the control experiment, the rice leaves were infected with *X. oryzae* pv. *oryzae*, and the plant roots were cultivated in water containing 0.02% MeOH in the absence of melatonin. After inoculation, pathogen-infected leaves were collected at 0 and 7 days after inoculation, and the bacterial population was measured according to the method reported by Song et al. (2015). Briefly, rice leaves were washed with 75% (vol/vol) ethanol for 1 min and homogenized. The diluted mixtures were evenly distributed on a nutrient agar plate and cultivated in darkness for 48 to 60 h. The CFUs on the plates were counted. Three independent experiments were performed.

Melatonin extraction. Melatonin was extracted via the isopropanol–chloroform method (Pan et al. 2010). The 5-week rice roots were submerged in hydroponic solutions containing melatonin (at 0 or 20 $\mu\text{g}/\text{ml}$), and the plant leaves were treated with water or *X. oryzae* pv. *oryzae* ($\text{OD}_{600} = 1.0$). The plants were collected, frozen in liquid nitrogen, and ground into powder. Fifty milligrams of powder was weighed and transferred into a 2-ml Eppendorf tube. Then, 500 μl of extraction buffer (isopropanol 66.62% vol/vol, ddH_2O 33.31% vol/vol and hydrochloric acid 0.07% vol/vol) was added, and the tube was placed in ice for 30 min. The tube was shaken for 10 s every 5 min. Next, 1 ml of chloroform was added to the tube, and the tube was placed in ice for another 30 min. The tube was also shaken for 10 s every 5 min. After centrifugation at 15,000 rpm and 4°C for 10 min, the organic phase was collected and dried with a nitrogen evaporator with nitrogen flow. The dried samples were reconstituted in 100 μl of methanol and filtered through a 0.1- μm membrane for high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) analysis. Three replicates were performed.

HPLC-MS/MS analysis. The plant extracts were analyzed with an HPLC-MS/MS system (Agilent HPLC 1290, Agilent, Santa Clara, CA, USA). Briefly, HPLC separation was performed on a Poroshell 120 SB–C18 column (2.1 $\times$ 150 mm, 2.7 μm , Agilent) at a flow rate of 0.3 ml/min. Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany), and formic acid (FA, HPLC

grade) was purchased from Aladdin (Shanghai, China). The gradient program using 0.1% formic acid in water (phase A) and MeOH (phase B) was applied as follows: from 80% A at 0 min to 50% A at 1 min, from 50% A at 1 min to 20% A at 9 min and held for 1.5 min, then returned to initial conditions. The column temperature was 30°C, and the injection volume was 2 μl .

MS analysis was performed in positive ion mode. The electrospray ionization source parameters were as follows: capillary voltage at 5.5 kV, capillary temperature at 500°C, auxiliary gas flow rate at 60 psi, curtain gas flow rate at 35 psi, and atomizing gas flow rate at 60 psi. Quantitative analysis was performed in multiple reaction monitoring mode with time-segmented scanings, as shown in Supplementary Table S1.

Quantification of the content of nitrate reductase, nitric oxide synthase, peroxidase, and H_2O_2 in rice leaves. Rice leaves (500 mg) were ground to powder in liquid nitrogen and then homogenized in 500 μl phosphate-buffered saline (PBS: 137 mmol/liter NaCl, 8.1 mmol/liter Na_2HPO_4 , 2.7 mmol/liter KCl and 2 mmol/liter KH_2PO_4 , pH = 7.4) (Kurppa et al. 2018). The homogenates were centrifuged at 12,000 rpm and 4°C for 20 min. For the nitrate reductase (NR) assay, the supernatants were collected and quantified with a specific enzyme-linked immunosorbent assay (ELISA) kit (Cat No: MM-094901, JiangSu KeTe Biotechnology, Yanchen, China). For the nitric oxide synthase (NOS) assay, the supernatants were collected and quantified with a specific ELISA kit (Cat No: KT0063401, JiangSu KeTe Biotechnology, Yanchen, China). For the peroxidase (POD) assay, the supernatants were collected and quantified with a POD ELISA kit (Cat No: KT3596001, JiangSu KeTe Biotechnology, Yanchen, China). For the H_2O_2 assay, the supernatants were collected and quantified with a specific H_2O_2 kit (Cat No: BC3595, Solarbio, China). Each experiment was conducted three times with three replicates.

