Eur J Plant Pathol (2021) 160:707–716
https://doi.org/10.1007/s10658-021-02279-8

Defense-related hormone signaling coordinately controls the role of melatonin during *Arabidopsis thaliana-Pseudomonas syringae* interaction

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Accepted: 8 April 2021 / Published online: 26 April 2021

Abstract Increasing evidence suggest that melatonin (*N*-acetyl-5-methoxytryptamine), an indolic compound identified from the pineal gland of mammals, regulates plant disease resistance. Here, we show that melatonin promoted susceptibility of salicylic acid (SA)-deficient *Arabidopsis* plants to the virulent bacterium *Pseudomonas syringae*, but enhanced resistance of jasmonic acid (JA)-insensitive mutants, ethylene (ET)-insensitive mutants, and abscisic acid (ABA)-biosynthetic mutants. However, melatonin had no effects on wild type *Arabidopsis* plants defending against *P. syringae*. In wild type *Arabidopsis* leaves, melatonin enabled to elevate endogenous SA and ABA levels and reduced JA and JA-isoleucine accumulation. In addition, melatonin induced the transcripts of SA-dependent pathogenesis-related protein 1 and JA/ET-dependent plant defensin 1.2. Furthermore, melatonin could affect neither pathogen-associated molecular pattern-triggered immunity nor avirulent effector-triggered immunity. Since ABA and JA/ET signaling antagonize SA-dependent disease resistance, our results thus clarify that defense-related hormone signaling, but not basal immune events, cooperatively determine the destiny of melatonin during *Arabidopsis thaliana-P. syringae* interaction.

Keywords Melatonin · SA · ABA · JA · *Arabidopsis thaliana* · *Pseudomonas syringae*

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Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), an indolic compound, was first identified from the bovine pineal gland in 1950s (Lerner et al. 1958; Lerner et al. 1959). In animals, melatonin regulates a series of physiological processes, including circadian rhythms, sleep, mood, seasonal reproduction, antioxidant, and innate immunity (Zawilska et al. 2009; Galano et al. 2011; Escames et al. 2012; Calvo et al. 2013; Reiter et al. 2014). Since melatonin was discovered in plants in 1995 (Dubbels et al. 1995; Hattori et al. 1995), its biological importance has drawn the extensive attention of plant biologists. Accumulating data suggest that melatonin functions as a positive regulator in various processes of plant growth and development, including seed germination, seedling growth, rooting, flowering, fruit ripening, and nutrient absorption (Arnao and Hernández-Ruiz 2014; Arnao and Hernández-Ruiz 2015; Nawaz et al. 2015). Melatonin has also been discovered to act as antioxidant and senescence retardation in plants (Park et al. 2013). Moreover, melatonin can improve plant adaptation to abiotic stresses, including salinity, drought, heavy metal, extreme temperature, radiation, and chemical

stimulus (Tan et al. 2007; Li et al. 2012; Tiryaki and Keles 2012).

The disease resistance mechanism in plants has been well established and described as a two-layer immune system (Chisholm et al. 2006; Jones and Dangl 2006). On the one hand, the surface membrane-localized receptors sense pathogen-associated molecular patterns (PAMPs) to activate PAMP-triggered immunity (PTI), such as callose deposition, reactive oxygen species (ROS) burst. On the other hand, the intracellular resistance proteins recruit those entered bacterial avirulent effectors to induce the resistance-enhanced effector-triggered immunity (ETI), which is often accompanied with programmed cell death at infected tissues.

Some phytohormones have been characterized in disease defense (Dong 1998). For example, salicylic acid (SA) promotes plant resistance to biotrophic or hemi-biotrophic pathogens, whereas the combination of jasmonic acid (JA) and ethylene (ET) signaling induces resistance against necrotrophic pathogens (Dong 1998; Thomma et al. 1998). Meanwhile, abscisic acid (ABA) has also been suggested to participate in plant disease defense (Asselbergh et al. 2008; Ton et al. 2009; Cao et al. 2011).

Interestingly, the role of melatonin in plant defense responses to pathogen attack has been recently demonstrated. For example, exogenous melatonin improves resistance of apple leaves to the fungus *Diplocarpon mali*-caused Marssonina apple blotch (Yin et al. 2013). The melatonin-mediated resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain (*Pst*) DC3000 in *Arabidopsis* depends on the phytohormone SA and ET signaling pathways (Lee et al. 2014). The serotonin *N*-acetyltransferase knockout *Arabidopsis* mutants reduce melatonin and SA levels and enhance susceptibility to the *Pst*DC3000(*avrRpt2*) strain expressing the avirulent effector protein AvrRpt2 (Lee et al. 2015). In addition, the melatonin-induced nitric oxide production is important for immune response to *Pst*DC3000 in *Arabidopsis* (Shi et al. 2015).

