Research Article

Cyclic 3-hydroxymelatonin exhibits diurnal rhythm and cyclic 3-hydroxymelatonin overproduction increases secondary tillers in rice by upregulating *MOC1* expression

Geun-Hee Choi, Kyoungwhan Back*

Department of Biotechnology, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 61186, Republic of Korea
*Correspondence: kback@chonnam.ac.kr; Tel: +82 62 530 2165

Running title: Diurnal rhythm of cyclic 3-hydroxymelatonin

Received: July 25, 2019; Accepted: August 21, 2019

ABSTRACT

Cyclic 3-hydroxymelatonin (c3OHM) is a major metabolite of melatonin in plants produced by the enzymatic action of melatonin 3-hydroxylase (M3H). However, the function of c3OHM in plants is unclear. Here, we report that *M3H* mRNA and c3OHM levels display diurnal rhythms with peaks at night, but not in a circadian manner. This diurnal rhythmicity occurred predominantly in the late vegetative growth stage (8 weeks after germination), but was absent in the early vegetative growth stage. Transgenic rice plants overexpressing or underexpressing *M3H* were generated to investigate the physiological roles of diurnal production of c3OHM. The *M3H*-overexpression (OE) line exhibited higher M3H activity and c3OHM production than the wild-type, and vice versa for the *M3H*-underexpression rice (RNAi). The seedling growth phenotype of the OE and RNAi lines was comparable to that of the wild-type but exhibited pleiotropic phenotypic defects at the reproductive stage, such as decreased height, biomass, grain yield, and fertility. Of note, the OE rice showed significantly increased numbers of secondary tillers and panicles. The increase in tiller number of the OE line was linked to increased expression of tiller-related genes, such as *MOC1* and *TB1*, suggesting that the diurnal rhythm of c3OHM production is associated with the tiller number, a pivotal agronomic trait governing grain yield in rice.

Key words: Cyclic 3-hydroxymelatonin; diurnal rhythm; M3H; melatonin; RNAi; tiller number; transgenic rice.

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1. INTRODUCTION

Considerable effort has focused on elucidating the functions of melatonin in plants. The first such study in 1969 reported that melatonin inhibits the mitotic spindle in endosperm cells of *Scadoxus multiflorus* (1). After thirty years, melatonin was reported to delay flowering in the short-day plant *Chenopodium rubrum* (2), suggesting involvement in plant reproductive growth. These initial findings on the mode of action of melatonin in plants led to the discovery of other physiological roles of melatonin in addition to the identification of melatonin in plants (3,4).

Exogenous melatonin application promotes normal plant growth and development such as germination (5, 6), root growth (7, 8), cell proliferation (9, 10), and night seedling growth (skotomorphogenesis) (11). It also attenuates the harmful effects of various stress conditions,

including salt (12, 13), senescence (14, 15), cold (16, 17), pathogens (18, 19), heavy metals (20, 21), and ER stress (22), among others (23, 24).

The above-mentioned functions of melatonin in plants have been confirmed by molecular genetics, including via transgenic approaches (7, 13, 20, 25-30), where almost all genes related to the melatonin biosynthetic pathway in plants have been cloned (31). In addition, many transcription factors essential for melatonin biosynthesis have been cloned from cassava (21, 32, 33).

In plants, melatonin is enzymatically converted into a variety of metabolites, such as 2-hydroxymelatonin (2OHM) and cyclic 3-hydroxymelatonin (c3OHM) (34), whereas in animals c3OHM is non-enzymatically formed from melatonin via the interaction with two hydroxyl radicals (·OH) (35). The key genes responsible for the production of these melatonin metabolites in rice have been cloned. These genes are melatonin 2-hydroxylase (*M2H*) and melatonin 3-hydroxylase (*M3H*), members of the 2-oxoglutarate-dependent dioxygenase family (36,37).

Treatment of the roots of rice seedlings with melatonin increases the production of 2OHM and c3OHM; the increase of c3OHM production is greater than that of 2OHM, suggesting that melatonin is rapidly metabolized to c3OHM in preference to 2OHM in rice plants (37). The presence of highly functional melatonin catabolic genes suggests that melatonin is regulated at the physiological low level, or that the functions of these metabolites in plants are different from those of melatonin. To determine whether these melatonin metabolites are degraded nonfunctional products, we first investigated the function of c3OHM by modulating the expression of *M3H* (Figure 1A). The *M3H* transgenic rice plants produced altered levels of c3OHM, which resulted in pleiotropic effects on tiller number, fertility, and grain yield by regulating a couple of genes controlling tiller number in rice such as MONOCULM1 (*MOC*1) and TEOSINTE BRANCHED1 (*TB1*). Also, the *M3H* transcript level exhibited a diurnal rhythm, with a peak at night in parallel with the peak c3OHM level, suggesting for the first time that the role of c3OHM in plant growth and development is different from that of melatonin.

2. MATERIALS AND METHODS

2.1. Generation of *M3H* transgenic rice plants.

Transgenic rice plants overexpressing rice *M3H* (GenBank accession number AK067086) were generated by cloning full-length *M3H* into the pIPKb002 binary vector, which is designed to constitutively express a transgene under the control of the maize ubiquitin promoter (38). In brief, the full-length *M3H* sequence was first amplified by PCR using specific primers (forward 5'-AAA AAG CAG GCT CCA TGG CGG GAG CAA GAT-3'; reverse 5'-AGA AAG CTG GGT CTA CTG GTCCAG CTT-3') and a cDNA provided by the National Institute of Agrobiological Sciences (37,39). The resulting *M3H* PCR product was further amplified using a second primer set containing 14 nt of the *attB1* sequence (forward 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CT-3'; reverse 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT-3') using the first PCR product as the template. The product of the second *M3H* PCR was gel-purified and cloned into the pDONR221 Gateway vector (Invitrogen, Carlsbad, CA, USA) via the BP recombination reaction. The pDONR221-M3H gene entry vector was next recombined with the pIPKb002 destination vector via LR recombination to yield the pIPKb002-M3H binary plasmid (Figure 1B).

