Received: 3 January 2020 | Revised: 6 February 2020 | Accepted: 11 February 2020

DOI: 10.1111/jpi.12640

ORIGINAL ARTICLE

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

WILEY

Daily rhythms of phytomelatonin signaling modulate diurnal stomatal closure via regulating reactive oxygen species dynamics in *Arabidopsis*

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Funding information
Ten Thousand Youth Talent Program of Yunnan Province; National Natural Science Foundation of China, Grant/Award Number: 31660595

Abstract

Melatonin is a well-studied neurohormone oscillating in a 24-h cycle in vertebrates. Phytomelatonin is widespread in plant kingdom, but it remains elusive whether this newly characterized putative hormone underlies the regulation by daily rhythms. Here, we report phytomelatonin signaling, as reflected by changes in endogenous concentrations of phytomelatonin and expression of genes associated with biosynthesis of phytomelatonin (*AtSNAT1*, *AtCOMT1,* and *AtASMT*) and its receptor (*AtPMTR1*), shows 24-h oscillations in *Arabidopsis*. The variation of reactive oxygen species (ROS) production and scavenging and expression of ROS-related genes significantly decrease in *pmtr1* and *snat* and increase in *PMTR1*-OE seedlings, indicating the rhythmicity in phytomelatonin signaling is required for maintenance of ROS dynamics. Additionally, the ROS signaling feedback influences the expression of *AtSNAT1*, *AtCOMT1*, *AtASMT,* and *AtPMTR1*, suggesting the phytomelatonin and ROS signaling are coordinately interrelated. The *pmtr1* mutant plants lose diurnal stomatal closure, with stomata remaining open during daytime as well as nighttime and mutants showing more water loss and drought sensitivity when compared with

Li, Wei and Peng are contributed equally to this work.

the wild-type Col-0 plants. Taken together, our results suggest that PMTR1-regulated ROS signaling peaks in the afternoon and may transmit the darkness signals to trigger stomatal closure, which might be essential for high water-use efficiency and drought tolerance.

KEYWORDS

drought tolerance, phytomelatonin rhythms, PMTR1, ROS signaling, stomatal closure

1 | INTRODUCTION

Living organisms have evolved adaptations to day/night cycles caused by the rotation of the Earth with a 24-h cycle. To anticipate the daily environmental changes (eg, light and temperature), plants use an endogenous circadian clock to adjust their growth, behavior, physiology, and metabolism. The daily stomatal rhythms are one of the typical plant behaviors controlled by the circadian clock that were first observed by Francis Darwin more than 120 years ago.[1] Stomatal opening anticipates dawn to underpin CO_2 assimilation, and the closure around dusk avoids water vapor loss at night in both C3 and C4 plants.[2,3] The morning stomatal opening is regulated by many components of circadian traditional transcription-translation feedback loops (TTFLs). For example, stomatal conductance rhythms are shortened by *toc1-1* (*TIMING OF CAB EXPRESSION1*).[4] In *CCA1-ox* arrhythmic plants (*CIRCADIAN CLOCK ASSOCIATED 1*; under the cauliflower mosaic virus 35S promoter), the stomata remain open until dark-initiated closure; in contrast, in wild-type plants stomatal conductance already decreases in the afternoon.[5] Additionally, *CCA1*-overexpressing *Arabidopsis* under the guard cell-specific promoter (*GC*) showed a loss of circadian-mediated stomatal opening.[6]

Reactive oxygen species (ROS), including superoxide (O_2^-), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) were initially thought to be toxic byproducts of aerobic metabolism. To cope with the oxidative damage caused by accumulating ROS, plants have evolved the enzymatic (eg, superoxide dismutase, peroxidases, and catalase) and non-enzymatic (eg, glutathione, ascorbate, and flavonoids) ROS-scavenging antioxidant mechanisms that maintain ROS homeostasis. The high turnover rates of ROS through production and scavenging make ROS ideal candidates for signaling molecules.[7,8] In plants, the cell membrane-located NADPH oxidase is the main source of extracellular O_2^- production that is essential for initiating the ROS signaling.[9] Additionally, ABA, melatonin and darkness induce stomatal closure via activating NADPH oxidase-dependent ROS production.[10-12] In *Arabidopsis*, ROS homeostasis (maintained by ROS accumulation and scavenging and ROS-related genes expression) shows daily rhythms that are regulated by CCA1.[13] Furthermore, the rhythms of ROS production peaking in the afternoon may act as a signal to promote the night stomatal closure in tobacco.[14]

Melatonin (N-*acetyl*-5-methoxytryptamine) is a well-studied neurohormone that shows daily oscillations.[15,16] Phytomelatonin was initially detected in land plants in 1995.[17,18] During the past two decades, various studies have confirmed the widespread distribution of phytomelatonin and its involvement in various aspects of plant growth and stress responses.[19] In *Arabidopsis*, genes encoding melatonin biosynthetic enzymes, namely serotonin N-acetyltransferase 1 (SNAT1), caffeate O-methyltransferase 1 (COMT1), and N-acetylserotonin methyltransferase (ASMT), have been cloned.[20-22] Recently, we identified the first phytomelatonin receptor (PMTR1, previously named CAND2) that regulates stomatal closure via NADPH oxidase-dependent ROS signaling pathway in *Arabidopsis*,[10] suggesting a possibility that this molecule is a new plant hormone.[10,19,23] Although it has been shown that the endogenous concentrations of phytomelatonin peak during darkness in *Chenopodium rubrum* as in vertebrates,[24,25] these observations were challenged lately as several publications found that the biosynthesis of melatonin is induced by light but decreased under darkness conditions in morning glory,[26] *Arabidopsis*[27] and rice.[28] Therefore, it remains controversial whether this newly characterized putative phytohormone is involved in regulation of daily rhythms as the endogenous concentrations of phytomelatonin are always affected by plant growth conditions and environmental factors (eg, temperature, UV light, and water status).[29]

Herein, we provided evidence for the rhythmic oscillations of phytomelatonin concentration and expression of genes associated with biosynthesis of phytomelatonin (*AtSNAT1*, *AtCOMT1,* and *AtASMT*) and its receptor (*AtPMTR1*) in *Arabidopsis* seedlings. Additionally, the daily rhythmicity in phytomelatonin signaling is required for maintenance of ROS dynamics through regulating expression of numerous genes involved in ROS generation and scavenging. We also found late day and night stomatal closure is an output of phytomelatonin signaling, which might be essential for high water-use efficiency and drought tolerance.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used throughout. The mutant lines used in this study were *pmtr1* (*cand2-1)*, *snat*, and *rbohD/F* as we described previously.[10,20] To generate *PMTR1*-OE transgenic plants, the coding sequence of *AtPMTR1* (*AtCand2*) was cloned into the pCAMBIA1300-35S:3 × FLAG vector at the *Sac*I and *Kpn*I sites. *Agrobacterium*-mediated transformation of *Arabidopsis* was performed as described previously.[30] Homozygous T3 lines of *PMTR1*-OE were selected for use in this study.

