Research Article

Rice *N*-acetylserotonin deacetylase regulates melatonin levels in transgenic rice

Kyungjin Lee, Ok Jin Hwang, 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

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ABSTRACT

A reverse melatonin biosynthetic pathway was recently discovered in plants, by which *N*-acetylserotonin (NAS) is converted into serotonin by *N*-acetylserotonin deacetylase (ASDAC) rather than into melatonin by *N*-acetylserotonin *O*-methyltransferase (ASMT). In this study, we generated transgenic rice plants in which *ASDAC* was either suppressed or overexpressed to determine whether *ASDAC* is functionally involved in melatonin biosynthesis. *ASDAC*-suppressed rice showed increased levels of NAS, 5-methoxytryptamine (5-MT), and melatonin, whereas *ASDAC*-overexpressed rice exhibited less melatonin synthesis than observed in the wild type. This finding is strong evidence of the role of *ASDAC* in melatonin biosynthesis in rice. The increased levels of 5-MT, which is produced either by ASDAC from melatonin or by serotonin *O*-methyltransferase (SOMT) from serotonin in *ASDAC*-suppressed rice, was ascribed to enhanced SOMT enzyme activity rather than increased transcripts, such as ASMT or caffeic acid *O*-methyltransferase (COMT) encoding SOMT activity.

Key words: *N*-acetylserotonin; 5-methoxytryptamine; melatonin; RNAi; serotonin; transgenic rice.

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1. INTRODUCTION

Melatonin is a multifunctional regulator that acts as a signal molecule or hormone in conjunction with potent antioxidants in both plants and animals (1-5). The alleviation of oxidative stress and endoplasmic reticulum (ER) stress by cadmium and tunicamycin treatment has been observed in animals and plants via pathways either dependent on or independent of melatonin receptors (6-10). Although melatonin was discovered in plants much later than in animals, considerable progress has been made in elucidating the physiological roles of melatonin and its biosynthetic pathway in plants (4, 11). Previous studies have shown that melatonin is clearly implicated in normal growth and development, as well as in plant defense responses (2, 12). The role of melatonin in growth and development includes the promotion of root growth (13), night seedling growth (14), sugar metabolism (15), secondary metabolite synthesis (16), senescence (17), and other processes (18). In plant defense, melatonin promotes protection against salt (4, 19), waterlogging (20), high temperature (21), and other stresses (22-24). In addition to the rapid progress in pharmacological studies of melatonin in plants, the successful cloning of genes encoding melatonin biosynthetic enzymes has almost completely elucidated the melatonin biosynthetic pathway (25). As opposed to animals, plants are capable of catalyzing melatonin into various melatonin metabolites including 2-hydroxymelatonin (2OHM) (26), cyclic 3-hydroxymelatonin (c3OHM) (27), and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (28). Among these melatonin metabolites, two distinct genes, melatonin 2-hydroxylase (*M2H*) and melatonin 3-hydroxylase (*M3H*), have been

successfully cloned from plants that catalyze melatonin into 2-OHM and c3OHM, respectively (26, 27). The 2OHM and c3OHM metabolites are reportedly associated with abiotic stress tolerance (29) and tiller production (30), respectively.

A reverse melatonin biosynthetic pathway was also recently reported in rice, such that *N*-acetylserotonin (NAS), the last substrate for melatonin biosynthesis, is deacetylated into serotonin by *N*-acetylserotonin deacetylase (ASDAC) (31). In rice, ASDAC also deacetylates melatonin into 5-MT, but with 10-fold less substrate affinity toward melatonin than observed in NAS (31). This ASDAC reaction is the opposite to that of serotonin *N*-acetyltransferase (SNAT) catalyzing the conversion of serotonin into NAS. Although the rice *ASDAC* gene has been cloned and its recombinant enzyme enzymatically characterized *in vitro*, its *in vivo* function remains completely unknown from the perspective of melatonin biosynthesis. In this study, to determine whether *ASDAC* is functionally linked to melatonin synthesis in rice, we generated transgenic rice plants in which the *ASDAC* gene was suppressed or overexpressed.