To downregulate *M3H*, the pTCK303 binary vector (40, gift from Dr. Kang Chong of the

Institute of Botany, Chinese Academy of Sciences, Beijing, China) was employed. A 540 bp *N*-terminal *M3H* fragment was amplified by PCR using specific primers (forward 5'-ACT AGT ATG GCG GGA GCA AGA-3' [*Spe*I site underlined]; reverse 5'-GAG CTC GCT TTT AGT CTC TGA-3' [*Sac*I site underlined]). The resulting *M3H* PCR product was first cloned into the T&A cloning vector (T&A:M3H; RBC Bioscience, New Taipei City, Taiwan), and the *M3H* insert (antisense) was digested by *Sac*I and *Spe*I and ligated into the pTCK303 vector, which had been digested by the same restriction enzymes. Thereafter, the sense fragments of the *M3H* insert were gel-purified from the T&A:M3H plasmid after digestion with *Kpn*I and *Bam*HI. These *Kpn*I and *Bam*HI inserts were further ligated into the above-mentioned pTCK303 vector harboring the corresponding antisense fragments, which were predigested with *Kpn*I and *Bam*HI. The resulting pTCK303:M3H RNAi binary vector (Figure 1C) and pIPKb002:M3H vector were transformed into *Agrobacterium tumefaciens* LBA4404, followed by transformation into rice as described previously (41).

2.2. Total RNA isolation and semi-quantitative reverse transcription PCR.

Total RNA was extracted from T_2 homozygous transgenic rice leaves (100 mg) using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) and treated with DNase I (Qiagen). RNA (1 µg) was reverse-transcribed using RevertAid reverse transcriptase (Thermo Scientific Fermentas, St. Leon-Ro, Germany) and 500 ng of an oligo(dT)18 primer at 42°C for 1 h. The resulting cDNA (0.2 µL) was amplified by PCR using the *M3H* forward primer 5'-AGG CTT AAG GAT GGC CTA-3' and reverse primer 5'-GGT ACC CTA CTG GTC CAG CTT-3'. The rice ubiquitin-5 gene (*UBQ5*) served as the loading control (28).

2.3. Quantification of c3OHM and melatonin.

To quantify c3OHM and melatonin levels, frozen rice samples (100 mg) were pulverized to a powder in liquid nitrogen using a TissueLyser II (Qiagen) and extracted with 1 mL of chloroform. The chloroform extracts were evaporated until dryness and dissolved in 100 µL of 40% MeOH. Aliquots of 10 µL were separated on a high-performance liquid chromatograph (HPLC) equipped with a fluorescence detector (Waters, Milford, MA, USA). We employed an Atlantis C18 column (Waters; 3.9 × 150 mm) with a methanol gradient from 30 to 45% within 12 min and isocratic elution of 45% for 30 min at a flow rate of 0.25 mL/min. c3OHM and melatonin were detected by excitation at 280 nm and emission at 348 nm. Under these conditions, c3OHM and melatonin were eluted at 19 and 31 min, respectively (37).

2.4. *In vitro* measurement of M3H activity.

Rice samples (100 mg) were homogenized in 10 mM Tris-HCl buffer (pH 8.0) and centrifuged for 15 min at 12,500 × *g* at 4°C. The soluble supernatants (40 µL) were utilized for assaying M3H activity. Each reaction consisted of a total volume of 100 µL of 10 mM Tris-HCl buffer (pH 8.0) containing 100 µM $FeSO_4$, 160 µmol/L α-ketoglutarate, 1 mM ascorbic acid, 100 µM melatonin, and 400 µg of catalase at 37°C for 1 h, as described previously (37). The reaction was stopped by the addition of 50 µL of MeOH containing 40% acetic acid. Aliquots of 10 µL were subjected to HPLC (Waters) as described above. All measurements were conducted in triplicate. The protein concentration was determined by the Bradford method using a protein assay dye (Bio-Rad, Hercules, CA, USA).

2.5. Plant materials and melatonin treatments.

Surface-sterilized rice seeds (*Oryza sativa* cv. Dongjin) were grown on half-strength Murashige and Skoog (MS) medium without sucrose (MB Cell, Seoul, Korea) in vertically oriented square polystyrene dishes (SPL Life Sciences, Pocheon-si, Korea) for 7 days at 28°C, under a 12/12 h light/dark (LD) cycle with a 150 µmol/m^2/s^1 photosynthetic photon flux density using white light-emitting diode (LED) lamps (21 W; Hyundai LED, Ansan-si, Korea). The 7-day-old seedlings were transferred to 50 mL conical polypropylene tubes containing various concentrations of melatonin and incubated for 24 h as described above. As for a dose response results of melatonin treatments, all three concentrations (0.1 mM, 1 mM, 5 mM) were tested and showed the same trends of results. Thus, we only showed the results of 0.1 mM melatonin treatment. The rice seedlings were next dissected into root and shoot parts, rapidly frozen in liquid nitrogen, and stored at −80°C for further analyses. For the field experiment, 25-day-old seedlings grown in the growth chamber were transplanted to the paddy field at Chonnam National University, Gwangju, Korea (53 m a.s.l.; 35^{O}09′N and 126^{O}54′W). Fifteen seedlings per independent transgenic line were grown in the paddy field in straight rows, with one seedling per hill. Fertilizer was applied at 70 N/40 P/70 K kg/ha. The agronomic traits height, leaf biomass, panicle number, panicle length, grain weight, and filling rate were recorded at harvest.

2.6. Quantitative real time-PCR analysis.

Real-time PCR was performed in a Mic qPCR Cycler system (Bio Molecular Systems, Queensland, VIC, Australia) with specific primers and the Luna Universal qPCR Master Mix (New England BioLabs, Ipswich, MA, USA). Gene expression levels were analyzed using Mic RQ software (Bio Molecular Systems) and normalized to that of *ACT1* as described previously (11). The primer sequences were as follows: *MOC1* (forward 5'-GCT CGC CCT CCC TAT TAA AC-3'; reverse 5'-GAA GAC GAC GAG GAG TGG AG-3') and *TB1* (forward 5'-CCT ACC ATG AGA GAA GAG ACC A-3'; reverse 5'-GTA GTG GGC TAT GAT CAG ATG TG-3').

2.7. Hydrogen peroxide and malondialdehyde assays.

Frozen leaves (0.05 g) were ground to a powder in liquid nitrogen and homogenized with 1 mL of homogenization buffer (50 mM Na-P [pH 6.5], 1 mM hydroxylamine). The extracts were centrifuged at 12,500 × *g* for 15 min at room temperature. The supernatants were collected and mixed with 0.5 mL of reaction buffer (20% H_2SO_4, 0.1% titanium chloride) and centrifuged at 12,500 × *g* for 10 min. The H_2O_2 content was quantified at a wavelength of 410 nm using a spectrophotometer (Optizen, Seoul, Korea) with a molar extinction coefficient of 0.28 µM/cm. To assay the malondialdehyde (MDA) level, frozen rice-leaf samples (50 mg) were ground to a powder in liquid nitrogen using a TissueLyser II (Qiagen) and extracted with 1.5 mL of reaction buffer containing 0.5% thiobarbituric acid (TBA) and 20% trichloroacetic acid (TCA). After centrifugation at 12,500 × *g* for 15 min, the supernatant was boiled at 95°C for 25 min and incubated on ice for 5 min. The MDA level was determined at wavelengths of 440, 532, and 600 nm using a spectrophotometer (Optizen), as described previously (42). MDA was quantified using a molar extinction coefficient of 156 nM/cm.