2.2 | Growth conditions

The seeds were surface-sterilized in 75% (v/v) ethanol for 5 min and 8% (w/v) sodium hypochlorite for 15 min, washed five times in distilled water, and sown on 1/6 Murashige and Skoog (MS, Sigma) agar medium [containing 1.0% (w/v) sucrose and 0.8% (w/v) agar, pH 5.7] in plates. After incubation at 4°C in the dark for 2-3 days, the plates were placed in a growth chamber at 22°C with 65% relative humidity under cool LED white light at light intensity of 100 μmol m^{-2} s^{-1} under 12-h light/12-h dark conditions. The seedlings were grown under 12-h light/12-h dark conditions for 10 days and then transferred into 12-h light/12-h dark cycles or continuous light conditions. In other experiments for measuring stomatal conductance and aperture, ROS production in guard cells, drought stress and water loss, plants were grown for 2-3 weeks in a mixed medium (vermiculite: pearl rock: peat soil = 3:1:1).

2.3 | UPLC-MS/MS Analysis

The samples (300 mg, 80-100 seedlings per sample) were extracted in 3 mL of 75% (v/v) methanol (Sigma), and 3 ng/g of melatonin-d4 (sc-207849, Santa Cruz Biotechnology) was added as the internal standard. The homogenates were mixed at 1500 rpm for 30 min followed by an ultrasonic treatment three times (30 min each) at room temperature. These procedures were repeated twice. After centrifugation at 13 000 *g* at 4°C for 15 min, the supernatants were vacuum-dried at 20°C in dark. The residue was reconstituted in 100 μL of 30% (v/v) acetonitrile–water for UPLC-MS/MS analysis.

The chromatography was done using an Acquity™ UHPLC system (Waters) equipped with an online vacuum degasser, a binary solvent pump, an autosampler, and a thermo-stated column compartment and column. For UPLC, the mobile phase A was 0.1% (v/v) formic acid in water, and the mobile phase B was acetonitrile. A UPLC BEH C18 column (Waters) was used to separate melatonin. The chromatography was carried out with a flow rate of 0.3 mL/min in the following gradient mode: 70%-50% A (0-3 min); 50%-5% A (3-3.1 min); 5% A (3.1-4.0 min); 5%-70% A (4.0-4.1 min); and 70% A (4.1-5.0 min). The injection volume was maintained at 10 μL and column temperature at 35°C. A triple quadrupole mass spectrometer (MS/MS) (Waters) was operated in a positive mode and electrospray ionization-multiple reaction-monitoring. The optimal conditions were set as follows: capillary voltage, 3500 V; drying gas (nitrogen) flow, 5 L/min; drying gas (nitrogen) temperature, 300°C; and nebulizer pressure, 2 bar. The melatonin and melatonin isotope were quantified at the mass-to-charge ratios (m/z) 233.1/174.1 and 237.1/163.0, respectively.

2.4 | Real time RT-PCR

Real time RT-PCR was performed as we described previously.[10] All primers used for RT-PCR are listed in Table S1. Total RNA was extracted from at least 100 seedlings per sample as a biological replicate. The experiments were conducted three times and showed similar results.

2.5 | Stomatal conductance and aperture measurements

Three-week-old plants grown under 12-h light/12-h dark cycle or constant darkness were used. Stomatal conductance was measured by a steady-state leaf porometer (Decagon Devices, model SC-1) on abaxial leaf surfaces. Stomatal movements in 24-h cycles were measured using the silicon polymer impression method.[31] Briefly, mature leaves from each genotype were covered with dental impression gel (1:1 freshly prepared mixture of the two components of dental condensation silicone; Zhermack). After 1 h, the material was removed and clear nail polish was placed on the impression gel for 2- to 4-h to obtain a positive impression of the leaf. The impression was analyzed by a bright-field microscope (Nikon, Optiphot-2).

To measure stomatal aperture in the presence of melatonin, the epidermal strips from 3- to 4-week-old plants were placed in the incubation medium containing 50 mM KCl, 0.1 mM $CaCl_2$, and 10 mM MES-KOH (pH 6.15) for 3 h under light (100 μmol m^{-2} s^{-1}). Then, various concentrations of melatonin were added to the stomatal incubation solution for 2-h incubation under light (100 μmol m^{-2} s^{-1}). Stomatal aperture was evaluated by measuring the width and the length of the stomatal aperture under a microscope (Nikon,

Optiphot-2), and the width/length ratio was calculated as described previously.[10]

2.6 | Reactive oxygen species (ROS) measurement in guard cells

We examined H_2O_2 production in guard cells using the H_2O_2-sensitive fluorescent probe H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Sigma-Aldrich) as described previously.[10]

2.7 | Nitroblue tetrazolium staining

Nitroblue tetrazolium (NBT) staining was used for histochemical detection of superoxide anions in ten-day-old seedlings as reported previously.[32]

2.8 | Hydrogen peroxide and superoxide anion measurements

Hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) concentrations were measured in 10-day-old seedlings grown on agar plates. H2O2 was measured by the titanium sulfate method and a Micro Hydrogen Peroxide (H_2O_2) Assay Kit (BC3595, Solarbio Life Sciences, China). O_2^- was measured by examining the oxidation of hydroxylamine to nitrite as described previously [33] and further confirmed using a Micro Superoxide Anion Assay Kit (BC1295, Solarbio Life Sciences, China).

2.9 | Antioxidant enzyme analyses

For measurement of SOD (EC 1.15.1.1), POD (EC 1.11.1.7), and CAT (EC 1.11.1.6) activities, the samples were homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 0.2 mM EDTA-Na_2, 0.1 mM ascorbic acid and 1% w/v PVPP in an ice bath using a mortar and pestle. The homogenate was centrifuged for 20 min at 12,000 g at 4C, and the supernatant was used for enzyme analyses.

SOD activity was measured according to the published method.[34] One unit of SOD activity was defined as the quantity of SOD enzyme protein required to produce 50% inhibition of NBT reduction under the experimental conditions. POD activity was measured according to the method of Maehly and Chance,[35] and CAT activity was assayed by monitoring the consumption of H_2O_2 at 240 nm.[36] One unit of POD activity was defined as a change of 0.005 at 470 nm per min per mg protein. The protein content was measured by the Bradford method.[37]

2.10 | Physiological characterization under drought stress and the water loss measurements

Water loss assays were performed at zeitgeber time 12 (ZT12). The leaves from 3-week-old plants under 12-h/12-h light/dark cycles were harvested at ZT12 for water loss measurements. The leaves were weighed in 30-min intervals at 22°C and 65% relative humidity in dark. The percentage of water loss was calculated on the basis of the initial weight of leaves.