2. MATERIALS AND METHODS

2.1. Generation of *ASDAC* transgenic rice plants.

The rice full-length *ASDAC* gene (GenBank accession no. AK072557) was provided by the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan) (32). To knock down rice *ASDAC* gene expression, we used the pTCK303 RNAi binary vector (kindly provided by Dr. Kang Chong at the Institute of Botany, Chinese Academy of Sciences, Beijing, China) (33). An N-terminal *ASDAC* fragment (ca. 301 bp) was amplified by polymerase chain reaction (PCR) using specific primers (forward 5′-ACT AGT ATG GAA CAG CTG TGG GTG-3′ [*Spe*I site underlined]; reverse 5′-GAG CTC ACC ACG ATG CTT CGA AGT-3′ [*Sac*I site underlined]). The resulting *ASDAC* PCR product was initially cloned into the T&A cloning vector (T&A:ASDAC; RBC Bioscience, New Taipei City, Taiwan), and the antisense *ASDAC* insert was obtained by *Sac*I and *Spe*I double digestion and ligated into the pTCK303 vector, which had been digested by the *Sac*I and *Spe*I restriction enzymes. Thereafter, the sense fragment of the *ASDAC* insert was obtained from *Kpn*I and *Bam*HI digestion of the T&A:ASDAC plasmid and gel purified using DE81 ion exchange paper (Whatman, Maidstone, UK). The purified *Kpn*I and *Bam*HI insert was further ligated into the pTCK303 vector harboring the corresponding antisense fragments, which were predigested with *Kpn*I and *Bam*HI.

To overexpress the *ASDAC* gene, the full-length *ASDAC* sequence was first amplified by PCR using specific primers (forward 5′-AAA AAG CAG GCT CCA TGG AAC AGC TGT GGG-3′; reverse 5′-AGA AAG CTG GGT TTA GAG CGA GTG GAG GTG-3′) and cDNA provided by the NIAS. The first PCR product of the *ASDAC* gene (ca. 1,332 bp) was further amplified using a second primer set containing 14 nt of the *attB* 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 a template. The second *ASDAC* PCR product was gel purified and cloned into the pDONR221 gateway vector (Invitrogen, Carlsbad, CA, USA) via the BP recombination reaction. The pDONR221-ASDAC gene entry vector was then recombined with the pIPKb002 destination vector via LR recombination to yield the pIPKb002-ASDAC binary plasmid, which was designed to constitutively express the *ASDAC* transgene under control of the maize ubiquitin promoter (34). The pTCK303:ASDAC RNAi binary vector (Figure 1B) and pIPKb002:ASDAC vector (Figure 5A) were both transformed into *Agrobacterium tumefaciens* LBA4404, followed by transformation into rice as previously described (35).

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

Total RNA (100 mg) was extracted from transgenic rice plants using the 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 using PCR with the *ASDAC* forward primer 5′-GGC GCG CCA TGG AAC AGC TGT GGG-3′ and reverse primer 5′-CCG CGC GGC GAG CGA GTG GAG GTG CTT-3′. The primer sets for *ASMT1* and *COMT* were *ASMT1-F* 5′-CGC CAA GGC TCC CAG TAA CAA C-3′, *ASMT1-R* 5′-TGA TCG TGC GCA CTA CTG ACT CCG GC-3′, *COMT-F* 5′-ACA TAT GGG TTC TAC AGC CGC-3′, and *COMT-R* 5′-GGG TAC CCT ACT TTG TGA ACT CGA-3′. The rice ubiquitin-5 gene (*UBQ5*) served as the loading control, as previously described (14).