In this study, we aim to investigate the potential mechanism that characterizes melatonin during plant-pathogen interaction. We will assess the role of melatonin in *Arabidopsis* plants responding to *Pst*DC3000 and examine the effects of exogenous melatonin on defense-related hormone signaling and basal immune events. This work contributes to improve our understanding for melatonin.

Materials and methods

Plants and pathogens

Bacterial strains used in this study include *Pst*DC3000 and *Pst*DC3000(*avrRpt2*) strain (Chen et al. 2000). All the used strains were cultivated on King's B (KB) medium (29 g Bacto™ Proteose peptone, 1.5 g K_2HPO_4, 0.74 g $MgSO_4$, 15 g Bacto™ agar and 8 g glycerol L^{-1}) supplemented with 100 μg mL^{-1} rifampicin at 28 °C. *Arabidopsis coi1–1* (Chen et al. 2020), *ein2–1* (CS65994), *sid2–2* (CS65996), and *NahG* transgenic line expressing a salicylate hydroxylase (Mei et al. 2016) that are all in the Columbia background (Col-0) were grown at a 65% relative humidity and a 12 h photoperiod at 23 °C. The homozygosity of *ein2–1* and *sid2–2* mutants was identified as previous reported (Tsuda et al. 2009).

Plant treatment and pathogenicity analysis

Four-weeks (wk) old *Arabidopsis* plants were sprayed with distilled water (DMSO, as control) and 10 μM or 50 μM melatonin (Dissolved in DMSO and further diluted with distilled water), respectively. After 4 h, treated plants were syringe-inoculated with a 10^6 colony-forming units (cfu)/mL suspension of virulent *Pst*DC3000 diluted in 10 mM $MgCl_2$. At 3 days post-inoculation (dpi), the bacterial populations in *Pst*DC3000-infected leaves were monitored as previously reported (Tan et al. 2014).

Hormone measurements

For hormonal analyses, 4-wk-old wild type *Arabidopsis* leaves were sprayed with distilled water and 50 μM melatonin, respectively. After 4 h, treated leaves were separately collected. Samples were prepared and the hormonal analysis for SA, JA, JA-Ile, and ABA was performed by HPLC-MS as previously reported (Pastor-Fernández et al. 2020; Xiong et al. 2014).

Aniline blue staining

To detect the effects of melatonin treatment on bacterial pattern-induced callose deposition, 4-wk-old wild type *Arabidopsis* leaves were syringe-infiltrated with 1 μM flg22, a 22 amino acid-contained bacterial flagellin peptide dissolved in distilled water (Zipfel et al. 2004).

After 24 h, syringe-infiltrated leaves were collected and stained with aniline blue (Hauck et al. 2003). Callose signal was observed under ultraviolet light with a fluorescence microscope.

Oxidative burst

For bacterial pattern-induced ROS burst, 4-wk-old health leaves from wild type *Arabidopsis* plants were sliced into approximately 1 mm strips and were further incubated in distilled water in 96-well plates for 12 h (Tan et al. 2014). 1 μM flg22 supplemented with 20 mM luminol (Sigma) and 1 μg horseradish peroxidase (Sigma) were added to 96-well plates. Luminescence was detected at once with a Luminometer (Promega).

Trypan blue staining

For detecting bacterial avirulent effector protein-induced cell death, 4-wk-old wild type Col-0 plants were inoculated with a 10^6 cfu/mL suspension of *Pst*DC3000(*avrRpt2*). After 24 h, inoculated wild type Col-0 leaves were collected and further stained with trypan blue as reported (Navarro et al. 2008).

Quantitative RT-PCR

Arabidopsis total RNA was extracted with RNeasy Plant Mini kit (Qiagen). RNA samples were digested with DNase Turbo DNAfree (Promega). 1 μg RNA of each samples was used for reverse transcription with SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using an ABI 7500 Fast RT-PCR instrument and SYBR Premix Ex Taq kit (TaKaRa, Otsu, Shiga, Japan). *ACTIN2* (Li et al. 2010) was used to standardize *PR1* and *PDF1.2*. The primers are listed in Supplementary Data Table S1.

Results

Melatonin does not alter bacterial resistance in wild type *Arabidopsis* plants

The virulent bacterium strain *Pst*DC3000 is a pathogen of *Arabidopsis* plants. Previously, it has been suggested that melatonin regulates *Arabidopsis* resistance to *Pst*DC3000. To investigate the undetermined role of melatonin during *Arabidopsis-P. syrinage* interaction, wild type *Arabidopsis* (Col-0) was separately pretreated with distilled water (as control) or 50 μM melatonin, and treated leaves were further syringe-inoculated with *Pst*DC3000. Pathogenicity analysis showed that no difference was observed either on *Pst*DC3000-induced leaf chlorosis or *Pst*DC3000 numbers between water and melatonin treatment at 3 dpi (Fig. 1A and B), indicating that melatonin has no effects on the *Arabidopsis-Pst*DC3000 system.