The drought treatment was performed using 2-week-old plants by withholding watering for additional 2 weeks under 12-h/12-h light/dark cycles. After the treatment, leaves were harvested and used for calculation of relative water content (RWC) as follows: (FW − DW)/(TW − DW) × 100, where, FW: fresh weight, DW: dry weight and TW: turgid weight, following the method reported previously.[38]

2.11 | Statistical analyses

All data were analyzed using Student's *t* test or ANOVA to determine the differences using the GraphPad Prism 8.

Determination of rhythmicity was performed using the online platform BioDare2 (Biological Data Repository 2, biodare2.ed.ac.uk).[39] $*P < .05$, $**P < .001$, $***P < .0001$, and ns = no significant difference.

3 | RESULTS

3.1 | The phytomelatonin signaling shows day/night rhythms in *Arabidopsis*

The endogenous concentrations of phytomelatonin showed significant rhythmicity in Col-0, with a main peak in the morning at zeitgeber time 4 h (ZT4; Figure 1A). The smaller second peak appeared at night (ZT20). Using the well-known clock gene *CCA1* as a positive control (Figure S1), we found the expression of *AtSNAT1* (At1G32070) and *AtCOMT1* (At5G54160) and *AtASMT* (At4G35160) genes showed significant time-of-day-specific rhythms in the morning in wild-type Col-0, peaking at ZT4 (Figure 1B), similarly to the changes in endogenous phytomelatonin concentrations (Figure 1A). Additionally, the *AtPMTR1* (At3G05010) expression also exhibited significant day/night rhythms ($P = .018$, *), but with a major peak later (about a 4-h delay) in the afternoon (ZT8, Figure 1C). Compared with Col-0, the *snat* mutant seedlings had significantly lower *AtPMTR1* expression (Figure 1C) and the peak melatonin concentration at ZT4 (Figure S2). The peak expressions of *AtSNAT1*, *AtCOMT1,* and *AtASMT* at ZT4 were significantly lower in *pmtr1* compared with Col-0

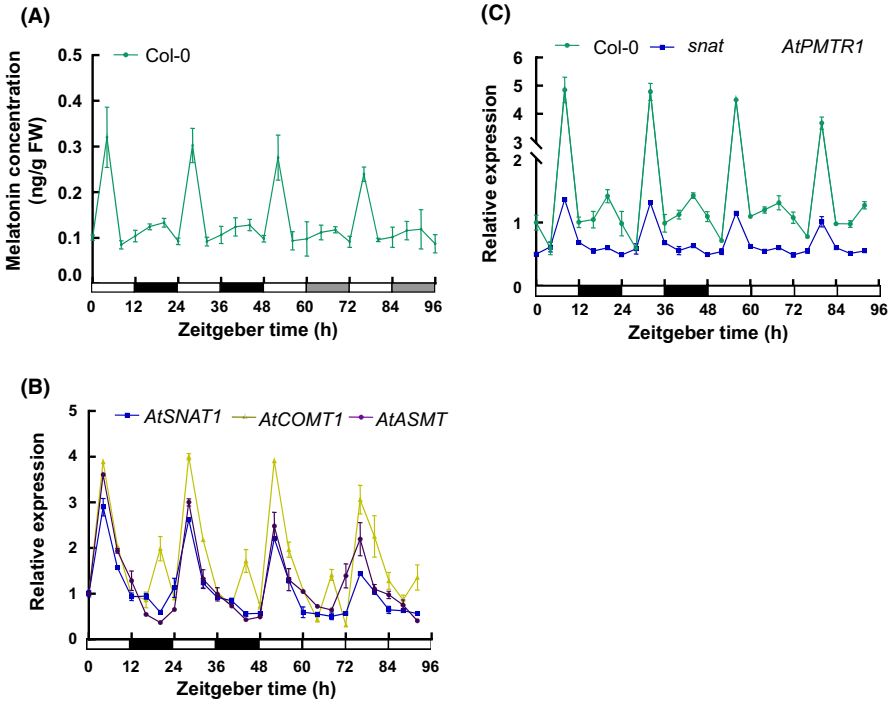

FIGURE 1 Rhythmicity in endogenous concentrations of phytomelatonin and gene expression of *AtSNAT1*, *AtCOMT1*, *AtASMT,* and *AtPMTR1*. (A) Endogenous melatonin concentration in Col-0 seedlings. The online platform BioDare2 (Biological Data Repository 2, biodare2.ed.ac.uk) was used to determine the significant rhythmicity of phytomelatonin concentration ($P = .013$, *). Values are means ± SEM (n = 6). (B) Rhythmicity in gene expression of *AtSNAT1*, *AtCOMT1,* and *AtASMT* in Col-0 seedlings. Significant rhythmicity of *AtSNAT1* ($P = .011$, *), *AtCOMT1* ($P = .00008$, ***), and *AtASMT* ($P = .0001$, **) was analyzed by BioDare2. Values are means ± SEM (n = 3). (C) Relative expression of *AtPMTR1* in Col-0 and *snat* seedlings. Paired Student's t test (Col-0 versus *snat*) found significant difference in *AtPMTR1* expression over time ($t = 4.165$, $df = 23$, $P = .0004$, ***). Values are means ± SEM (n = 3). Ten-day-old seedlings were grown under two cycles of 12-h light/12-h dark and two cycles of continuous light conditions. Actual and subjective days are denoted by white bars. Actual and subjective nights are denoted by black and gray bars, respectively

(Figure 2), indicating that the expression of genes related to phytomelatonin synthesis and receptor include a positive feedback regulatory loop.

3.2 | Daily rhythms of phytomelatonin signaling are required for maintenance of ROS dynamics

Significant rhythmicity in concentrations of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) and activities of antioxidant enzymes superoxide dismutase (SOD), and catalase (CAT) was observed in Col-0 peaking in the afternoon (ZT8; Figure 3A,B; Figure S3), similarly to previous studies.[13,14] However, the changes in POD activity did not show significant rhythms in Col-0 ($P = .42$, ns). Compared with Col-0, the concentrations of O_2^- and H_2O_2, coupled with activities of the antioxidant enzymes, markedly decreased in the *pmtr1* and *snat* mutant lines (Figure 3A). In contrast, concentrations of these two signaling molecules and activities of the antioxidant enzymes were higher in the transgenic *PMTR1-OE* seedlings (overexpressing *AtPMTR1*; Figure S4) than Col-0 (Figure 3A).