2.8. Quantification of 2OHM levels.

Frozen rice samples (100 mg) were ground to a powder in liquid nitrogen using TissueLyser II (Qiagen) and extracted with 1 mL of chloroform. The chloroform extracts were evaporated until dryness and dissolved in 100 µL of 40% MeOH. Aliquots (10 µL) were subjected to HPLC with UV detection (Waters), as described previously (37). In brief, the samples were separated on a Sunfire C18 column (Waters; 4.6 × 150 mm) with isocratic elution using 35% MeOH in 0.3% trifluoroacetic acid for 25 min at a flow rate of 0.25 mL/min. The 2OHM detected at 254 nm was eluted at 18.9 min.

2.9. Statistical analysis.

Data were analyzed by analysis of variance using IBM SPSS Statistics software (ver. 23.0; IBM Corp., Armonk, NY, USA). Means with different letters or asterisks indicate significantly different values at $P < 0.05$ according to a *post hoc* Tukey's honestly significant difference (HSD) test. Data are presented as means ± standard deviation.

3. RESULTS

3.1. Generation of *M3H* transgenic rice plants.

To examine the physiological role of c3OHM in rice (Figure 1A), we generated transgenic rice plants with an altered c3OHM level. From the initial nine and six T_0 transgenic lines, three independent homozygous transgenic lines (T_2) with *M3H* overexpression (OE) or underexpression (RNAi) were generated by *A. tumefaciens*-mediated transformation. Semi-quantitative RT-PCR indicated that *M3H* transcript was overexpressed in the three OE lines and significantly reduced in the three RNAi lines (Figure 1D). Indeed, the in vitro M3H activity was lower and higher in the RNAi and OE lines, respectively, compared to the wild-type (Figure 2A). To determine whether these molecular and biochemical data were associated with the level of c3OHM, 7-day-old seedlings were subjected to rhizospheric treatment with 100 µM melatonin for 24 h, and the c3OHM level in the corresponding shoot was determined. The OE and RNAi lines had higher and lower (except line 2), respectively, c3OHM levels than the wild-type (Figure 2B). The reason for the aberrant result with line 2 is unclear; thus, line 2 was excluded from subsequent field experiments. Seven-day-old seedlings were treated with 0.5 mM cadmium for 3 days to induce melatonin synthesis. All RNAi lines produced more melatonin than the wild-type due to suppression of the conversion of melatonin to c3OHM. However, the OE lines had melatonin contents comparable to that of the wild-type (Figure 2C). Overall, *M3H* is implicated in the production of c3OHM in vivo.

To gain insight into the role of c3OHM in the plant response to oxidative stress, 7-day-old seedlings treated with 100 µM melatonin for 24 h were subjected to rhizospheric application of 0.2 m cadmium. As shown in Figure 2D, the c3OHM level was decreased at 3 h after cadmium treatment, and was 50% and 400% lower at 6 and 24 h, respectively, suggesting that c3OHM production is suppressed by oxidative stress. In contrast, the 2OHM level fluctuated throughout the time course, suggesting that 2OHM is unlikely to be related to oxidative stress (Figure 2E). This result is consistent with a previous report that 2OHM possesses very low reactivity towards free radicals (43). In sum, although both c3OHM and 2OHM are major melatonin metabolites in plants, they play different roles in the response to oxidative stress. c3OHM, rather than 2OHM, is likely involved in the scavenging of reactive oxygen species

(ROS) upon oxidative stress. In contrast, c3OHM may be readily metabolized to other metabolites in the presence of oxidative stress, but 2OHM is stable or its conversion to its indolinone (oxindole) tautomer. These data require further investigation.

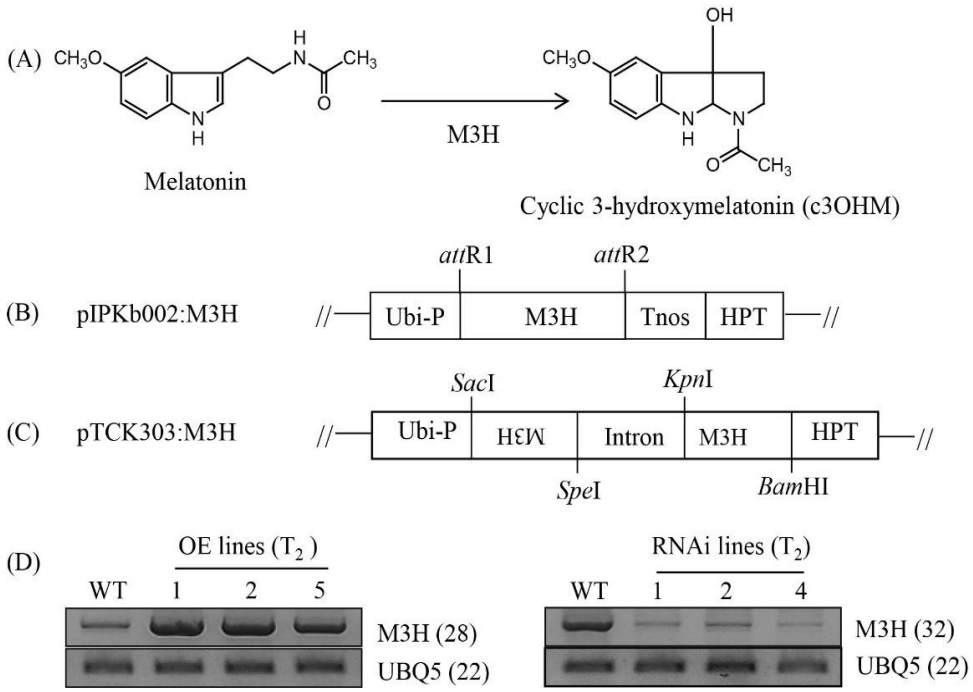

Fig. 1. Schematic diagram of M3H activity, binary vectors, and RT-PCR analysis results.

(A) Reaction scheme of M3H. (B) Binary vector used for M3H overexpression. (C) Binary vector used for M3H RNAi. (D) RT-PCR analyses of homozygous T_2 transgenic lines grown for 8 weeks in the growth room. M3H, melatonin 3-hydroxylase; Ubi-P, maize ubiquitin promoter; HPT, hygromycin phosphotransferase; attR1 and attR2, att recombination sites; WT, wild-type; UBQ5, rice ubiquitin5 gene; OE, M3H-overexpression line; RNAi, M3H-underexpression line. The GenBank accession numbers of M3H and UBQ5 are AK067086 and Os03g13170.