To clarify this, we further tested the role of 10 μM melatonin that is previously reported to successfully restrict the growth of *Pst*DC3000 in wild type Col-0 leaves (Lee et al. 2014). Unfortunately, 10 μM melatonin could still not alter *Pst*DC3000-induced leaf chlorosis and bacterial numbers (Fig. 1S). In addition, we also detected the effects of melatonin on Ws-2 ecotype *Arabidopsis* plants. The result showed that melatonin did not affects *Pst*DC3000-induced leaf chlorosis and bacterial numbers on Ws-2 (Fig. 1C and D).

Impairment of JA signaling, ET signaling, or ABA biosynthesis confers melatonin to induce bacterial resistance

SA, JA, ET, and ABA are important factors to regulate plant disease resistance. To detect the possible mechanism that affects the function of melatonin, we next performed the pathogenicity analysis by using these defense-related hormone signaling/biosynthesis *Arabidopsis* mutants.

For SA signaling, *sid2–2* mutants (Fig. 2S) and *NahG* transgenic lines were pretreated with distilled water and 50 μM melatonin, respectively, and were further inoculated with *Pst*DC3000. At 3 dpi, melatonin treatment displayed severe chlorosis on *sid2–2* and *NahG* leaves compared with water treatment (Fig. 2A). In addition, bacterial numbers increased by almost five-fold, compared to water treatment (Fig. 2B). These results indicate that SA deficiency leads to the negative role of melatonin in bacterial resistance.

For JA, ET, and ABA signaling, JA-insensitive *coi1–1* mutants, ET-insensitive *ein2–1* mutants (Fig. 2S), and ABA-deficient *aba2–1* mutants were treated with water and melatonin and inoculated with virulent *Pst*DC3000. At 3 dpi, melatonin treatment showed severe leaf chlorosis on these *Arabidopsis* mutants compared with water treatment (Fig. 2C). Bacterial numbers under melatonin treatment decreased by almost five-fold compared to