Using the known rhythmic genes *CATs* [40-42] as positive controls, we found that most of the ROS-generating (NADPH oxidase, *RbohA-I*) and ROS-scavenging (*CATs*, *CSD1*, *FSD2, PRXs*, and *AOXs*) genes showed the time-of-day-specific expression patterns, with peak values in the afternoon (ZT8; Figure 3B, Figures S5-S7), except for *CAT2* that peaked at dawn in wild-type Col-0 (Figures S6 and S7). However, compared with Col-0, the expression of these genes was strongly reduced in the *pmtr1* and *snat* mutant lines (Figure 3B and Figures S5-S7), but were mostly higher in the latter than the former, coinciding with the peak concentrations of H_2O_2 and O_2^- and the activities of antioxidant enzymes (Figure 3A). These findings imply the daily rhythms of phytomelatonin signaling are required for maintenance of ROS dynamics.

3.3 | ROS signaling feedback influences the expression of phytomelatonin signaling-related genes

We further analyzed the effect of ROS signaling on expression of phytomelatonin-related genes in the NADPH oxidase double mutant *rbohD/F*. Figure 4 shows the peak gene

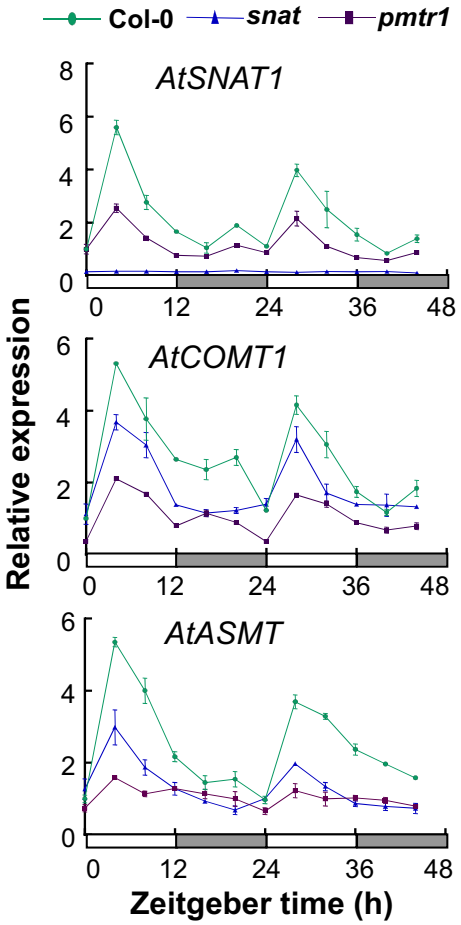

FIGURE 2 Relative expression of *AtSNAT1*, *AtCOMT1,* and *AtASMT* in Col-0, *pmtr1,* and *snat* seedlings. Ten-day-old seedlings grown under 12-h light/12-h dark conditions were transferred to continuous light conditions. Actual daytime is denoted by white bars, and subjective nighttime is denoted by gray bars. The seedlings were sampled at 4-h intervals. Two-way ANOVA was used to determine the significant differences among genotypes at various time periods. *AtSNAT1*, F = 19.74, *df* = 22, *P* < .0001, ***; *AtCOMT1*, F = 5.94, *df* = 22, *P* < .0001, ****; *AtASMT*, F = 14.09, *df* = 22, *P* < .0001, ****. Values are means ± SEM (n = 3)

expression of *AtSNAT1*, *AtCOMT1*, *AtASMT,* and *AtPMTR1* decreased in *rbohD/F* mutant seedlings compared with Col-0, suggesting phytomelatonin and ROS signaling might be interrelated.

3.4 | Night stomatal closure is an output of rhythmic phytomelatonin signaling

The application of 10 μM melatonin promoted stomatal closure and ROS production in Col-0, *snat* and *PMTR1-OE,* but not in *pmtr1* (Figure 5), confirming PMTR1 plays a key role in melatonin-induced stomatal closure via inducing ROS production as we recently described.[10] We therefore analyzed the possible interactions between phytomelatonin signaling and daily stomatal rhythms. The dynamics of stomatal aperture were significant altered in the mutant and transgenic lines (Figure 6A). Stomatal closure started from afternoon (ZT8) to its strongest level at nighttime (ZT16), and then stomata opened around dawn (ZT24) in Col-0, *snat* and *PMTR1-OE*. Col-0 and *PMTR1-OE* stomata were closed already at ZT12/ZT16, while *pmtr1* and *snat* stomata remained mostly open (Figure 6A). Student's t test found significant difference in stomatal aperture in Col-0, *snat,* and *PMTR1-OE,* but not in *pmtr1,* at nighttime (ZT12, ZT16, and ZT20; Figure 6A). The stomatal conductance data (Figure 6B) further confirmed that the *pmtr1* plants even lost night stomatal closure under continuous darkness conditions (free-running), for example, the stomatal conductance still remained high levels until 72 h of darkness (from 12 to 84 h, Figure 6B). These results suggested that PMTR1-mediated phytomelatonin signaling is required for promoting the night stomatal closure.

3.5 | Rhythmic phytomelatonin signaling-modulated stomatal closure is essential for minimizing night water loss and promoting drought tolerance

We then measured the night water loss and drought tolerance in Col-0, *pmtr1,* and *PMTR1-OE*. A larger water loss during nighttime (Figure 7A) and higher drought sensitivity (Figure 7B-D) were observed in *pmtr1* mutant plants in comparison with Col-0. In contrast, plants overexpressing *AtPMTR1* (*PMTR1-OE*) showed a smaller water loss (Figure 7A) and higher drought tolerance (Figure 7 B-D), probably because of smaller stomatal apertures (Figure 6A) and lower conductance (Figure 6B) compared with Col-0. The stomata of *PMTR1*-OE were hypersensitive to exogenous melatonin, eg. 0.01-1 μM melatonin-induced greater stomatal closure and ROS production compared with Col-0 (Figure 8A,B). Smaller stomatal aperture and lower stomatal conductance during daytime (Figure 6A,B) and higher sensitivity of stomata to melatonin (Figure 8) in *PMTR1*-OE than *pmtr1* might be related to higher ROS concentration in the former (Figure 3 and Figures S5-S7).