RNA isolation and quantitative reverse transcription PCR. Primers were designed by Primer 5 (version 5.0) with the gene sequences in the Rice Genome Annotation Project database (Supplementary Table S2). The total RNA was extracted using TRIzol reagent (Invitrogen, Germany). Then, 2 μg of total RNA was treated with 1 μl gDNA Eraser (Takara, Japan) to eliminate genomic DNA. Finally, complementary DNA (cDNA) was synthesized using a PrimerScript RT reagent kit (Takara, Japan). Reverse transcription PCR (RT-PCR) was performed with diluted cDNA and SYBR Green PCR Master MIX (TakaRa, Japan) on a QuantStudio 6 RT-PCR system (Thermo Fisher Scientific, USA). The PCR program consisted of 95°C for 3 min, 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Dissociation curves were generated at the end of the PCR cycles, and relative gene expression was calculated via the $2^{-\Delta(\Delta\text{Ct})}$ method. *OsActin* gene was used as the internal reference (AK 060893) (Mitsuhara et al. 2008), and all experiments were repeated three times with cDNA prepared from different biological samples.

Results

Endogenous melatonin was induced by *X. oryzae* pv. *oryzae* infection. To determine whether melatonin is involved in the response to bacterial pathogens in rice, we decided to measure the content of endogenous melatonin in leaves and roots after *X. oryzae* pv. *oryzae* inoculation. To achieve this goal, melatonin was extracted and analyzed by HPLC-MS/MS. The rice plants were inoculated with *X. oryzae* pv. *oryzae*, and the concentration of melatonin was measured 24 h after inoculation. As a control experiment, mock-inoculated plants were incubated under the same conditions, but the pathogen inoculation was replaced by sterile water. The melatonin standard curve with $r = 0.9901$ is shown in Supplementary Figure S1. The peak corresponding to melatonin was detected at 5.69 min with the standard sample. A main mass/charge ratio (m/z) peak at 233.0 Da was observed, which is in well agreement with the expected molecular weight of melatonin, $[\text{M}+\text{H}]^+ = 233.13$ (Supplementary Fig. S2). The same m/z peak was detected in the rice leaves at the same retention time. MS/MS spectrometry analysis of the m/z peak revealed fragments at 153.0 and 225.1 Da, demonstrating the presence of melatonin. It was observed that endogenous melatonin significantly increased in the *X. oryzae* pv. *oryzae*-infected leaves from 0 h (16.53 ng/g fresh weight [FW]) to 24 h (21.44 ng/g FW) (Fig. 1). In

the control plants, endogenous melatonin in leaves increased only slightly at 24 h (17.58 ng/g FW), compared with that at 0 h (16.53 ng/g FW). Interestingly, we found that endogenous melatonin also increased in the roots when the rice leaves were challenged with *X. oryzae* pv. *oryzae* (Fig. 1). In this sense, 23.98 ng/g FW melatonin was detected in the inoculated plants, whereas only 19.54 ng/g FW melatonin was observed in the roots of the control plants. Thus, the melatonin content in plant roots, when the leaves of these plants were infected by the pathogen, was 1.22 times higher than that in the control plants. The excess of melatonin in plant roots may be either transported from other parts of the plant or synthesized in the same roots.