3.2. Diurnal rhythms of *M3H* transcript and c3OHM levels.

According to the Rice XPro Database (44; http://ricexpro.dna.affrc.go.jp), *M3H* expression shows a diurnal rhythm with a peak at night in the late vegetative growth stage (around 8 weeks after germination), but has no diurnal rhythm during early vegetative growth. RT-PCR analyses showed that the *M3H* mRNA level did not exhibit a diurnal rhythm in 10-day-old rice seedlings, but peaked at night and troughed during the day in 14-week-old rice plants grown in the field (Figure 3A). qRT-PCR analyses also confirmed the diurnal rhythm of *M3H* expression during these growth stages in the wild-type (Figure 3B). The *M3H* transcript level was higher in the OE lines relative to the wild-type and did not show a diurnal rhythm (Figure 4C), also in the RNAi lines the *M3H* transcript level exhibited a disrupted diurnal rhythm unlike that of the wild-type and its expression level was markedly lower (Figure 4D). The diurnal rhythm of the *M3H* mRNA level was closely associated with that of its product c3OHM in the wild-type (Figure 4E). The peak c3OHM level at night was about twofold higher than that during the day. However, the diurnal rhythm of the c3OHM level was disrupted in the OE and RNAi lines.

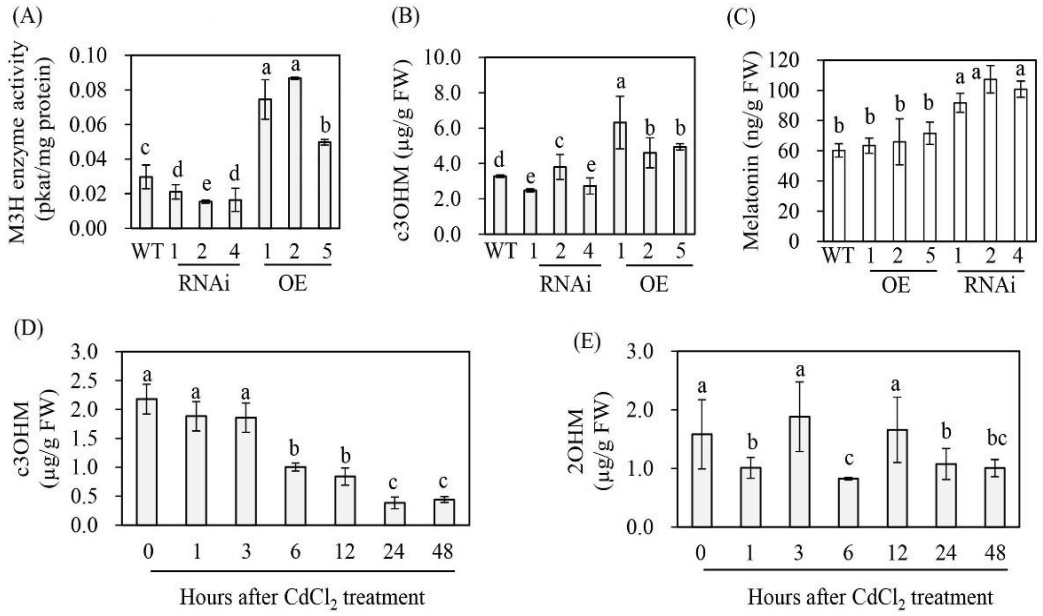

Fig. 2. Biochemical analyses of *M3H* transgenic rice plants.

(A) Measurement of in vitro M3H activity in rice plants. (B) Cyclic 3-hydroxymelatonin (c3OHM) level in rice plants treated with 100 μM melatonin for 24 h. (C) Melatonin level in rice plants. (D) Effects of cadmium treatment on the c3OHM level. (E) Effects of cadmium treatment on the 2-hydroxymelatonin (2OHM) level. Seven-day-old rice seedlings were used for in vitro measurement of M3H activity. The roots of these seedlings were treated with 100 μM melatonin for 24 h for measurement of the c3OHM level (B) or with 0.5 mM cadmium for 3 days under a 12/12 h light/dark (LD) cycle for measurement of the melatonin level (C). Seven-day-old rice seedlings pretreated with 100 μM melatonin for 24 h were transferred to new plastic tubes containing 0.5 mmol/L cadmium for the indicated times under continuous light (D, E). Values are means ± SD of three independent experiments. Different letters indicate significant differences (Tukey's post hoc HSD test; $P < 0.05$).

We next quantified the *M3H* transcript level to determine whether its diurnal rhythm was linked to a free-running internal circadian clock (45). Fourteen-week-old rice plants grown in the paddy field were subjected to continuous light or continuous darkness (Figure 4). RNA samples from the aerial parts of rice plants were prepared at 4 h intervals. The *M3H* mRNA level was determined by qRT-PCR using the actin gene as an internal control. The *M3H* mRNA level was low during the day at Zeitgeber (ZT) 11 and ZT 15, but was induced sevenfold at ZT 23 during the night, which is a typical diurnal rhythm (Figure 4A,C). However, the *M3H* mRNA level did not show a diurnal rhythm during the subsequent continuous light (Figure 4A) or darkness (Figure 4C), indicating that it is not regulated by a circadian clock. Notably, the *M3H* transcript level was elevated during the continuous dark condition, suggesting induction of its expression by darkness. Of interest, the diurnal expression of *M3H* was linked to enhanced synthesis of c3OHM during continuous darkness (Figure 4D). This indicates that c3OHM is involved in the dark-induced rhythm in plants, as is melatonin in animals. Thus, c3OHM is likely the functional counterpart in plants of melatonin in animals in terms of its induction at night, although the c3OHM level does not show a circadian rhythm in plants.