2.3. Quantification of serotonin, NAS, melatonin, and 5-methoxytryptamine

For serotonin, NAS, and 5-methoxytryptamine (5-MT) quantification, 100 mg powder was extracted with 1 mL methanol. The homogenates were centrifuged (11,500 × *g*, 5 min), evaporated to dryness, and dissolved in 0.1 mL 100% methanol. The samples were analyzed using reverse-phase high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA). Compounds were separated in a SunFire C18 column (Waters; 4.6 × 150 mm) with an isocratic elution profile of 6% (v/v) methanol for serotonin and NAS or 10% (v/v) methanol for 5-MT in water containing 0.3% trifluoroacetic acid at a flow rate of 1 mL/min. Detection of the compounds was monitored at 280 nm. All analyses were performed in triplicate. For melatonin detection, frozen rice samples (100 mg) were pulverized to a powder in liquid nitrogen using a TissueLyser II (Qiagen) and extracted with 1 mL chloroform. The chloroform extracts were evaporated until dryness and dissolved in 100 μL 40% MeOH. Aliquots of 10 μL were separated on an HPLC system equipped with a fluorescence detector (Waters, Milford, MA, USA). We used an Atlantis C18 column (Waters; 4.6 × 150 mm) with a methanol gradient of 42–50% within 27 min and isocratic elution of 52% for 35 min at a flow rate of 0.15 mL/min. Melatonin was detected by excitation at 280 nm and emission at 348 nm. Under these conditions, melatonin was eluted at 31 min.

2.4. 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 at a 150 μmol/m^2/s photosynthetic photon flux density using white light-emitting diode (LED) lamps (21 W; Hyundai LED, Ansan-si, Korea). Rice seeds were also germinated and grown in pots for 5 weeks under the same conditions. We transferred 7-day-old seedlings to 50-mL conical polypropylene tubes containing various chemicals such as NAS and melatonin and incubated for 24 h as described above. Samples were rapidly frozen in liquid nitrogen, and stored at −80°C for further analyses.

2.5. Measurement of *O*-methyltransferase activity.

Rice seedlings (0.1 g) were ground in 100 mM potassium phosphate buffer (pH 7.8) containing β-mercaptoethanol. The homogenates were centrifuged (13,500 × *g*, 5 min, 4°C), and the resulting supernatants were used as a crude enzyme solution to measure the activity of *O*-methyltransferase enzymes such as *N*-acetylserotonin *O*-methyltransferase (ASMT), caffeic acid *O*-methyltransferase (COMT), and serotonin *O*-methyltransferase (SOMT). Soluble extracts (30 μL) were incubated in a total volume of 100 μL 100 mM potassium phosphate buffer (pH 7.8) containing 0.5 mM NAS (or caffeic acid and serotonin) and 0.5 mM S-adenosyl-L-methionine at 37 °C for 1 h, and terminated by the addition of 50 μL methanol. A 10-μL aliquot was subjected to HPLC for quantification of *O*-

methylated products such as melatonin, ferulic acid, and 5-MT, depending on the added substrates. Ferulic acid was measured by HPLC with a gradient elution profile of 20–60% MeOH containing 0.3% trifluoroacetic acid at a flow rate of 0.8 mL/min (36). All measurements were performed in triplicate. Protein concentration was determined using the Bradford method with a kit (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analysis

Asterisks indicate significantly different values at $P < 0.05$, according to *post hoc* Tukey's honestly significant difference (HSD) tests. Data are presented as means ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Generation of *ASDAC*-suppressed transgenic rice plant.

To examine the functional role of the *ASDAC* gene in rice from the perspective of melatonin biosynthesis (Figure 1A), we generated transgenic rice plants in which the *ASDAC* gene was downregulated using RNAi technology. Three independent homozygous transgenic lines (T_2) with *ASDAC* underexpression (RNAi) were generated via *A. tumefaciens*-mediated transformation. *ASDAC* transcript levels were lower in the *ASDAC* RNAi transgenic rice lines than in the wild type (WT) as determined by RT-PCR analysis (Figure 1C), indicating the successful generation of *ASDAC* knockdown transgenic rice plants.