Exogenous melatonin improves rice resistance against *X. oryzae* pv. *oryzae* infection. To screen the optimal rice resistance, we cultivated the rice plants in hydroponic cultures containing 5, 10, or 20 μ g/ml of melatonin while *X. oryzae* pv. *oryzae* was inoculated in the rice leaves. Melatonin at 20 μ g/ml provided the highest disease resistance and was used in the study. To calculate melatonin-induced resistance, we measured the lesion length produced by the pathogen after 7 days. As a control experiment, the rice leaves were infected with *X. oryzae* pv. *oryzae*, and the plant roots were cultivated in the absence of melatonin. The results indicated that treating rice roots with melatonin induces disease resistance against *X. oryzae* pv. *oryzae*. As shown in Figure 2, the lesion length in the presence of 0 and 20 μ g/ml melatonin was 4.9 and 2.2 cm, respectively (Fig. 2A and B). The bacterial population in melatonin treated plants was less than that in the control plants (Fig. 2C), which is consistent with the lesion length results. Furthermore, the addition of melatonin (100 μ g/ml) into soil-cultivated rice plants reduced the incidence of BB by 86.21% (Supplementary Fig. S3). Our results suggested that rice resistance is increased when the plant roots are treated with exogenous melatonin.

Changes in endogenous melatonin in response to exogenous melatonin. To study the variations in the concentration of endogenous melatonin in the presence of exogenous melatonin, we treated the roots of the plants with melatonin (20 μ g/ml) while the plant leaves were inoculated with *X. oryzae* pv. *oryzae*. Then the melatonin content in plant leaves was measured via HPLC-MS/MS. For the control experiment, the roots of the rice plants were submerged in a hydroponic solution with 0 μ g/ml melatonin, and the rice leaves were mock-inoculated with water in the absence of pathogen. In the control plants, the melatonin levels increased from 1 to 24 h (Fig. 3A), and the maximum content of melatonin (29.41 ng/g FW) was detected at 24 h. When the plant

leaves were infected with the pathogen in the absence of melatonin, the maximum melatonin content (39.28 ng/g FW) was detected after 1 h. Then, melatonin levels decreased from 1 to 12 h. In the plants treated with melatonin in the absence of pathogen infection, the maximum melatonin content (50.97 ng/g FW) was detected after 1 h (Fig. 3B). The combination of melatonin and pathogen infection allowed a maximum content of 45.62 ng/g FW melatonin, which was detected after 12 h. It must be noted that the content of endogenous melatonin increased in the leaves when exogenous melatonin was applied in the roots, in the presence and in the absence of pathogen.

Changes in the enzymatic activity of NR, NOS, and POD in response to exogenous melatonin. The enzymatic activity of NR, NOS, and POD in the leaves of the rice plants was determined with commercial ELISA kits. The plant roots were submerged in hydroponic solutions containing 0 μ g/ml of melatonin, and the plant leaves were treated with water in the absence of pathogen as the control experiment. When the plant roots were not treated with melatonin and the leaves were not inoculated with the pathogen, the maximum enzymatic activity of NR (124.38 U/g FW) was detected at 12 h. Then, NR activity decreased (Fig. 4A). When the leaves were infected by the pathogen in the absence of melatonin, the enzymatic activity of NR increased from 0 to 24 h, achieving the maximum enzymatic activity (164.24 U/g FW) at 24 h (Fig. 4B). For the NOS assay, the maximum enzymatic activity (575.63 μ U/g FW) was detected at 0 h in the control experiment. Then, NOS activity decreased over time (Fig. 4C), which is consistent with the concentration of melatonin in the leaves (Fig. 3A). On the other hand, when the leaves were infected by the pathogen in the absence of melatonin, the enzymatic activity of NOS increased from 0 to 24 h, achieving the maximum enzymatic activity (749.94 μ U/g FW) at 24 h (Fig. 4D). These results indicated that infection with the pathogen produces a relevant

Fig. 1. Endogenous melatonin is induced by *Xanthomonas oryzae* pv. *oryzae* infection. The content of endogenous melatonin in rice leaves and roots was measured by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). Rice leaves were inoculated with PXO99 at an optical density at 600 nm of 1.0, whereas water was used for the control plants. Melatonin was extracted from leaves and roots at 0 and 24 h and analyzed via HPLC-MS/MS. Significance at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ levels. FW, fresh weight.