4 | DISCUSSION

Melatonin is an ancient small molecule present in bacteria, plants and animals; its structure has never changed throughout evolution.[43] The sequence similarities of the melatonin receptors from *Arabidopsis* (PMTR1) and humans (MT1, MT2 and GPR50) are poor, but they are all membrane proteins with seven predicted helices.[10] This indicates that melatonin receptors have evolved independently in different lineages to achieve a similar purpose,

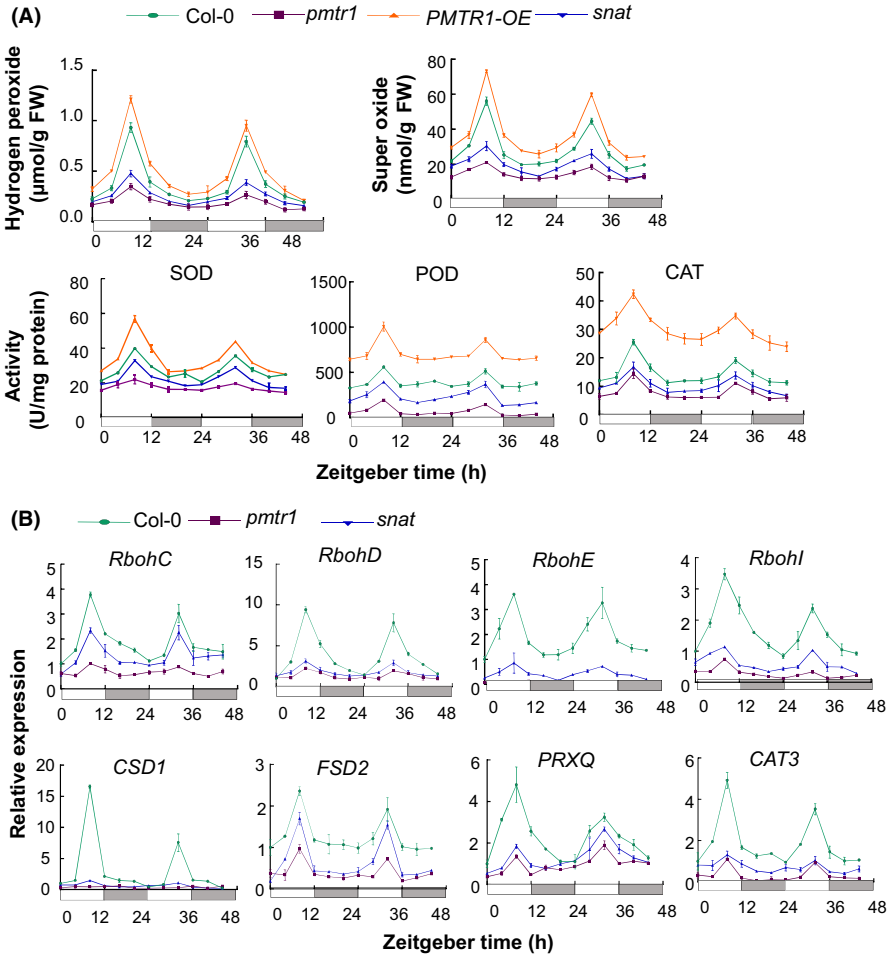

FIGURE 3 Rhythms of phytomelatonin are required for maintenance of ROS homeostasis. (A) Concentrations of H_2O_2 and O_2^- and the activities of SOD, POD and CAT in Col-0, *snat*, *pmtr1,* and *PMTR1-OE* seedlings. Significant rhythmicity of H_2O_2 ($P = .00003$, ***), O_2^- ($P = .0001$, **), SOD ($P = .003$, *), POD ($P = .42$, ns), and CAT ($P = .00005$, ***) in Col-0 was analyzed by BioDare2. Two-way ANOVA was used to determine the significant difference in H_2O_2 ($F = 17.71$, $df = 33$, $P < .0001$, ***), O_2^- ($F = 16.82$, $df = 33$, $P < .0001$, ***), SOD ($F = 12.41$, $df = 33$, $P < .0001$, ***), POD ($F = 2.776$, $df = 33$, $P < .0001$,***), and CAT ($F = 1.464$, $DF = 33$, $P = .0783$, ns) among different genotypes over time. Values are means ± SEM (n = 6). (B) Rhythmicity in the expression of ROS-related genes in wild-type Col-0, *snat,* and *pmtr1* seedlings. The expression of *RbohC* ($P = .0001$, ***), *RbohD* ($P = .00005$, ***), *RbohE* ($P = .0049$, *), *RbohI* ($P = .0068$, *), *CSD1* ($P = .00543$, *), *FSD2* ($P = .01955$, *), *PRXQ* ($P = .0005$, **), and *CAT3* ($P = .0049$, *) was significant in Col-0 (using Biodare2 analysis). Two-way ANOVA was used to determine the significant difference in gene expression in different genotypes over time: *RbohC*, $F = 6.81$, $df = 22$, $P < .0001$, ***; *RbohD*, $F = 20.47$, $df = 22$, $P < .0001$, ***; *RbohE*: $F = 7.08$, $df = 22$, $P < .0001$, ***; *RbohI*: $F = 14.35$, $df = 22$, $P < .0001$, ***; *CSD1*, $F = 98.05$, $df = 22$, $P < .0001$, ***; *FSD2*, $F = 2.42$, $df = 22$, $P < .05$, *; *PRXQ*, $F = 6.83$, $df = 22$, $P < .0001$, ***; and *CAT3*, $F = 13.12$, $df = 22$, $P < .0001$, ***. Values are means ± SEM (n = 3). Ten-day-old seedlings were grown under two cycles of continuous light conditions. White and gray bars represent day and night, respectively

for example, mediating phytomelatonin signaling via direct binding. In vertebrates, melatonin is high at night [15,16]; conversely, several reports suggest that phytomelatonin biosynthesis is induced by light and peaks in the morning under light/dark cycles.[26-28,44] In this study, we found that the major peaks of the endogenous concentrations of phytomelatonin coupled with the expression of *AtSNAT1*, *AtCOMT1*, *AtASMT,* and *AtPMTR1* genes showed the time-of-day-specific rhythms during the daytime (Figures 1 and 2). The transcript amounts of *AtPMTR1* peaked later (about a 4-h delay) than the expression of *AtSNAT1*, *AtCOMT1* and *AtASMT* (Figures 1 and 2). Additionally, the expression of *AtPMTR1* was induced by exogenous melatonin,[10] but decreased in *snat* seedlings with lower endogenous phytomelatonin concentration (Figure 1C and Figure S2), indicating that daily rhythms of *AtPMTR1* are tightly regulated by the temporal expression of phytomelatonin.