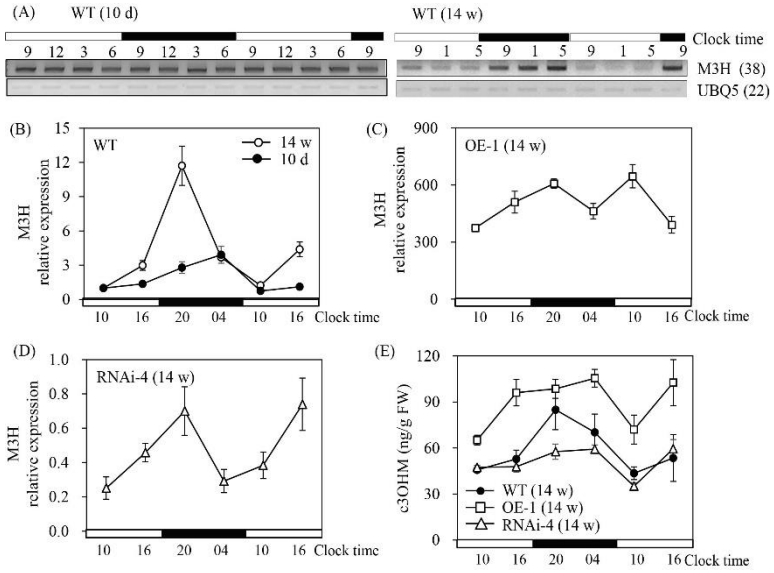

Fig. 3. Diurnal rhythm of the *M3H* mRNA and c3OHM levels in transgenic and wild-type rice plants.

(A) RT-PCR analyses of the diurnal rhythm of the M3H mRNA level in 14-week-old and 10-day-old rice leaves. (B) qRT-PCR analysis of the diurnal rhythm of the M3H mRNA level in 14-week-old and 10-day-old rice leaves. (C) qRT-PCR analysis of the diurnal rhythm of the M3H mRNA level in 14-week-old OE rice leaves. (D) qRT-PCR analysis of the diurnal rhythm of the M3H mRNA level in 14-week-old RNAi rice leaves. (E) Diurnal rhythm of the c3OHM level in 14-week-old transgenic and wild-type rice leaves. Fourteen-week-old rice leaves were harvested from the paddy field at the indicated day and night time points. Ten-day-old rice leaves were from the growth chamber. The GenBank accession numbers of M3H and Actin1 are AK067086 and Os03g50885, respectively.

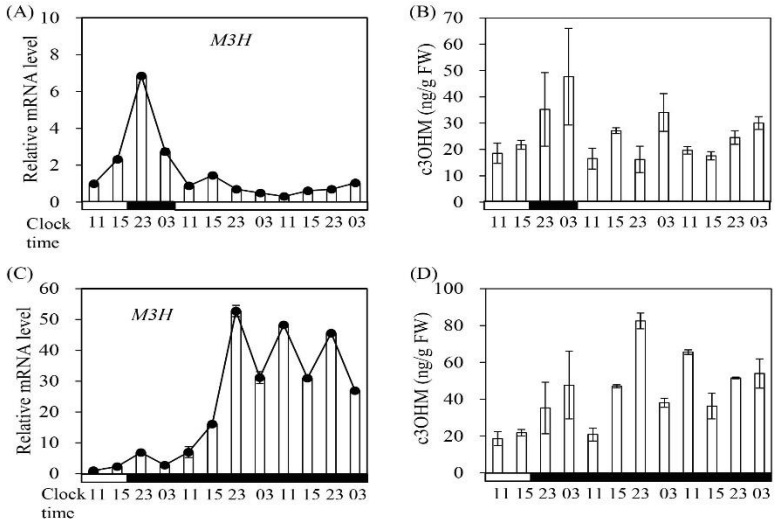

Fig. 4. Diurnal rhythms of *M3H* and c3OHM.

Diurnal rhythms under a 12/12 h LD cycle followed by 46 h of continuous light of the (A) M3H mRNA level and (B) c3OHM level. Diurnal rhythm under a 12/12 h LD cycle followed by 46 h of continuous darkness of the (C) M3H mRNA level and (D) c3OHM level. The samples were harvested from 14-week old rice leaves grown in the paddy field.

3.3. Growth phenotypes of transgenic rice seedlings.

Based on the darkness-induced synthesis of c3OHM, we hypothesized that the day/night cycle influences seedling growth. Thus, transgenic and wild-type rice seeds were seeded and grown on half-strength MS medium under continuous light (LL) (150 µmol/m^2/s^1), continuous dark (DD), or a 12/12 h LD cycle for 7 days at 28°C. As shown in Figure 5, the shoot lengths of the OE and RNAi lines were not different from those of the wild-type under any of the growth conditions. Also, root growth of the OE and RNAi lines was indistinguishable from that of the wild-type. Therefore, the altered *M3H* mRNA level did not exert a visible detrimental effect on 7-day-old seedlings. In summary, the *M3H* RNAi rice plants, which have a lower c3OHM level, are phenotypically different from the *SNAT2* RNAi rice lines, which have a lower melatonin level and exhibited the semi-dwarf seedling phenotype (11,30). Thus, melatonin and c3OHM play different physiological roles in seedling growth under different light regimes.

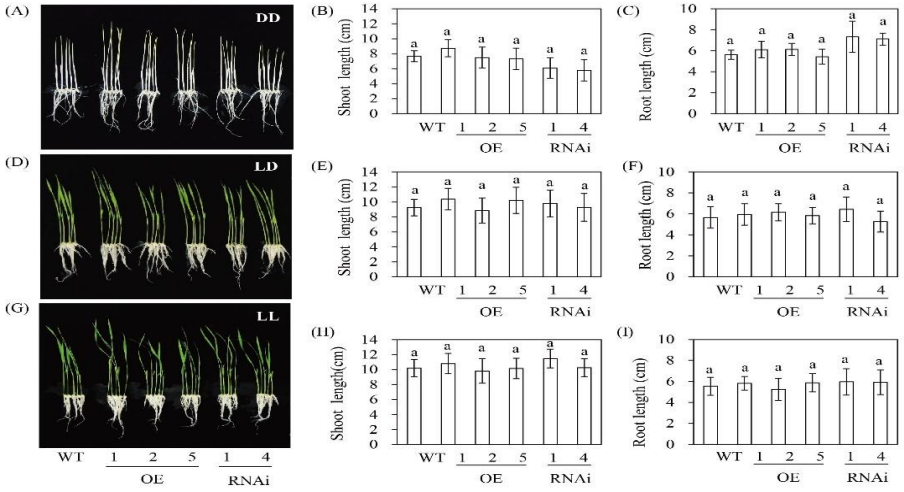

Fig. 5. Effect of light regimes on the growth of wild-type and transgenic rice seedlings.

(A) Phenotype, (B) shoot length (n = 20), and (C) root length (n = 20) of rice seedlings grown under continuous darkness (DD). (D) Phenotype, (E) shoot length (n = 20), and (F) root length (n = 20) of rice seedlings grown under a 12/12 h LD cycle. (G) Phenotype, (H) shoot length (n = 20), and (I) root length (n = 20) of rice seedlings grown under continuous light (LL). WT, wild-type; OE, M3H overexpression lines; RNAi, M3H suppression lines. Dehusked seeds were surface-sterilized and seeded on half-strength Murashige and Skoog (MS) medium for 7 days. Different letters indicate significant differences (Tukey's post hoc honestly significant difference [HSD] test; P < 0.05).