Fig. 2. Exogenous melatonin improves rice resistance against *Xanthomonas oryzae* pv. *oryzae* infection. **A**, Disease symptoms in melatonin-treated plants and control plants after inoculation with *X. oryzae* pv. *oryzae*. The symptoms were measured 7 days after the inoculation. Five-week rice plants were cultivated in hydroponic cultures with different melatonin concentrations (0 and 20 μ g/ml). The leaves were inoculated with *X. oryzae* pv. *oryzae* for the disease resistance assay. **B**, Lesion length of leaves in melatonin-treated plants and control plants at 7 days after inoculation. **C**, Population of PXO99 in melatonin-treated plants and control plants in 6-cm² Petri dishes at 0 and 7 days after inoculation. Bar = 2 cm. Significance at * $P < 0.05$ and **** $P < 0.0001$ levels.

increase in NR and NOS activity. As shown in Figure 4E, the changes in POD activity were similar to the changes in NOS activity when the plants were treated with melatonin in the absence of pathogen infection. In this sense, the enzymatic activity of POD and NOS increased over time, achieving a maximum at 24 h (Fig. 4D and F). On the other hand, when the plant roots were treated with melatonin and the leaves were inoculated with the pathogen, the maximum enzymatic activity of NOS (789.94 μ U/g FW), which was detected at 24 h, was significantly higher than that detected when the leaves were inoculated with the pathogen in the absence of melatonin (771.23 μ U/g FW). Thus, the obtained results demonstrated that the enzymatic activity of NR, NOS, and POD increased in the presence of exogenous melatonin.

H₂O₂ was induced by exogenous melatonin. The concentration of H₂O₂ in the leaves was determined with a commercial kit. The obtained results indicated that the H₂O₂ levels of the control plants did change significantly from 0 to 24 h, showing an average content of 6.40 μ mol/g FW H₂O₂ (Fig. 5A). On the other hand, when the leaves were infected with the pathogen and the roots were submerged in hydroponic cultures in the absence of melatonin, the maximum H₂O₂ content was 9.06 μ mol/g FW and was detected at 24 h, which may be related to the changes in melatonin concentration in the same leaves (Fig. 3A). Treating the plant roots with melatonin in the absence of pathogen infection allowed a maximum content of 9.23 μ mol/g FW H₂O₂, which was detected at 24 h (Fig. 5B). Then, H₂O₂ levels decreased, which is proportional to the variations in the enzymatic activity of POD in the same rice leaves (Fig. 4D). When the plant roots were treated with melatonin and the leaves inoculated with the pathogen, the maximum H₂O₂ content (9.11 μ mol/g FW), which was detected at 12 h, was slightly lower than that detected in plant roots with melatonin in the absence of pathogen infection. These results indicated that both the addition of exogenous melatonin and inoculation with the pathogen produce a relevant increase in the concentration of H₂O₂.

Melatonin induced the expression of key genes of the salicylic acid and jasmonic acid signaling pathways. To investigate the mechanistic role of melatonin on rice innate immunity, we determined the messenger RNA (mRNA) expression of the key genes in the SA- and jasmonic acid (JA)-mediated signaling pathways. The dissociation curves of defense relevant genes are shown in Supplementary Figure S4. As shown in Figure 6A, both *EDS1* and *NPR1* were upregulated at 12 h. It must be noticed that the mRNA level of *NPR1*, a key regulator in the SA signaling network, was 10 times higher than those at 0 h. In the JA pathway, *LOX1*, *LOX2*, *AOS*, and *PDF1.2* were also upregulated at 12 h (Fig. 6B). The mRNA

levels of *LOX1* and *AOS2* were 20 times higher than those at 0 h. Similarly, the mRNA level of *PDF1.2*, a JA-inducible plant defensin marker gene, was 15 times higher than those at 0 h. These results suggest that SA and JA pathways may be activated after the addition of exogenous melatonin.

Melatonin induced the expression of PRs. Because *NPR1* is a common activator of PRs, we decided to determine the mRNA levels of PRs in the melatonin-treated plants. We found that all the PRs that are present in rice plants were upregulated after 12 h of treatment in leaves and in roots of the treated plants (Fig. 7). The mRNA levels of *PR1b*, *PR8a*, and *PR9* were 20 times higher at 12 h than those at 0 h, whereas the mRNA levels of *PR8b* and *PR8c* were 10 times higher at 12 h than those at 0 h. It was also observed that the mRNA levels of *PR6a*, *PR4a*, and *PR2* were 5 times higher at 12 h than those at 0 h. Our results suggest that PRs were induced by melatonin.