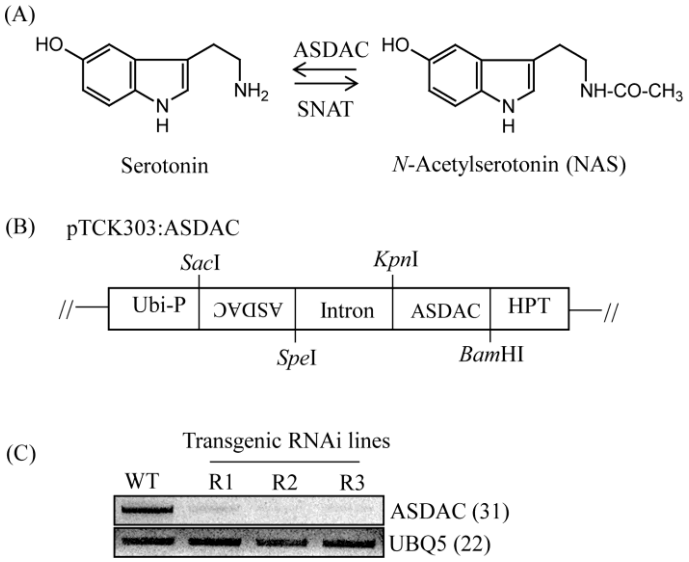

Fig. 1. Schematic diagram of ASDAC enzyme reaction, RNAi binary vector, and reverse-transcription polymerase chain reaction (RT-PCR) analysis results.

(A) ASDAC reaction scheme. (B) Binary vector used for ASDAC suppression. (C) RT-PCR analyses of homozygous T2 transgenic lines grown for 5 weeks in a growth room. ASDAC, N-acetylserotonin deacetylase; SNAT, serotonin N-acetyltransferase; Ubi-P, maize ubiquitin promoter; HPT, hygromycin phosphotransferase; WT, wild type; UBQ5, rice ubiquitin 5 gene; R1–R3, ASDAC underexpression line. The GenBank accession numbers for ASDAC and UBQ5 are AK072557 and Os03g13170, respectively.

To determine whether *in vivo* ASDAC enzyme activity was decreased in the transgenic lines, 7-day-old rice seedlings were rhizospherically treated with 1 mM NAS for 24 h; serotonin levels in

corresponding shoots were then determined. The RNAi lines had lower serotonin levels than the WT (Figure 2A) due to *ASDAC* suppression. However, 5-MT levels following 1 mM melatonin application in the RNAi transgenic lines were similar to those of the WT, perhaps due to the 10-fold lower ASDAC enzyme activity toward the melatonin substrate than the NAS substrate (31). These findings indicate that *ASDAC* is involved in regulating the balance between serotonin and NAS rather than the balance between melatonin and 5-MT *in vivo*. Because both *SNAT* and *ASDAC* are nuclear-encoded chloroplast-targeted enzymes (31), we propose that chloroplasts play a pivotal role in regulating melatonin synthesis in plants. Unlike plants, animals utilize mitochondria as key organelles for melatonin control because the melatonin biosynthetic enzyme ASMT and melatonin demethylase enzyme cytochrome P450 ($CP_{450}1B_1$) are localized in the mitochondria (37). $CP_{450}1B_1$ catalyzes melatonin into NAS, a reverse reaction of melatonin synthesis. Thus, the balance between NAS and melatonin plays a key role in melatonin production in animals (37).