3.4. Alleviation of high-dose melatonin-mediated root inhibition in the OE lines.

Application of a high concentration of melatonin inhibits root growth in rice (8) and *Arabidopsis* (6). Here, we found that 5 mmol/L melatonin reduced root growth by 30% compared to the untreated control, whereas 1 and 2.5 mM melatonin did not significantly reduce root growth (Figure 6A, B). In contrast, the MDA level was increased in a melatonin concentration-dependent manner, suggesting that a high concentration of melatonin generates oxidative stress in rice as the level of MDA increased with exogenous application of increasing

concentrations of melatonin (Figure 6C). In contrast to the wild-type, *M3H* OE rice seedlings were tolerant to 5 mM melatonin (20% inhibition of root growth) (Figure 6D, E). However, in the RNAi lines, melatonin application inhibited root growth by 45% compared to the wild-type. The attenuation of melatonin toxicity was associated with its rate of conversion to c3OHM (Figure 6F). Thus, the effect of melatonin on root growth was ameliorated by its conversion to c3OHM in the OE lines. Therefore, c3OHM is not a waste product and *M3H* is directly involved in melatonin metabolism.

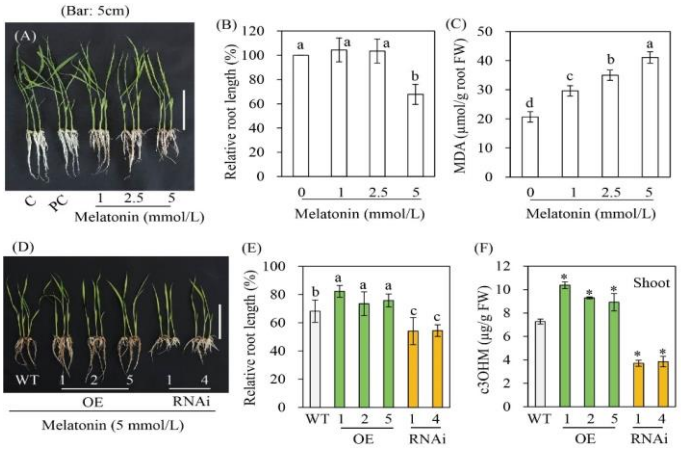

Fig. 6. Inhibition of the growth of rice root by exogenous melatonin.

(A) Phenotype, (B) root length (n = 20), and (C) malondialdehyde (MDA) level in response to melatonin in the wild-type. (D) Phenotype, (E) root length (n = 20), and (F) c3OHM level in response to 5 mM melatonin. C, water treatment; PC, 1% ethanol treatment; WT, wild-type; OE, M3H-overexpression lines; RNAi, M3H-suppression lines. Dehusked seeds were surface-sterilized and seeded on half-strength MS medium containing melatonin for 7 days. Melatonin was dissolved in ethanol. All melatonin-treated samples contained 1% ethanol, except C. Different letters indicate significant differences (Tukey's post hoc HSD test; P < 0.05). The root length of the untreated wild-type control was set as 100%.

3.5. Growth characteristics of transgenic rice plants in the paddy field.

We next assessed the effect of alteration of *M3H* on the growth parameters of rice plants in the paddy field at 9 weeks after germination (tillering [late vegetative] stage). The biomass, height, and tiller number were similar between the wild-type and *M3H* transgenic rice lines (Figure 7A, C–E). However, the main stem (culm) thickness of the wild-type was about 10.5 mm, whereas that of the OE lines was considerable thinner (Figure 7B, F). The culm thickness of the RNAi lines was slightly but non-significantly smaller than that of the wild-type.

Because c3OHM is an antioxidant (35), we measured the H_2O_2 and MDA levels of the rice plants. The H_2O_2 and MDA levels were similar between the wild-type and transgenic plants (Figure 7G, H). Therefore, alteration of c3OHM levels was unrelated to ROS scavenging activity during normal growth. In addition, the detached leaves of 10-week-old rice plants grown in the growth chamber were subjected to senescence treatments under a 12/12 h LD cycle. The response of the OE and RNAi lines to senescence was identical to that of the wild-type in terms of their MDA and chlorophyll levels (data not shown). These data indicate that a high level of c3OHM is not related to senescence tolerance. This is in contrast to the senescence tolerance of melatonin-rich transgenic rice lines (20).

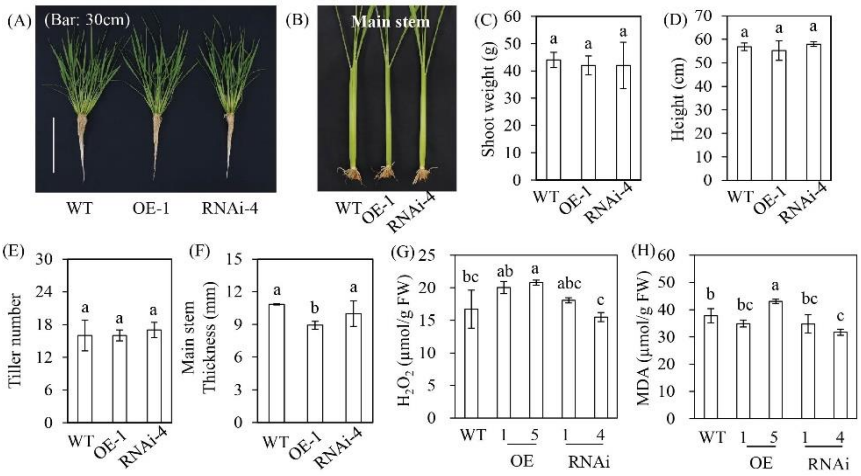

Fig. 7. Phenotypes of *M3H* transgenic lines grown for 9 weeks in the field.

(A) Representative phenotypes of wild-type and transgenic rice plants. (B) Photograph of the main stem. (C) Shoot weight (n = 3). (D) Plant height (n = 20). (E) Tiller number (n = 20). (F) Main stem thickness (n = 20). (G) Hydrogen peroxide level (n = 4). (H) MDA level (n = 4). Rice samples were collected from a paddy field after growth for 9 weeks post-germination. Different letters indicate significant differences (Tukey's post hoc HSD test; P < 0.05).