Discussion

Melatonin was identified in plants more than two decades ago (Dubbels et al. 1995; Hattori et al. 1995). Recently, numerous studies have revealed that melatonin is widely distributed in many species, and its contents vary from picograms to micrograms per gram tissue weight (Kolár and Machackova 2005; Posmyk and Janas 2009). In morning glory, the melatonin content in roots was reported to be higher than that in leaves (Van Tassel et al. 2001). Similarly, our results indicated that the content of melatonin is also higher in the root than in the leaves of rice plants.

On the other hand, previous reports confirmed that the concentration of melatonin is easily affected by pathogens. The increase in melatonin levels in watermelon and other cucurbits was found to be related to resistance against diverse filamentous pathogens (Mandal et al. 2018). Moreover, the application of exogenous melatonin to plant roots increased host defense responses against pathogen infection (Liu et al. 2018; Zhang et al. 2018a; Zhang et al. 2017). A key enzyme in melatonin production was reported to be upregulated in *X. oryzae* pv. *oryzae*-infected leaves of rice (Wei et al. 2016). In this work, it was observed that the concentration of melatonin was increased in both leaves and root tissues and that the content of melatonin in roots was higher than that in leaves challenged with *X. oryzae* pv. *oryzae* (Fig. 1). Thus, we can speculate that roots may be an important place for melatonin synthesis in plants. However, how the infected leaves induced melatonin biosynthesis in roots warrants further investigation.

Recently, melatonin was shown to work as a positive regulator in plant innate immunity against pathogen infection (Fan et al. 2018). The application of exogenous melatonin improved apple defense

Fig. 3. Concentration of endogenous melatonin after application of exogenous melatonin. Five-week rice plants were cultivated in hydroponic cultures with melatonin (0 and 20 μ g/ml). Plants leaves were inoculated with PXO99 at an optical density at 600 nm of 1.0. The melatonin content in plant leaves was determined at 0, 1, 3, 12, and 24 h. **A**, Plant roots were submerged in hydroponic cultures without melatonin, or **B**, plant roots were submerged in hydroponic cultures with 20 μ g/ml of melatonin. Significance at $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ levels. FW, fresh weight.

against *Diplocarpon mali*, the causal agent of Marssonina apple blotch (Yin et al. 2013), and increased disease resistance against *PstDC3000* infection in *Arabidopsis* and tobacco (Lee et al. 2014). Similarly, we found that the application of exogenous melatonin, only by treating the roots, can increase rice resistance (Fig. 2). To

compare between the effects of the addition of melatonin on roots and leaves, we sprayed the melatonin solution on rice leaves, and we found that the lesion length in the treatment group was similar to that in the control group. However, when the roots were submerged in a solution containing melatonin, 20 $\mu\text{g/ml}$, the disease

Fig. 4. Changes in the enzymatic activity of nitrate reductase (NR), nitric oxide synthase (NOS), and peroxidase (POD) in melatonin-treated plants. Five-week rice plants were cultivated in hydroponic cultures with melatonin (0 and 20 $\mu\text{g/ml}$). Plants leaves were inoculated with PXO99 at an optical density at 600 nm of 1.0. The activity of NOS and POD in plant leaves was quantified at 0, 1, 3, 12, and 24 h. **A**, The activity of NR in leaves when plant roots were submerged in hydroponic cultures without melatonin or **B**, when plant roots were submerged in hydroponic cultures with 20 $\mu\text{g/ml}$ of melatonin. **C**, The activity of NOS in leaves when plant roots were submerged in hydroponic cultures without melatonin or **D**, when plant roots were submerged in hydroponic cultures with 20 $\mu\text{g/ml}$ of melatonin. **E**, The activity of POD in leaves when plant roots were submerged in hydroponic cultures without melatonin or **F**, when plant roots were submerged in hydroponic cultures with 20 $\mu\text{g/ml}$ of melatonin. Significance at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ levels. FW, fresh weight.

resistance of the rice plants significantly increased. It must be noted that previous results indicated that melatonin at 20 $\mu\text{g/ml}$ has no obvious antibacterial effects on the proliferation of *X. oryzae* pv. *oryzae* (X. Chen et al. 2018). Thus, our results indicated that treating the root with melatonin may be an effective way to improve plant disease resistance.