ROS are initially thought to be toxic byproducts of aerobic metabolism, but they are also important signaling molecules.[7,9] In plants, NADPH oxidase is the main source of extracellular O_2^- production and is regarded as the engine of ROS signaling.[9] The extracellular O_2^- produced by NADPH

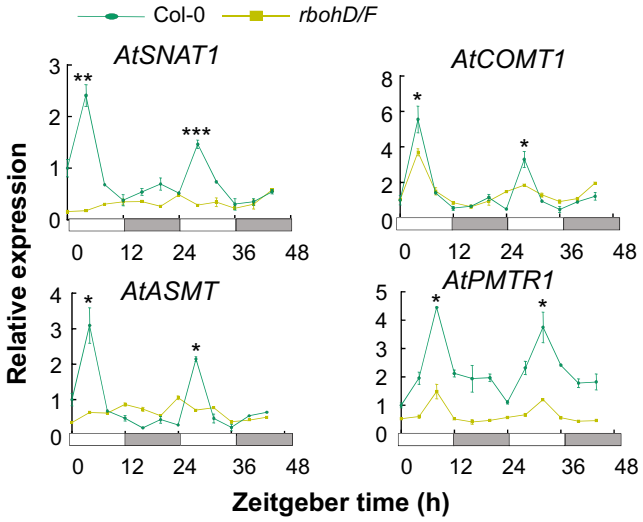

FIGURE 4 Relative expression of genes related to biosynthesis of phytomelatonin (*AtSNAT1, AtCOMT1,* and *AtASMT*) and its receptor (*AtPMTR1*) in wild-type (Col-0) and *rbohD/F* mutant seedlings. Paired Student's *t* test (Col-0 versus *rbohD/F*) found significant difference in the expression of *AtSNAT1* ($t = 2.52$, $df = 11$, $P = .03$,*), *AtCOMT1* ($t = 3.69$, $df = 11$, $P = .004$,*), and *AtPMTR1* ($t = 7.69$, $df = 11$, $P < .0001$, ***) over time. For *AtASMT* ($t = 0.82$, $df = 11$, $P = .43$, ns), significant difference was found for peak values (ZT4 and ZT28). Values are means ± SEM (n = 3). White and gray bars represent subjective day and night, respectively

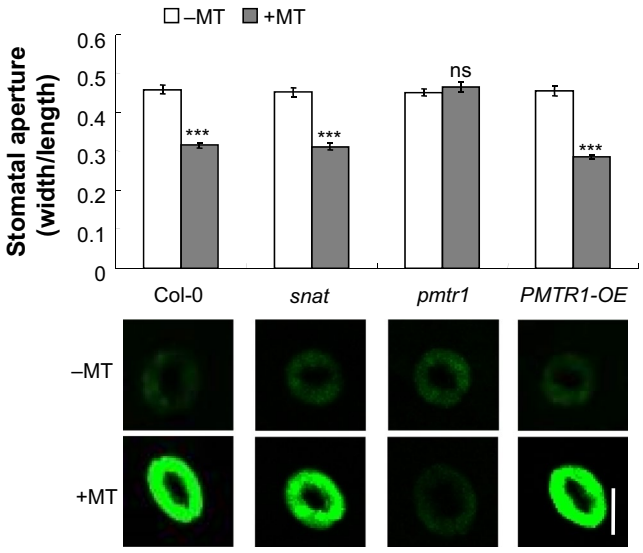

FIGURE 5 Effect of melatonin on stomatal aperture (upper panel) and ROS production (lower panel) in Col-0, *snat*, *pmtr1,* and *PMTR1-OE*. The epidermal strips obtained at ZT4 from 3- to 4-week-old plants were treated by 0 or 10 μM melatonin (MT) for 2 h. White bar = 10 μm. ***$P < .0001$ and ns (no significant difference) between –MT and + MT in each genotype using Student's *t* test. Values are means ± SEM (n = 30)

oxidase is converted spontaneously, or catalytically by SOD to H_2O_2 and then transported into cell via aquaporins, initiating intracellular and extracellular signal transduction

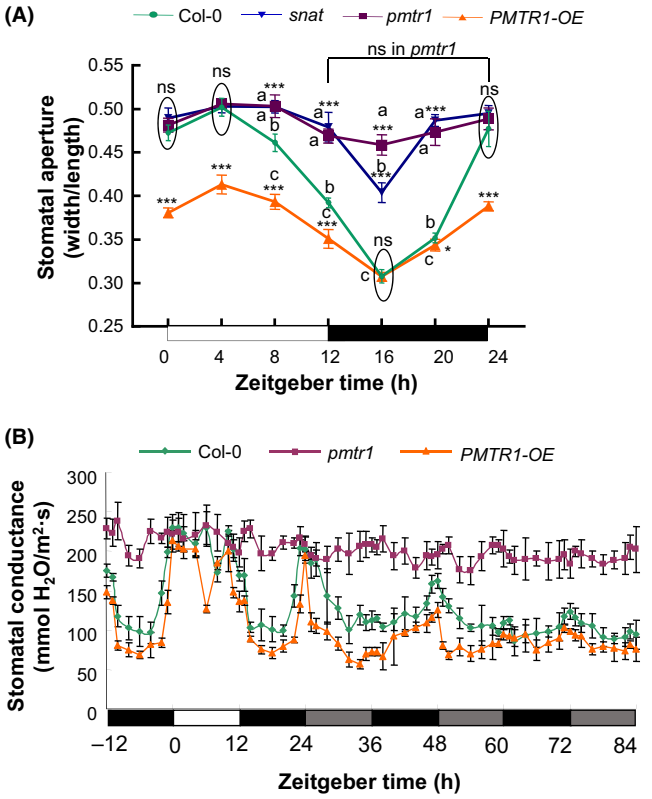

FIGURE 6 Phytomelatonin rhythms are essential for night stomatal closure. (A) Changes in stomatal aperture under 12-h light/12-h dark conditions. White and black bars represent day and night, respectively. Significant difference was analyzed by paired Student's *t* test (*, $P < .05$; **, $P < .001$; ***, $P < .0001$, ns, no significant; n = 40). Different letters indicate significant difference at each time point (*t* test, $P < .05$). Values are means ± SEM (n = 50). (B) Rhythms of stomatal conductance in Col-0, *pmtr1,* and *PMTR1-OE*. Two-week-old plants were grown under 12-h/12-h light/dark cycles and then were shifted to constant darkness conditions. The time at which the lights were turned on was termed Zeitgeber time 0 (ZT0). Actual and subjective days are denoted by white bars and gray bars, respectively. Actual nights are denoted by black bars. Two-way ANOVA was used to determine the significant differences among the genotypes over time ($F = 2.64$, $df = 128$, $P < .0001$,***). Values are means ± SEM (n = 6)

[45,46] and regulating redox balance[47] and stomatal closure.[48] For example, NADPH oxidase-mediated ROS production activated the antioxidant enzymes and defense response in *Arabidopsis*,[49,50] maize,[51,52] and *Panax ginseng*[53] and was associated with human heart failure.[54] Additionally, exogenous melatonin-induced ROS production and stomatal closure were dependent on PMTR1-mediated activation of NADPH oxidase.[10] In this study, we found that the expression of most genes from the NADPH oxidase family and concentrations of O_2^- showed significant rhythmicity (Figure 3 and Figures S5-S7). Additionally, the variation in gene expression and activity of the antioxidant enzymes were significantly decreased in *pmtr1,* and *snat,* which had impaired ROS production and the expression of NAPDH oxidase (Figure 3; Figures S5-S7).