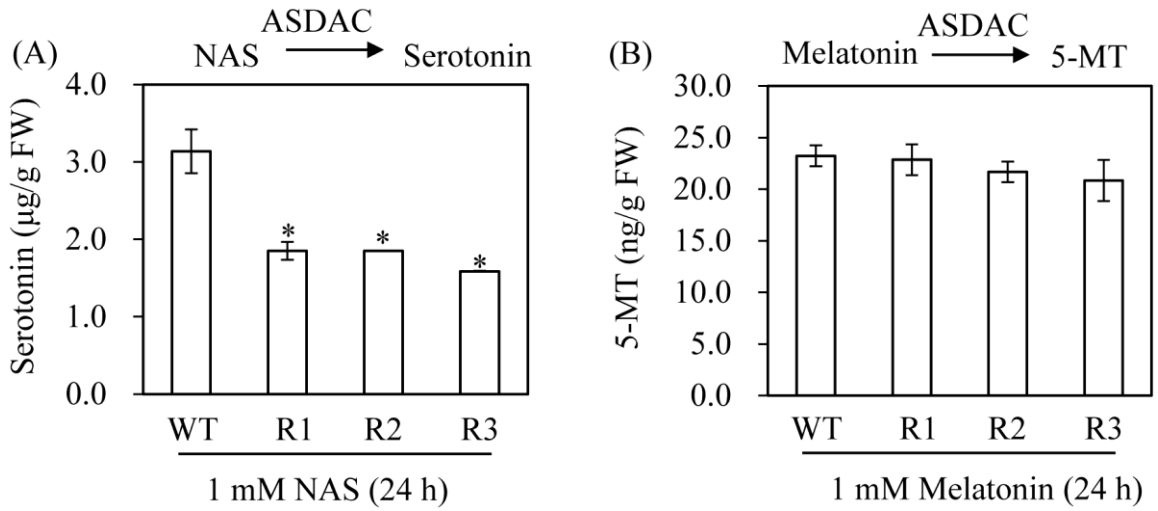

Fig. 2. Measurement of ASDAC activity *in vivo*.

(A) Serotonin content following 1 mM NAS treatment. (B) 5-MT content following 1 mM melatonin treatment. Roots of 7-day-old rice seedlings were treated with 1 mM NAS or melatonin for 24 h. Values are means ± standard deviation (SD) of three independent experiments. Asterisks indicate significant differences from the WT (Tukey's post hoc honestly significant difference [HSD] test; $P < 0.05$). NAS, N-acetylserotonin; 5-MT, 5-methoxytryptamine; WT, wild type; R1–R3, ASDAC suppression rice. Vertical SD bars were not shown due to the identical values of three replicates (A, R2 and R3).

3.2. Quantification of serotonin, NAS, 5-MT, and melatonin in RNAi transgenic rice

Detached rice leaves from 5-week-old rice plants were treated with 0.5 mM cadmium chloride for 3 days to induce melatonin biosynthesis under continuous light. Following cadmium treatment, serotonin levels reached 30 mg/g fresh weight (FW) in all rice plants including the WT (Figure 3A), whereas untreated rice leaves produced serotonin at around 20 μg/g FW in the WT and RNAi lines. The equal production of serotonin between WT and RNAi lines may be attributable to the far lower activity of ASDAC than of tryptamine 5-hydroxylase (T5H), a serotonin biosynthetic enzyme (25). In contrast, NAS levels were much higher in the RNAi lines, which produced 18,300 ng/g FW on average, than in the WT, which produced 8,000 ng/ g FW. This result suggests that *ASDAC* suppression led to enhanced NAS production in the RNAi lines. The increased NAS levels in the *ASDAC* RNAi lines resulted in the overproduction of melatonin compared to the WT (Figure 3D). Surprisingly, 5-MT levels were also enhanced in the RNAi lines because ASDAC possesses enzyme

activity to catalyze melatonin into 5-MT, although 5-MT synthesis activity was far lower than serotonin synthesis activity (31). Thus, the increased 5-MT in the RNAi lines may have been due to an increase in *O*-methyltransferase enzymes such as ASMT and COMT, which catalyze conversion of serotonin into 5-MT (25,38).