It is widely known that NO is one of the most important signaling molecules involved in the regulation of plant defense (Del Rio 2015; Turkan 2018). In this sense, Shi et al. (2015a) reported that melatonin induced NO production and, subsequently, high NO concentration upregulated the mRNA levels of SA-related genes, which are essential for the innate immunity of *Arabidopsis* against *Pst*DC3000 infection. Similarly, we observed in this study that the enzymatic activity of NR and NOS was upregulated in melatonin-treated plants, detecting the maximum enzymatic activity of NR and NOS when the rice plants were challenged with *X. oryzae* pv. *oryzae* in the presence of exogenous melatonin (Fig. 4A and C). It has been reported that in some plant development stages, NO might act as an upstream signal of diverse plant hormones (Mur et al. 2013). However, it remains to be confirmed whether this response activates innate immunity and increases host resistance.

Previous reports found in the literature demonstrated that POD plays a significant role in defense systems of plants against bacterial infections (Wang et al. 2013). In an antioxidant defense system, POD is an enzyme that catalyzes conversion of the excess H_2O_2 into H_2O and O_2 (Teixeira et al. 2004). The enzymatic activity of POD was increased in apple leaves infected by pathogen *D. mali* (Yin et al. 2013). When the fruit was treated with exogenous melatonin together with pathogen infection, the enzymatic activity of POD was also increased in kiwifruit (Liang et al. 2018). When lychee fruit was treated with exogenous melatonin, the mRNA levels of four antioxidant genes were found to be upregulated (Zhang et al. 2018c). Similarly, we found that the enzymatic activity of POD was increased by exogenous melatonin in rice plants. The reported results indicate that melatonin may improve plant antioxidant capacity by modulating the activity of redox enzymes.

Hydrogen peroxide is an important member of ROS and plays a crucial role in a variety of defense responses against biotic stress in higher plants (Wei et al. 2018a). It was observed that ROS production was induced when plants were challenged with melatonin (Z. Chen et al. 2017, 2018; Gong et al. 2017). It was reported that H_2O_2 exerts strong antibacterial effects on the growth of bacterial pathogens (Ohlin et al. 2018). In *Arabidopsis*, a rapid induction of H_2O_2 was

Fig. 5. Concentration of H_2O_2 in melatonin-treated plants. Five-week rice plants were grown in hydroponic cultures with melatonin (0 and 20 $\mu\text{g/ml}$). Plants leaves were inoculated with PX099 at an optical density at 600 nm of 1.0. The content of H_2O_2 in plant leaves was quantified at 0, 1, 3, 12, and 24 h. **A**, Plant roots were submerged in hydroponic cultures without melatonin, or **B**, plant roots were submerged in hydroponic cultures with 20 $\mu\text{g/ml}$ of melatonin. Significance at $*P < 0.05$ level. FW, fresh weight.

Fig. 6. Quantitative reverse transcription PCR (qRT-PCR) analysis of key genes in the salicylic acid (SA) and jasmonic acid (JA) pathways. **A**, Expression analysis of *EDS1* and *NPR1* in the SA pathway. **B**, Expression analysis of *LOX1*, *LOX2*, *AOS2*, and *PDF1.2* in the JA pathway. Five-week rice plants were cultured in hydroponic cultures with melatonin (0 and 20 $\mu\text{g/ml}$). The total RNA of rice leaves was extracted at 0 and 12 h, and the relative gene expression was quantified by qRT-PCR. The relative transcriptions were normalized according to the transcription level of *OsActin*. Significance at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ levels.