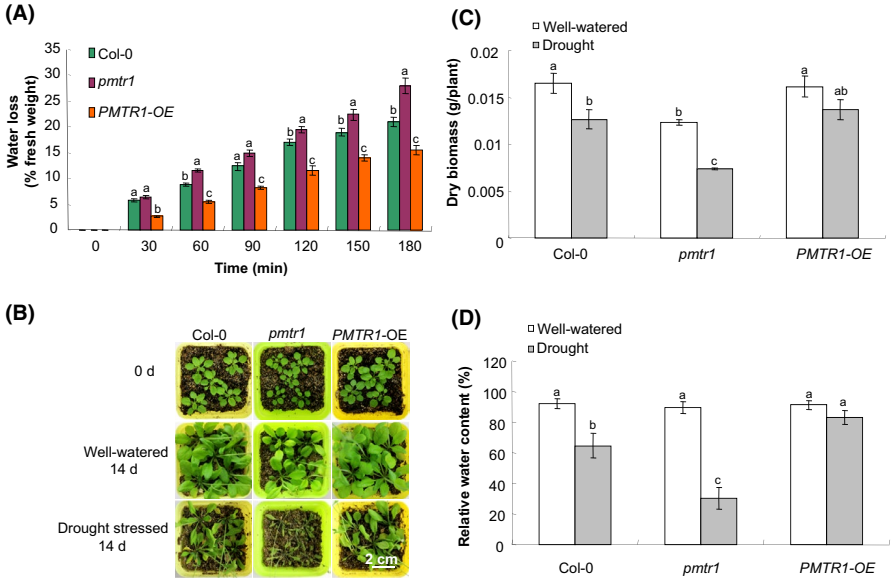

FIGURE 7 Phytomelatonin signaling is essential for avoiding night water loss and promoting drought tolerance. (A) Water loss of rosette leaves excised from 3-week-old plants at ZT12 during the subsequent 3 h. Different letters indicated significantly different values at $P < .05$ using Student's *t* test (n = 3). Images (B), dry biomass (C) and relative water content (D) of Col-0, *pmtr1,* and *PMTR1-OE* under drought or well-watered conditions. Two-week-old plants were treated by withholding watering for additional 2 weeks under 12-h/12-h light/dark cycles. Different letters indicated significantly different values at $P < .05$ using Student's *t* test (n = 12-15)

The opposite results were observed in *PMTR1-OE* (Figure 3; Figures S5-S7). These findings indicated that phytomelatonin rhythms are essential for maintenance of ROS signaling homeostasis.

A primary function of melatonin as a direct free-radical scavenger and a broad-spectrum antioxidant with receptor-independent actions extends back about 3.6 billion years.[55] How has this antioxidant molecule evolved such a complex signaling function to act as a superior ROS signaling regulator? *Arabidopsis AtPMTR1* homologues are found from aquatic green alga (*Micromonas commoda* and *Coccomyxa subellipsoidea*) and the common land plant ancestor charophyte

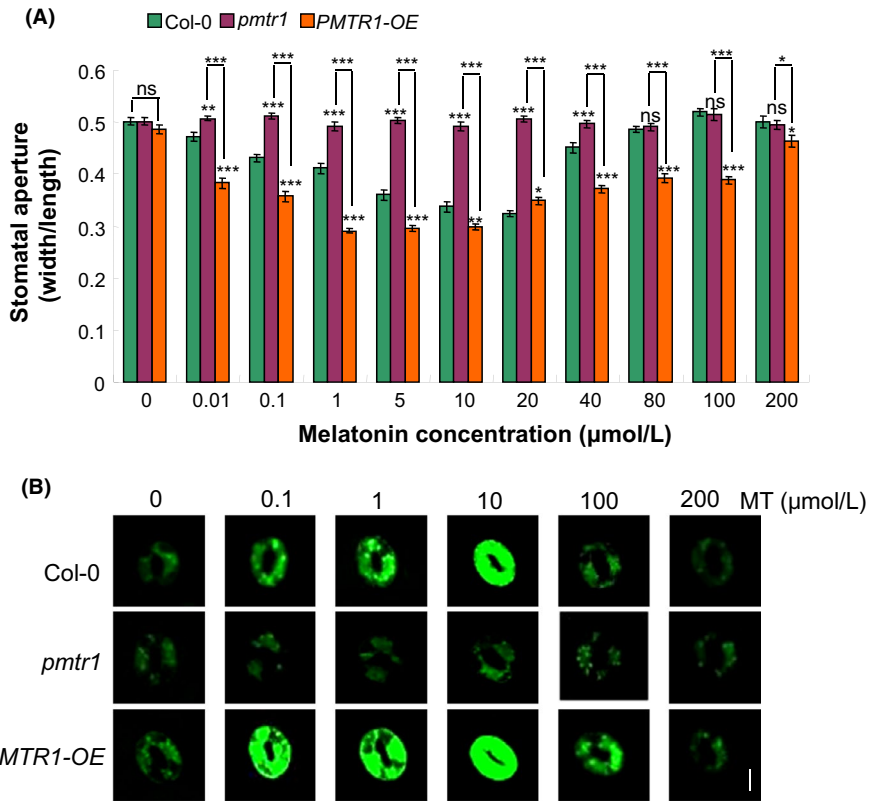

FIGURE 8 Effect of different concentrations of exogenous melatonin (MT) on stomatal aperture (A) and ROS production (B). The epidermal strips obtained at ZT4 from 3- to 4-week-old plants were treated by 0-100 μM melatonin (MT). White bar in (B) indicates 10 μm. Student's *t* test was used to evaluate the significant difference among various treatments. $*P < .05$, $**P < .001$, $***P < .0001$, and ns = no significant difference. For (A), values are means ± SEM (n = 25-30)