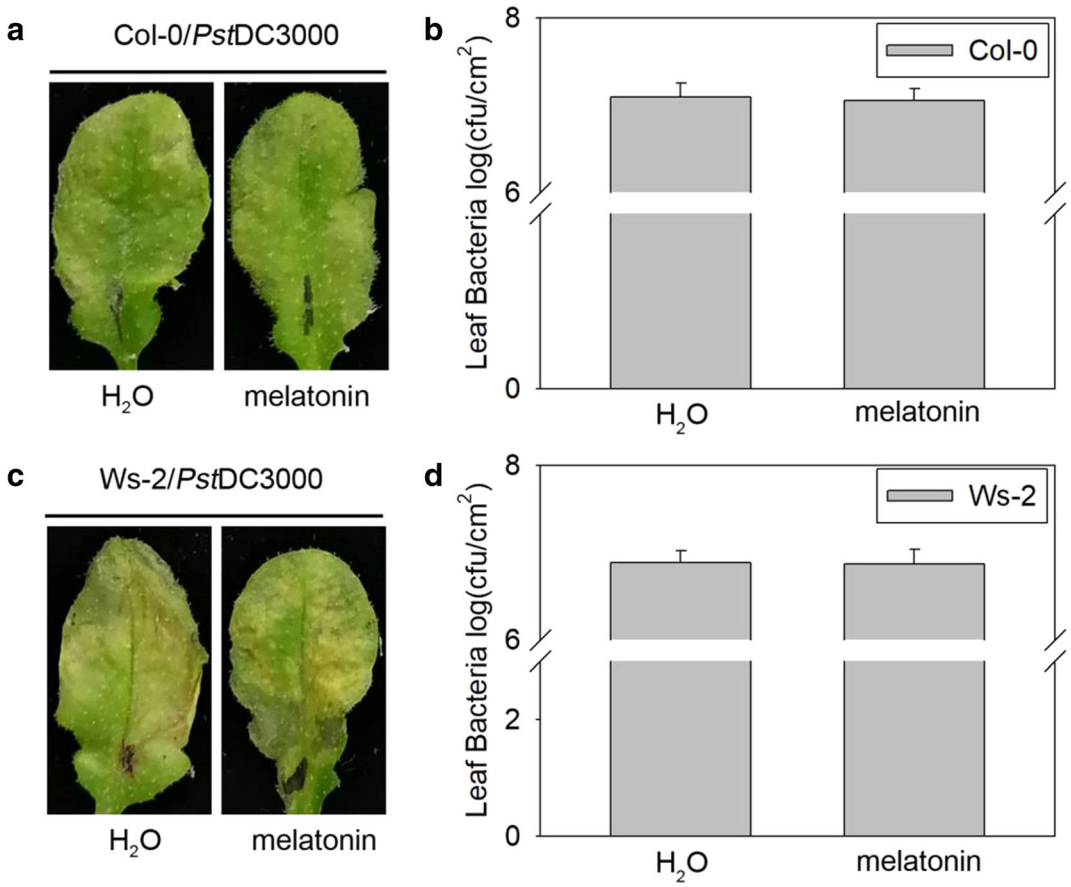

Fig. 1 Melatonin treatment does not alter resistance to virulent bacterium *Pst*DC3000 in wild type Col-0 and Ws-2 *Arabidopsis* plants. Four-wk-old *Arabidopsis* plants were sprayed distilled water (H_2O) or 50 μM melatonin for 4 h prior to inoculation with *Pst*DC3000. The pathogenicity analysis was performed at 3 dpi. (A and C) The disease symptoms in *Pst*DC3000-inoculated *Arabidopsis* leaves. (B and D) The growth of *Pst*DC3000 in *Arabidopsis* leaves. Error bars indicate the standard error of eight independent samples. These experiments were performed at least four times

water treatment (Fig. 2D). These results indicate that disruption of JA, ET, or ABA signaling induces the positive role of melatonin in bacterial resistance.

Melatonin induces SA and ABA biosynthesis but represses JA accumulation

We next examined the effects of melatonin on biosynthesis of SA, ABA, JA, and JA-Ile. Wild type Col-0 plants were treated with distilled water or 50 μM melatonin. Treated leaves were used for detection of the level of the phytohormones. The result showed that the average SA content of fresh leaves under distilled water and melatonin treatment was 516.20 and 723.40 ng/g, respectively (Fig. 3A). The average JA content of fresh leaves under distilled water and melatonin treatment was 59.80 and 22.80 ng/g, respectively (Fig. 3B). The average JA-Ile content of fresh leaves under distilled water and melatonin treatment was 10.20 and 3.15 ng/g, respectively (Fig. 3C). The average ABA content of fresh leaves under distilled water and melatonin treatment was 12.42 and 48.96 ng/g, respectively (Fig. 3D). These results indicate that melatonin regulates biosynthesis of defense-related hormones.

In *Arabidopsis* plants, pathogenesis-related protein 1 (PR1) and JA/ET-dependent plant defensin PDF1.2 are well known markers, whose transcript may separately reflect SA and JA/ET signaling. To better understand the function of melatonin on defense-related hormone signaling, we examined the transcriptional profiling of SA-dependent *PR1* and JA/ET-dependent *PDF1.2* in melatonin-treated wild type Col-0 plants. Compared to distilled water treatment, melatonin treatment separately increased the expression of *PR1* (Fig. 3E) and *PDF1.2* (Fig. 3F) by 6.34- and 1.26-fold.