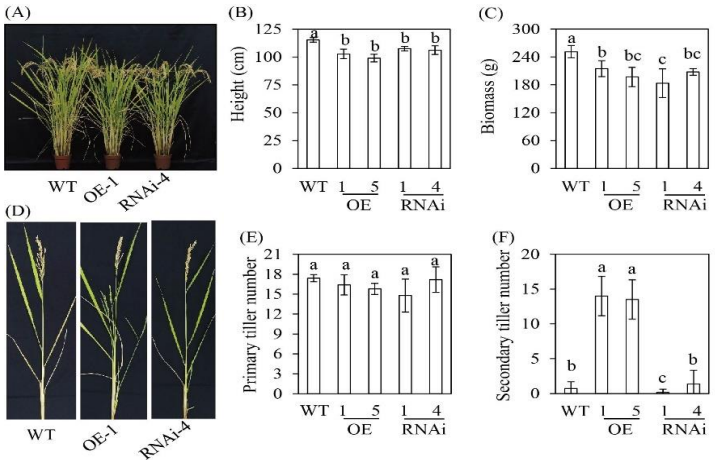

Fig. 8. Phenotypes of *M3H* transgenic lines grown for 17 weeks in the field.

(A) Representative phenotypes at the ripening stage. (B) Height (n = 20). (C) Shoot weight (n = 3). (D) Photograph of a tiller-bearing panicle. (E) Primary tiller number (n = 20). (F) Secondary tiller number (n = 20). Rice samples were collected from a paddy field after growth for 17 weeks post-germination. Different letters indicate significant differences (Tukey's post hoc HSD test; P < 0.05).

3.6. c3OHM overproduction increases secondary tiller number in the field.

We investigated the yield components of the transgenic rice plants to examine whether alteration of c3OHM production affects rice reproduction. Rice plants in the paddy field were harvested at 17 weeks after germination. The yield parameters were evaluated for 20 plants per transgenic line and the wild-type. The OE and RNAi lines were shorter and had a lower shoot weight than the wild-type (Figure 8A–C). In contrast, the primary tiller number was similar

between the wild-type and transgenic plants (Figure 8E). Surprisingly, the secondary tiller number was significantly greater in the OE lines compared to the wild-type and RNAi lines (Figure 8F), suggesting that c3OHM enhances tillering activity.

Due to their increased secondary tiller number, the panicle number was greatly increased in the OE lines relative to the wild-type and RNAi lines (Figure 9A, B). However, the OE lines had significantly reduced spikelet number, fertility, and grain yield compared to the wild-type (Figure 9C–F). Of note, the main stem of the OE and RNAi lines was thinner than that of the wild-type (Figure 9G), suggesting involvement of c3OHM in main culm growth.

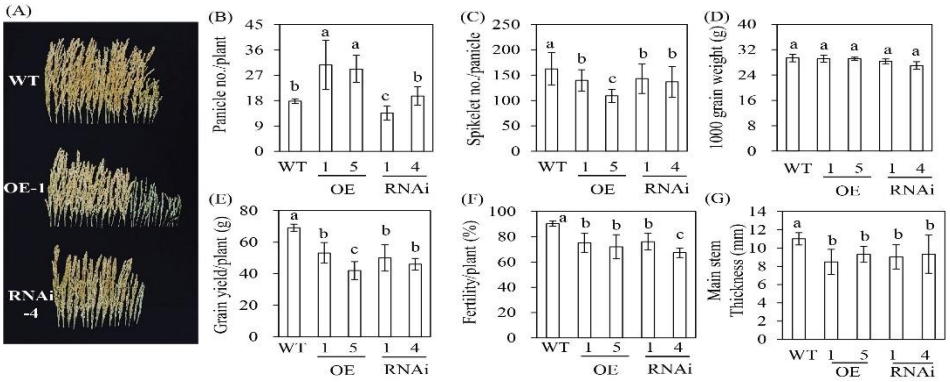

Fig. 9. Yield components of wild-type and transgenic rice seeds.

(A) Photographs of representative panicles. (B) Panicle number per plant (n = 20). (C) Spikelet number per panicle (n = 20). (D) One-thousand-grain weight (n = 20). (E) Grain yield per plant (n = 20). (F) Fertility (n = 20). (G) Main-stem thickness (n = 20). Rice samples were collected from a paddy field after growth for 17 weeks post-germination. Different letters indicate significant differences (Tukey's post hoc HSD test; P < 0.05).

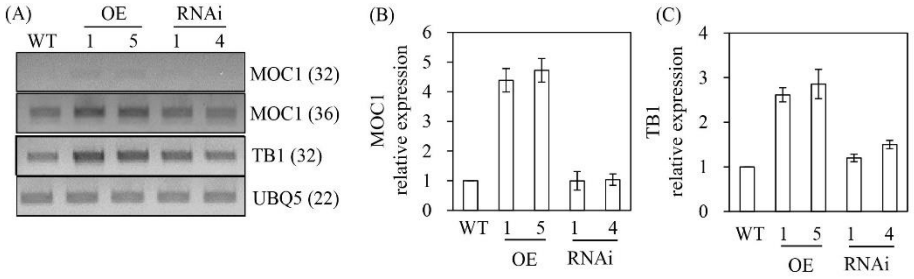

Fig. 10. Expression level of *MOC1* and *TB1* by RT-PCR.

(A) Semi-quantitative RT-PCR analysis of MOC1 and TB1 expression. (B) qRT-PCR analysis of MOC1 expression in wild-type and transgenic rice plants. (C) qRT-PCR analysis of TB1 expression in wild-type and transgenic rice plants. Rice leaves were collected from a paddy field after growth for 14 weeks post-germination. The GenBank accession numbers of MOC1, TB1, and UBQ5 are AY242058, AK107083, and Os03g13170, respectively.

Several genes are known to regulate tiller number in plants (45), among which MONOCULM1 (*MOC1*) and TEOSINTE BRANCHED1 (*TB1*) play a particularly important role. The *MOC1* and *TB1* transcript levels were enhanced in the OE lines, and not in the RNAi lines or the wild-type, suggesting that the increased tiller number in the OE lines is associated with enhanced expression of *MOC1* and *TB1* (Figure 10). Thus, c3OHM is functionally implicated in tiller production in rice.

4. DISCUSSION

4.1. Melatonin metabolites and corresponding genes in plants.

c3OHM is a metabolite of melatonin produced by its interaction with two hydroxyl radicals; thus, the c3OHM level is regarded as an *in vivo* biomarker of the hydroxyl radical level (47). In fact, the urinary c3OHM level increased significantly relative to that of controls when rats were exposed to ionizing radiation (47). In addition, c3OHM repairs DNA damaged by oxidative stress and inhibits injury to mitochondrial cytochrome C *in vitro* (48, 49). Although initially regarded as an end-product, c3OHM is further oxidized by two hydroxyl radicals to form N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (50). AFMK is produced enzymatically from melatonin by indoleamine 2,3-dioxygenase and myeloperoxidase (51-53). c3OHM is a key melatonin metabolite responsible for scavenging peroxyl radicals (·OOH), and exhibited its activity at a level two orders of magnitude higher than that of trolox in aqueous solution (35).