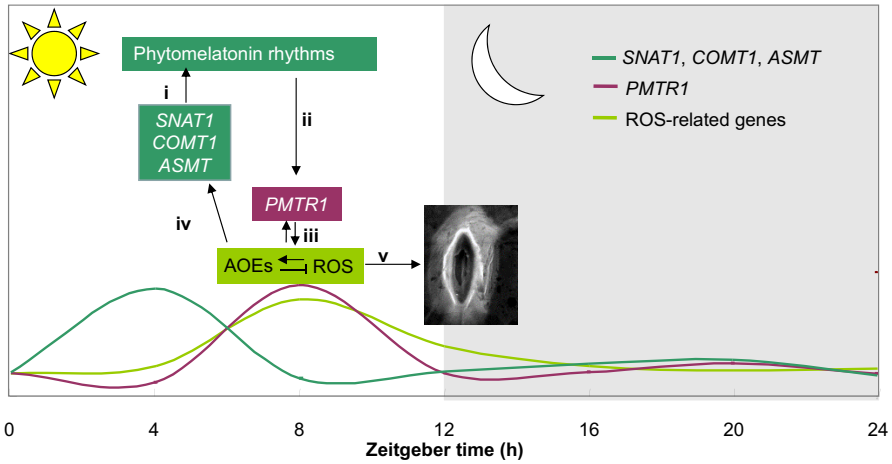

FIGURE 9 A proposed model that depicts the coordinated regulation of phytomelatonin rhythms and ROS signaling in the maintenance of the 24-h stomatal rhythms in *Arabidopsis*. AOEs, antioxidant enzymes

(*Klebsormidium nitens*) to semi-terrestrial plants bryophytes and current seed plants, but not in red algae or prokaryotic cyanobacteria (Figure S8). It is likely that PMTR1 evolved after the separation of red algae and green algae, and likely about 1.2 billion years ago because the earliest fossils of green algae date back to the *Precambrian*.[56] The Great Oxidation Event (GOE) occurred about 2.5 billion years ago,[57] later than the origin of melatonin but earlier than the evolution of the PMTR1. The physiological levels of endogenous phytomelatonin are too low (eg, nM range in *Arabidopsis*, Figure 1A) to be a sole antioxidant. Therefore, it is presumed that the selective pressure associated with oxidative stress in plants facilitated evolution of the phytomelatonin receptor-mediated regulation of ROS homeostasis, as an effective and economical strategy to cope with ROS stress by regulating antioxidant systems. Indeed, phytomelatonin and ROS signaling are coupled by interrelated regulation of each other (Figure 4; also see Ref.[58]).

Stomata are the main gateways of transpiration. More than 90% of the water lost via transpiration is released through stomata on leaf surface,[59] which is an inevitable consequence of photosynthesis during daytime.[3] Diurnal stomatal closure is an effective strategy to avoid water vapor loss at night when there is no opportunity for carbon fixation by photosynthesis. Although the well-studied components controlling daily stomatal movements are mostly traditional TTFLs and phytohormones,[4-6,60] the ROS signaling also plays important roles in these rhythmic processes. For example, NAPDH oxidase-dependent ROS production is involved in ABA-, melatonin-, and darkness-induced stomatal closure.[10-12] The endogenous rhythms in ROS production serve as a signal to trigger late day and night stomatal closure in tobacco.[14] Similarly, we found that the stomatal closure was lost (Figure 6) and showed high water loss and drought sensitive phenotype (Figure 7) in *pmtr1* mutant plants with low concentration of ROS production and scavenging (Figure 3 and Figures S5-S7). In contrast, the *PMTR1*-OE plants with higher ROS concentrations showed smaller stomatal aperture and lower stomatal conductance during daytime (Figure 6) and higher drought tolerance (Figure 7) compared with Col-0, which might be due to the stomata are hypersensitive to melatonin-induced stomatal closure and ROS production (Figure 8). These results indicated that a PMTR1-regulated ROS oscillatory peak in the afternoon might serve as a darkness signal to stimulate stomatal closure, which is essential for avoiding night water loss and conferring drought tolerance.

Taken together, our data demonstrate that concentration of phytomelatonin and the expression of its biosynthesis-related genes show significant rhythmic changes, peaking in the morning (Figure 1 and 9 i). The rhythmic expression of *AtPMTR1* peaks later in the afternoon, which might be regulated by changes in the endogenous phytomelatonin concentration (Figures 1, 2, and 9 ii). The rhythms in phytomelatonin signaling are required for maintenance of ROS homeostasis via regulating the expression of numerous ROS-related genes (Figure 3, Figures S5-S7; Figure 9 iii). The ROS signaling could also feedback influence the expression of phytomelatonin-related genes (Figures 4 and 9 iv). Furthermore, the PMTR1-mediated ROS signaling peaked in the afternoon and might provide a darkness signal to promote the late day and night stomatal closure (Figures 6 and 9 v), which is essential for avoiding night water loss and promoting drought tolerance (Figure 7); moreover, it is likely required for evolutionary plant adaptation to dry-land environments. The endogenous concentrations of phytomelatonin and expression of genes related to biosynthesis of phytomelatonin and its receptor persist under constant light (Figures 1 and 2), indicating that the phytomelatonin signaling might be regulated by the internal biological clock. Furthermore, the rhythmicity in phytomelatonin signaling is required for maintenance of the diurnal stomatal closure (Figure 6) through regulating the ROS dynamics (Figure 3 and Figures S5-S7). Therefore, the possible relationships of phytomelatonin rhythms, redox network and the circadian clock genes are needed for further investigation. Additionally, it would be also interest to investigate what changes occur in this field in CAM (crassulacean acid metabolism) plants with different stomatal behaviors.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 31660595 to Q. C.) and Ten Thousand Youth Talent Program of Yunnan Province (to Q. C.).

CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHORS CONTRIBUTIONS

Qi Chen conceived the studies and designed the work, analyzed and interpreted the data, and wrote and revised the manuscript. Dongxu Li, Jian Wei, and Zhongping Peng performed most of the experiments and analyzed the data. Wenna Ma, Qian Yang, Wei Sun, Wei Yang, Wei Chang, and Dashi Yu performed parts of some experiments or provided technical assistance for this work. Zed Rengel, Xiaodong Xu, Li Yuan, Zhongbang Song, Jianbo Shen, and Xiuming Cui analyzed the data and commented on the writing of the manuscript. Qi Chen, Zed Rengel, Xiaodong Xu, and Russel J. Reiter interpreted the data and revised the manuscript. All authors discussed the results and commented on the manuscript.

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SUPPORTING INFORMATION

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How to cite this article: Li D, Wei J, Peng Z, et al. Daily rhythms of phytomelatonin signaling modulate diurnal stomatal closure via regulating reactive oxygen species dynamics in *Arabidopsis*. *J Pineal Res*. 2020;68:e12640. https://doi.org/10.1111/jpi.12640