observed in leaves under *Pst*DC3000 infection (Lee and Back 2017). In apple, the level of H_2O_2 in melatonin-treated leaves was similar to that in melatonin- and pathogen-treated leaves (Yin et al. 2013). Several reports indicated that melatonin and POD act as free radical scavengers (Tamura et al. 2013; Teixeira et al. 2004; Wang et al. 2013). In this work, the induction of H_2O_2 was also observed in the leaves of rice plants after challenge with *X. oryzae* pv. *oryzae* and in the presence of melatonin. Thus, our results suggested that melatonin and POD may modulate ROS levels by maintaining steady-state intracellular H_2O_2 concentrations. Melatonin might act as a balance signaling molecule between ROS and NO (Arnao and Hernández-Ruiz 2019b; Mukherjee 2019). Recently, the first phyto-melatonin receptor (PMTR1), which is able to regulate stomatal closure, was identified in *A. thaliana* (Wei et al. 2018a). This finding of PMTR1 opens a door for melatonin to be considered as a new plant hormone (Arnao and Hernández-Ruiz 2019a). Whether melatonin-induced BB resistance and H_2O_2 production in rice depend on PMTR1-mediated signaling pathways must be further explored.

It is well known that pathogen-associated molecular patterns, pattern-triggered immunity, and effector-triggered immunity play essential roles in plant–pathogen interactions (Klessig et al. 2018; Li et al. 2018; Proietti et al. 2018). Among pattern-triggered immunity and effector-triggered immunity, endogenous SA and JA, with a concomitant upregulation of a variety of defense genes, play crucial roles in plant innate immunity (Hickman et al. 2017; Kaur et al. 2015; Klessig et al. 2000). In the SA pathway, *NPR1* is a key regulator in signaling transduction (Ding et al. 2018; Zhang et al. 2018b). *PR1*, *PR2*, and *PR5* are marker genes of the SA pathway in plants (Uknes et al. 1992). In the JA pathway, *LOX* and *AOS* genes are involved in JA biosynthesis, and *PDF1.2*, *PR3*, and *PR4* are marker genes of the JA pathway (Farmer and Ryan 1992; Le Berre et al. 2017; Thatcher et al. 2018). Peroxidase gene *PR9* is known to be induced by *X. oryzae* pv. *oryzae* infection (Chittoor et al. 1997). Lee and co-workers reported that the mRNA levels of *PR1*, *EDS1*, and *NPR1* were upregulated in *Arabidopsis* under melatonin treatment (Lee and Back 2017; Shi et al. 2015a). In agreement with these previous results, the mRNA levels of *PR1*, *EDS1*, and *NPR1* in rice plants were upregulated under melatonin treatment. Moreover, we also observed that three marker genes (*PDF1.2*, *PR3*, and *PR4*) of the JA pathway were upregulated in the melatonin treatments. These results indicated that melatonin may increase the rice resistance by activating the SA/JA pathways.

In summary, we have investigated for the first time the effect of melatonin on the disease resistance of rice against *X. oryzae* pv. *oryzae* infection. Endogenous melatonin concentration in rice plants was determined by HPLC-MS/MS. The application of exogenous melatonin induced the accumulation of H_2O_2 and NO, which enhanced the expression of key regulators in the SA and JA signaling pathways and elicited the expression of PRs. Overall, this study confirmed that exogenous melatonin increases rice resistance and may lead to the

development of novel cultivation strategies with dual resistance to pathogens.

Acknowledgments

We thank Jialei Wang for her excellent technical assistance at the central lab of Jiangsu Academy of Agricultural Sciences.

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Fig. 7. Quantitative reverse transcription PCR (qRT-PCR) analysis of defense-related genes in rice plants after treatment with melatonin. Five-week rice plants were cultured in hydroponic cultures with melatonin (0 and 20 μ g/ml). The total RNA of rice leaves was extracted at 0 and 12 h, and the relative gene expression was quantified by qRT-PCR. The relative transcriptions were normalized according to the transcription level of *OsActin*. Significance at $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ levels.

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