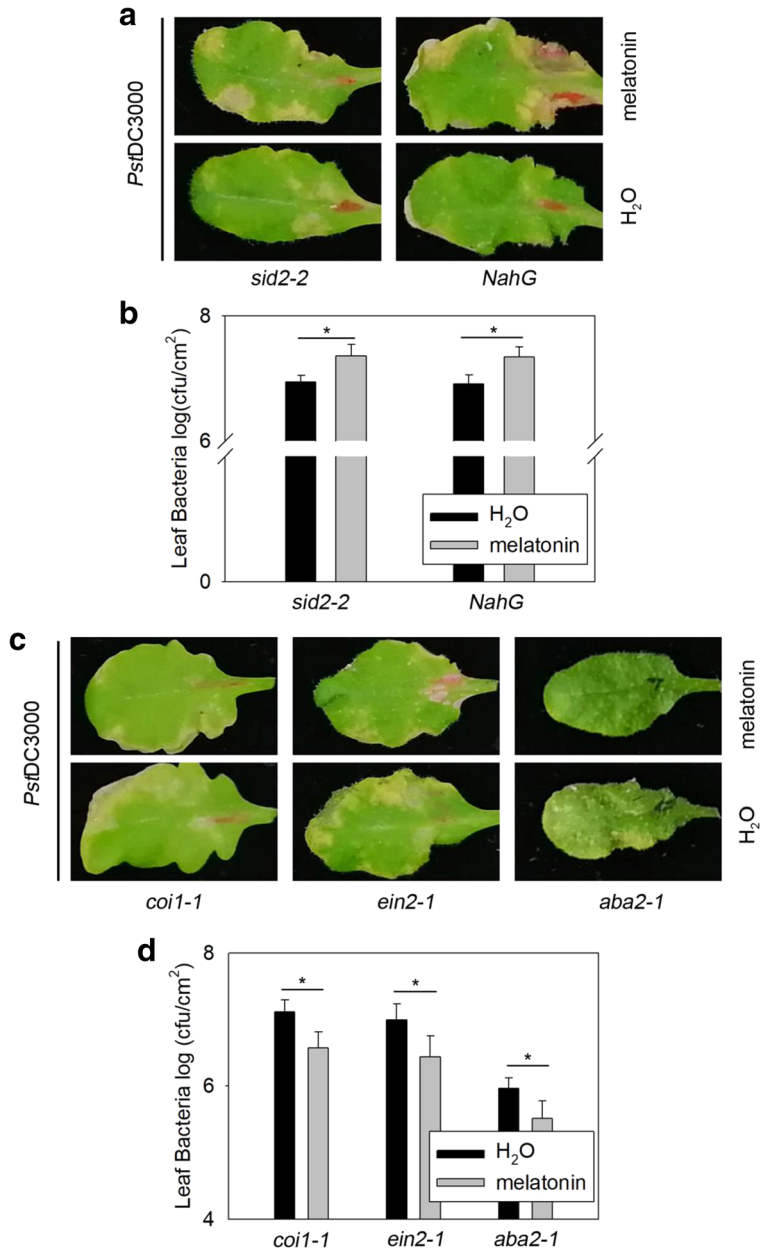

Fig. 2 Melatonin treatment alters resistance to virulent *Pst*DC3000 in SA-deficient *sid2–2* and *NahG* plants, JA-insensitive *coi1–1* mutants, and ET-insensitive *ein2–1* mutants, and ABA-deficient *aba2–1* mutants. Four-wk-old *Arabidopsis* plants were sprayed distilled water (H_2O) or 50 μM melatonin for 4 h prior to inoculation with *Pst*DC3000. The pathogenicity analysis was performed at 3 dpi. (A) and (C) The disease symptoms in *Pst*DC3000-inoculated *Arabidopsis* leaves. (B) and (D) The growth of *Pst*DC3000 in *Arabidopsis* leaves. FW means fresh weight. Error bars indicate the standard error of eight independent samples. Asterisks indicate a significant difference (*$p < 0.05$). These experiments were performed at least four times

Melatonin has no effects on PTI and ETI

PTI and ETI are the two basal immune strategies to defend against pathogen attack in plants (Chisholm et al. 2006; Jones and Dangl 2006). To investigate the effects of melatonin on PTI, wild type Col-0 leaves were separately treated with distilled water or 50 μM melatonin, and treated leaves were further syringe-infiltrated with flg22, a well-known bacterial PAMP that may induce cell wall enhancement of some *Arabidopsis* plants (Zipfel et al. 2004). Aniline blue staining showed that callose fluorescence in stained leaves had no observed difference between distilled water and melatonin treatment (Fig. 4A). In addition, we also detected PAMP-triggered ROS burst, another events among PTI responses, and found that pretreatment with melatonin did not affect flg22-induced transient H_2O_2 levels in wild type Col-0 plants, compared with distilled water treatment (Fig. 4B). For ETI, trypan blue staining demonstrated that melatonin treatment did not alter