In water hyacinth, AFMK production shows a diurnal rhythm, with the peak level (20 ng/g leaves) occurring in the early dark phase of the LD cycle (50). The second plant melatonin metabolite discovered was 2OHM, the level of which is many orders of magnitude higher than that of melatonin (34). Rice *M2H*, which is responsible for 2OHM synthesis, has been cloned, and belongs to the 2-oxoglutarate-dependent dioxygenase (2-ODD) superfamily (36). Its expression is localized in chloroplasts and is induced in response to cadmium (54). Exogenous 2OHM application to both monocotyledonous and dicotyledonous plants enhanced their tolerance of combined cold and drought stress (55, 56). However, the physiological roles of 2OHM during plant growth and development remain to be investigated.

In contrast to animals, in plants c3OHM is mainly produced from melatonin in a reaction catalyzed by the 2-ODD M3H (37). Based on the *in vivo* conversion rate and catalytic efficiency of recombinant proteins (V_{max}/K_m), c3OHM rather than 2-OHM is the predominant melatonin metabolite in rice seedlings, but M3H is localized to the cytoplasm unlike M2H (chloroplasts) (37,54).

4.2. Possible roles of c3OHM diurnal rhythm and tiller production.

In this study, we first investigated the *M3H* mRNA and c3OHM levels during a LD cycle. Surprisingly, the *M3H* mRNA level exhibited a diurnal rhythm, with a peak at night during the late vegetative growth stage (about 8 weeks after germination) but not during early seedling growth (until 4 weeks after germination) (44). The diurnal rhythm of the c3OHM level was associated with that of the *M3H* mRNA level, suggesting involvement of c3OHM in late development. Of note, melatonin production exhibits no such diurnal rhythm, although rice serotonin *N*-acetyltransferase 2 (*SNAT2*) expression showed a diurnal rhythm with a peak at night (11). In cherry and *Arabidopsis*, the melatonin level peaks during the day (18, 57) whereas animals exhibit melatonin peak at night (58). However, a nocturnal melatonin peak was reported in *Chenopodium rubrum* (59). The diurnal rhythm of *M3H* expression and the c3OHM level was altered in the OE and RNAi lines, and to a greater degree in the OE lines than in the RNAi lines (Figure 3). Based on its potent scavenging of hydroxyl and peroxyl radicals (35, 60), the physiological roles of c3OHM may lie in regulating the cellular levels of ROS as melatonin does in plants (23) and animals (61, 62). However, the OE lines did not show greater tolerance to oxidative stresses, such as senescence and cadmium treatment, compared to the wild-type, suggesting that c3OHM does not scavenge ROS. In analogy, the levels of H_2O_2 and MDA at the tillering stage in the OE and RNAi lines were comparable to those in the wild-

type, and the transgenic lines had no seedling growth defect with the exception of a thin culm in the OE lines. During the tillering stage, rice produces a number of tillers on the bottom of the main culm. Thus, a mature rice plant possesses a main stem and multiple tillers (63). However, the transgenic lines exhibited defects in reproductive growth compared to the wild-type. The OE and RNAi lines at the harvest stage showed decreased grain yield, spikelet number, and fertility (Figure 9). These negative effects may be due to the altered c3OHM diurnal rhythm in those lines (Figure 3). The OE lines also had an increased number of tillers, particularly of the secondary tiller. The wild-type, OE, and RNAi lines each had 18 primary tillers, and the OE lines had up to 12 secondary tillers; however, the wild-type and the RNAi lines had none (Figure 8F). Thus, the panicle number in the OE lines was 1.6-fold higher than that in the wild-type (Figure 9B).

In summary, c3OHM is involved in tiller production, and is a determinant of the number of secondary tillers. In rice, tillering ability is governed by a series of parameters including light, plant density, and genetic factors (46, 63). For example, shade and a high plant density reduce the tiller number in rice. The major genes controlling tiller number in rice include *MOC1* and *TB1* (64, 65). A *moc1* knockout mutant lost tillering activity, yielding a main culm lacking tillers; in contrast, *MOC1* OE leads to a significant increase in tiller numbers, particularly secondary and tertiary tiller numbers (64). However, *TB1* OE or suppression has little effect on rice tiller formation (66). Thus, *MOC1* is responsible for the formation of secondary, tertiary, quaternary, and quinternary tillers (64). In accordance with the key role of *MOC1* in tiller promotion in rice, the *M3H* OE lines had a higher *MOC1* transcript level than the wild-type. Therefore, c3OHM, a major melatonin metabolite, plays a role in regulating formation of the secondary, but not the primary tiller. Consistent with our findings, transgenic rice plants expressing sheep serotonin *N*-acetyltransferase (*SNAT*) or rice *SNAT1* produced slightly more panicles than the wild-type (20, 67), indicating that the increase in panicle numbers is attributable to an increased level of c3OHM rather than melatonin. In fact, the *M3H* RNAi line produced more melatonin than the wild-type, but a similar number of tillers, confirming that melatonin is unrelated to tiller formation.

Another possible link between diurnal c3OHM production and the regulation of tiller production is the onset of the diurnal rhythm of *M3H* expression at the tillering stage (9 weeks after germination), and its disappearance at the ripening stage (43; http://ricexpro.dna.affrc.go.jp). The data indicate that the diurnal rhythm of c3OHM production by *M3H* is regulated in the developmental stages in rice plants, in parallel with tiller production. Based on our results, we postulate that the rhythmic c3OHM production during the day/night cycle plays a role in modulating tiller production by modulating the expression of *MOC1* possibly via a plant melatonin receptor (68). Further study is required to determine the mechanism underlying the function of the diurnal rhythm of c3OHM production in plants.

ACKNOWLEDGMENTS

This research was supported by grants from the Next-Generation BioGreen 21 Program (SSAC Project No. PJ1325501) and the Basic Science Research Program of the National Research Foundation of Korea (2017R1A2A2A05069253) funded by the Ministry of Education, Republic of Korea.

AUTHORSHIP

Geun-Hee Choi carried out the experiments. Kyoungwhan Back designed, advised and

wrote the article.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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Please cite this paper as:
Choi, G.-H. and Back, K. 2019. Cyclic 3-hydroxymelatonin exhibits diurnal rhythm and cyclic 3-hydroxymelatonin overproduction increases secondary tillers in rice by upregulating MOC1 expression. Melatonin Research. 2, 3 (Aug. 2019), 120-138. DOI:https://doi.org/https://doi.org/10.32794/11250034.