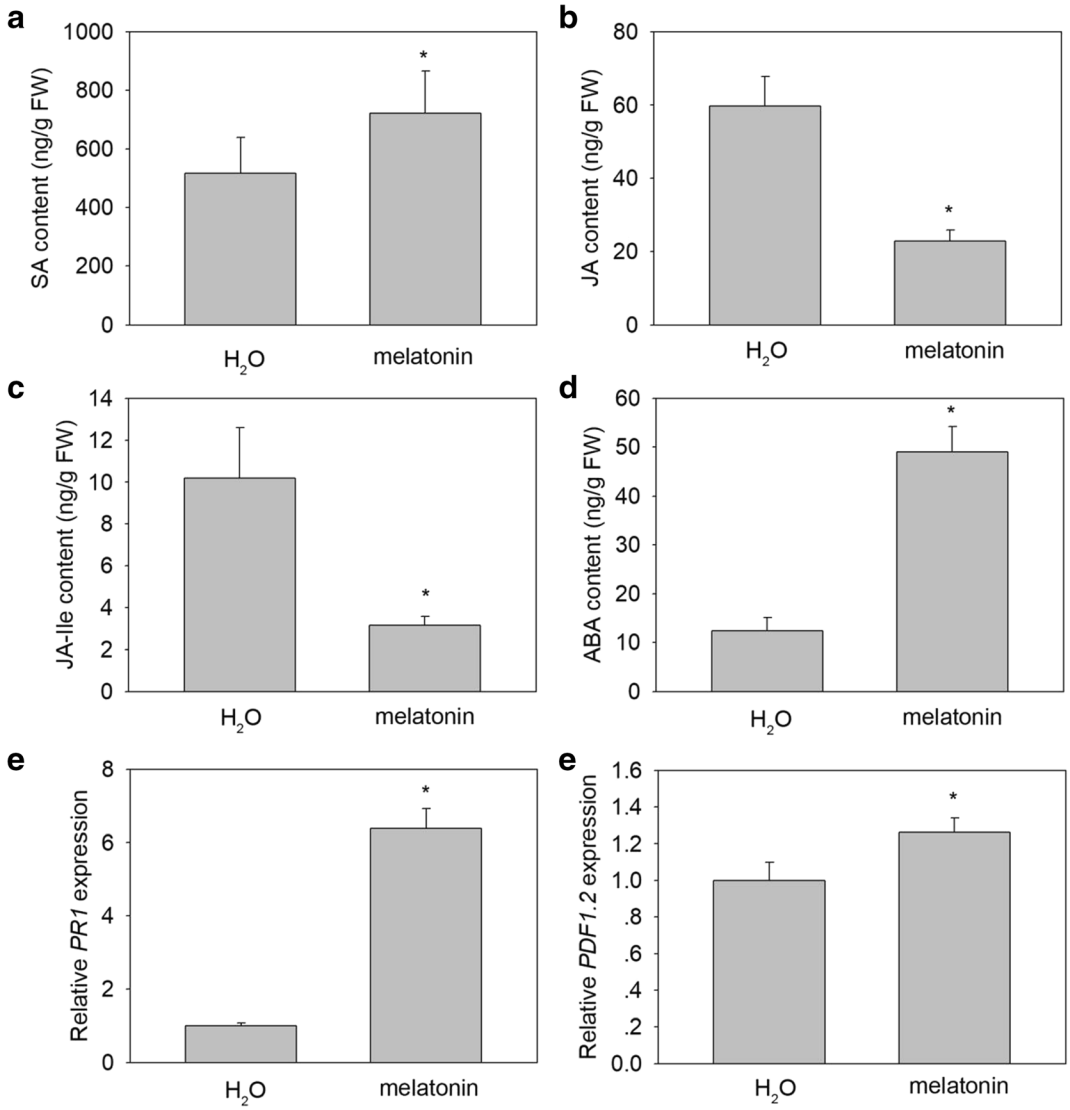

Fig. 3 Melatonin treatment affects defense-related hormone signaling in *Arabidopsis*. Four-wk-old wild type Col-0 *Arabidopsis* plants were sprayed with distilled water (H_2O) or 50 μM melatonin for 4 h. The contents of SA, JA, JA-Ile, and ABA and the transcripts of *PR1* and *PDF1.2* were measured. (A) SA content. (B) JA content. (C) JA-Ile content. (D) ABA content. (E) Relative *PR1* gene expression. (F) Relative *PDF1.2* gene expression. Error bars represent the standard error of three independent samples. Similar results were obtained in three independent experiments. Asterisks indicate a significant difference (*$p < 0.05$)

*Pst*DC3000(*avrRpt2*)-induced cell death in wild type Col-0 leaves (Fig. 4C). These results indicate that melatonin has no effects on either PTI or ETI in *Arabidopsis*.

Discussion

The *Arabidopsis-P. syringae* model system has been widely used for investigation of the mechanism of plant disease defense (Chisholm et al. 2006; Jones and Dangl 2006). Just as melatonin contributes to the innate immune response in animals (Calvo et al. 2013), the role of melatonin in plant defense responses has been recently reported. In this study, we demonstrated that melatonin had no ability to alter disease resistance of wild type *Arabidopsis* plants to *P. syringae*. By contrast, melatonin reduced bacterial resistance of SA-deficient *Arabidopsis* plants and enhanced bacterial resistance of JA-insensitive mutants, ET-insensitive mutants, and

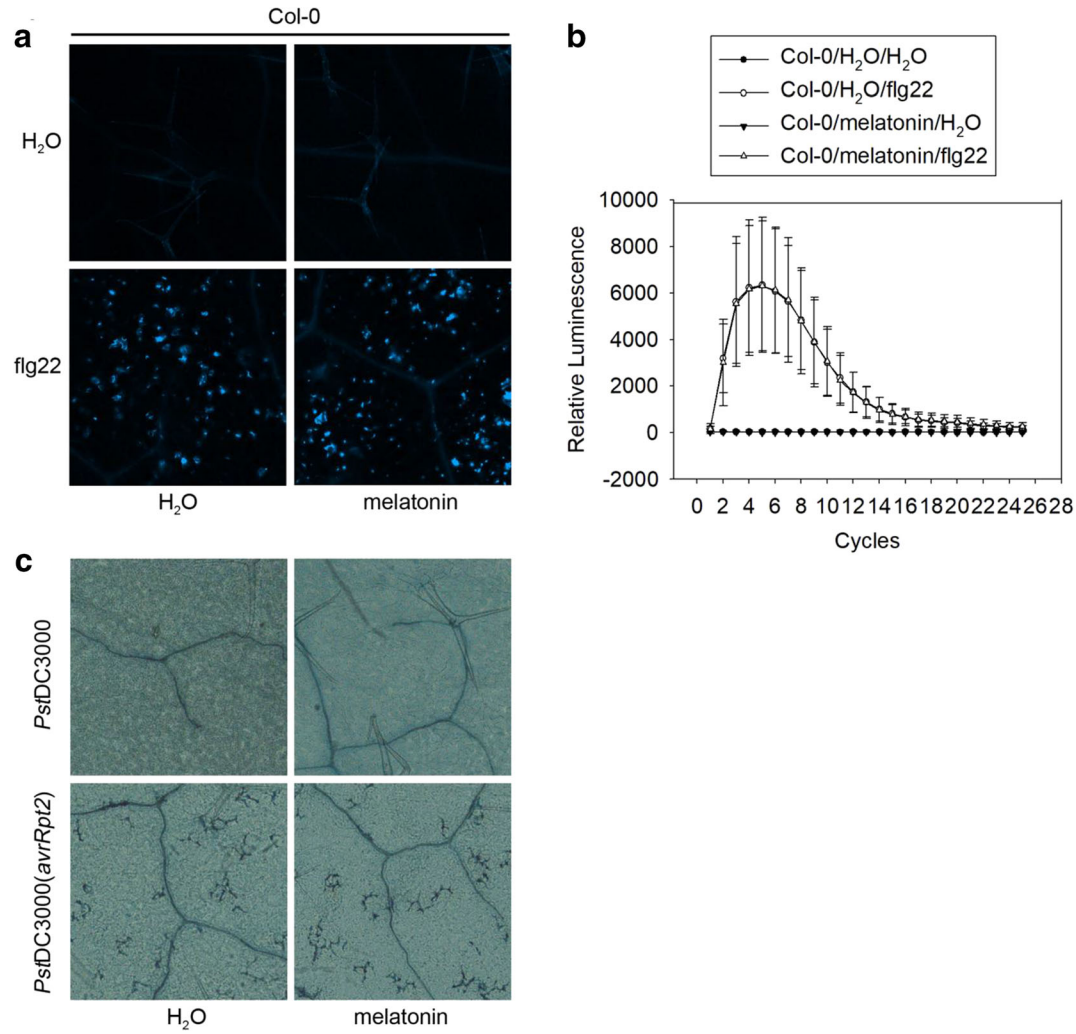

Fig. 4 Melatonin treatment does not affect PTI and ETI in *Arabidopsis*. Four-wk-old wild type Col-0 *Arabidopsis* plants were sprayed with distilled water (H_2O) or 50 μM melatonin for 4 h. (A) Flg22-induced callose deposition. Treated leaves treated were injected with 1 μM flg22 for 24 h, and aniline blue staining was performed. (B) Flg22-induced ROS burst. Treated Leaves were sliced into approximately 1 mm strips and were incubated in distilled water for 12 h. Distilled water (H_2O) and flg22-induced H_2O_2 levels were measured. Error bars indicate the standard error of eight independent samples. (C) Avirulent effector protein AvrRpt2-induced cell death. Treated Leaves were inoculated with virulent *Pst*DC3000 or avirulent *Pst*DC3000(*avrRpt2*) for 24 h, and trypan blue staining was performed. These experiments were performed at least four times

ABA-biosynthetic mutants. Further investigations showed that melatonin could elevate SA and ABA but reduce JA and JA-Ile accumulation. In addition, melatonin may up-regulate SA-dependent *PR1* and JA/ET-dependent *PDF1.2* but could not affect PTI and ETI.

Previously, it has been reported that 10 μM melatonin decreases the propagation of the virulent bacterial pathogen *Pst*DC3000 on wild type Col-0 *Arabidopsis* plants (Lee et al. 2014). However, our data demonstrated that pretreatment with 50 μM melatonin does not alter disease symptoms and bacterial numbers in the wild type Col-0 and Ws-2 plants syringe-inoculated with *Pst*DC3000. It seems to be possible that the role of exogenous melatonin in plant disease resistance is related to its content. Unfortunately, we still failed to detect an enhanced disease resistance in the wild type Col-0 plants when a low concentration of melatonin is used. Considering that Lee et al. performed pathogenicity analysis by spraying *Pst*DC3000 suspension on *Arabidopsis* leaves, we deduced that different method of bacterial inoculation might be a key determination for melatonin action in plant disease resistance.

Interestingly, our data uncovered that melatonin enhanced susceptibility to *Pst*DC3000 in SA-deficient *sid2–2* and *NahG* transgenic plants. Particularly, melatonin increased resistance to *Pst*DC3000 in JA-insensitive *coi1–1* mutants, ET-insensitive *ein2–1* mutants, and ABA-biosynthetic *aba2–1* mutants, namely melatonin contributes to disease resistance of JA, ET, and ABA deficient plants. In fact, with the SA signaling mutant *npr1* (non-expressor of *PR1*) melatonin-mediated resistance to *Pst*DC3000 disappears (Lee et al. 2014), which is largely consistent with our data that melatonin increases the susceptibility of *sid2–2* and *NahG* plants to *Pst*DC3000. However, with *ein2* melatonin-mediated resistance to *Pst*DC3000 disappears (Lee et al. 2014), which is in contradiction with our results that melatonin increases resistance of *ein2–1* mutants to *Pst*DC3000. We suspected that different inoculation methods might contribute to the diverse effects of melatonin on *ein2–1*.

Plant hormones participate in plant defense responses. The best well-studied hormones are SA, JA, and ET (Dong 1998; Thomma et al. 1998). SA is the major signaling molecule implicated in plant resistance to biotrophs (Kunkel and Brooks 2002). The endogenous level of SA is elevated in various plants under pathogen attack. SA induces disease resistance and expression of the *PR* genes, whereas impairment of SA accumulation compromises disease resistance and *PR* gene expression. JA/ET is responsible for resistance to necrotrophs (Kunkel and Brooks 2002). The expression of defensin PDF1.2 gene is induced by JA and ET, and JA and ET are required for the induction of *PDF1.2*. Based on the regulation of *PR1* and *PDF1.2*, the antagonistic relationship between SA and JA/ET has been established. In addition, the abiotic stress-related hormone ABA regulates plant disease defense and antagonizes SA signaling (Asselbergh et al. 2008; Ton et al. 2009; Cao et al. 2011). Particularly, the negative role of ABA and the positive role of SA in disease resistance to *P. syringae* are confirmed. Furthermore, ABA accumulation represses the onset of JA accumulation during virulent *P. syringae*-induced lesion development (Fan et al. 2009), and ABA also antagonizes JA/ET signaling in plant disease defense (Cao et al. 2011).

Our data showed that melatonin treatment elevated SA and ABA content, but repressed JA and its conjugate JA-Ile levels. Considering SA and ABA antagonize mutually (Asselbergh et al. 2008; Ton et al. 2009; Cao et al. 2011), the potential role of melatonin in disease resistance of wild type *Arabidopsis* plants is probably resulted from the canceling effect of SA and ABA. Because SA and ABA separately contribute to *Arabidopsis* susceptibility to *Pst*DC3000 (Tan et al. 2019), the melatonin-induced susceptibility of *sid2–2* mutants and *NahG* transgenic plants to *Pst*DC3000 might be largely resulted from a high ABA content, whereas the resistance of *aba2–1* mutants is probably caused by the enhanced SA content. Because ABA accumulation precedes JA accumulation (Fan et al. 2009), it may explain that the low levels of JA and JA-Ile are probably derived from suppression of melatonin-induced ABA accumulation. The resistance of *coi1–1* and *ein2–1* mutants to *Pst*DC3000 implies the role of JA/ET signaling to antagonize SA-mediated disease resistance (Kunkel and Brooks 2002). In addition, our data showed that melatonin induced the expression of SA-dependent *PR1* gene and JA/ET-dependent *PDF1.2* gene, which is consistent with a previous report (Lee et al. 2014). Because the transcripts of *PR1* is SA-dependent (Dong 1998), the activation of *PR1* might be resulted from the melatonin-induced SA accumulation. Although our data showed that both SA and ABA levels were induced by melatonin, both hormone signaling systems are antagonistic. It is implied that the activation of *PDF1.2* is melatonin dependent.

Furthermore, our data showed that melatonin treatment had no effect on bacterial pattern-induced callose deposition and ROS burst, as well as avirulent effector protein AvrRpt2-induced cell death in *Arabidopsis*, which further indicated that melatonin did not affect *Arabidopsis* PTI and ETI. Interestingly, it has been reported that lack of melatonin may enhance susceptibility to avirulent bacterium pathogen *Pst*DC3000(*avrRpt2*) (Lee et al. 2015), which is contradiction with our results. Obviously, the role of melatonin in ETI still remains undetermined because of limited references. Despite SA is closely related to PTI and ETI in *Arabidopsis* (Durrant and Dong 2004), the well-known fact is that ABA antagonizes SA-regulated immune responses (Asselbergh et al. 2008; Ton et al. 2009; Cao et al. 2011; Tan et al. 2019). Considering our data that SA and ABA contents were improved together under melatonin treatment, SA-promoted PTI and ETI might be correspondingly cancelled by elevated ABA.

In conclusion, we re-characterized the role of melatonin during *Arabidopsis-P. syringae* interaction. Importantly, we further clarified why melatonin treatment only alters bacterial resistance in some defense-related hormone signaling mutants but not in wild type *Arabidopsis* plants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-021-02279-8.

Acknowledgments This study was funded by Natural Science Foundation of Guizhou Province of China ([2020]1Y107), Provincial Program on Platform and Talent Development of the Department of Science and Technology of Guizhou ([2019]5661, [2019]5617), the Joint Fund of the National Natural Science Foundation of China, the Karst Science Research Center of Guizhou (U1812401) and the Doctoral Starting up Foundation of Guizhou Normal University (GZNUD2018-4).

Author contributions Q.L. and Q.W. planned and designed the research; Q.L., U.R.A., R.W., K.L., and X.M. performed the experiments; Q.L. and R.W. analyzed the data; Q.L. and Q.W. wrote the manuscript.

Declarations

Ethics declarations This article is not submitted elsewhere for publication and this manuscript complies with the Ethical Rules applicable for this journal.

Ethical statement This article does not involve any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interest.

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