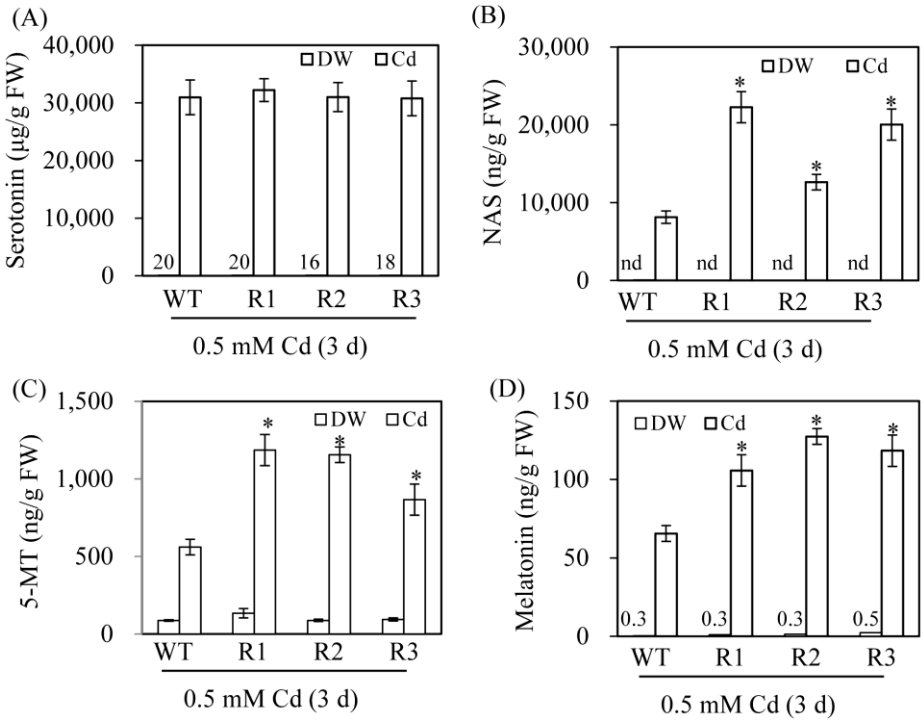

Fig. 3. Melatonin and melatonin intermediate content in *ASDAC*-suppressed rice.

(A) Serotonin content. (B) N-Acetylserotonin (NAS) content. (C) 5-Methoxytryptamine (5-MT) content. (D) Melatonin content. We challenged leaves detached from 5-week-old rice plants with 0.5 mM $CdCl_2$ for 3 days under continuous light and then subjected them to high-performance liquid chromatography (HPLC) analysis. Asterisks indicate significant differences from the WT (Tukey's HSD; $P < 0.05$). DW, distilled water control; nd > 1 ng/g FW.

To address this possibility, we measured the activity of several *O*-methyltransferase enzymes in the WT and RNAi lines challenged with cadmium stress. As shown in Figure 4, COMT activity showed no difference between the WT and RNAi lines (Figure 4A), whereas SOMT and ASMT were higher in the RNAi rice than in the WT (Figure 4B, C). However, the transcript levels encoding *ASMT*1 and *COMT* did not increase correspondingly in RNAi rice, indicating the irrelevance of transcriptional control of *ASMT*1 and *COMT* in the increase in 5-MT. These two transcripts were shown to be differently regulated by exogenous melatonin treatment in rice seedlings (Figure 4E). Collectively, these results suggest that the increase in 5-MT in RNAi rice was due to enhanced activity of *O*-methyltransferase enzymes such as SOMT. In view of melatonin synthesis, two *O*-methyltransferase enzymes have been involved depending on substrates such as NAS and serotonin. ASMT catalyzes NAS into melatonin whereas SOMT catalyzes serotonin into 5-MT. Thus far, it was known that both *ASMT* and *COMT* genes encode both ASMT and SOMT enzyme activity (25).

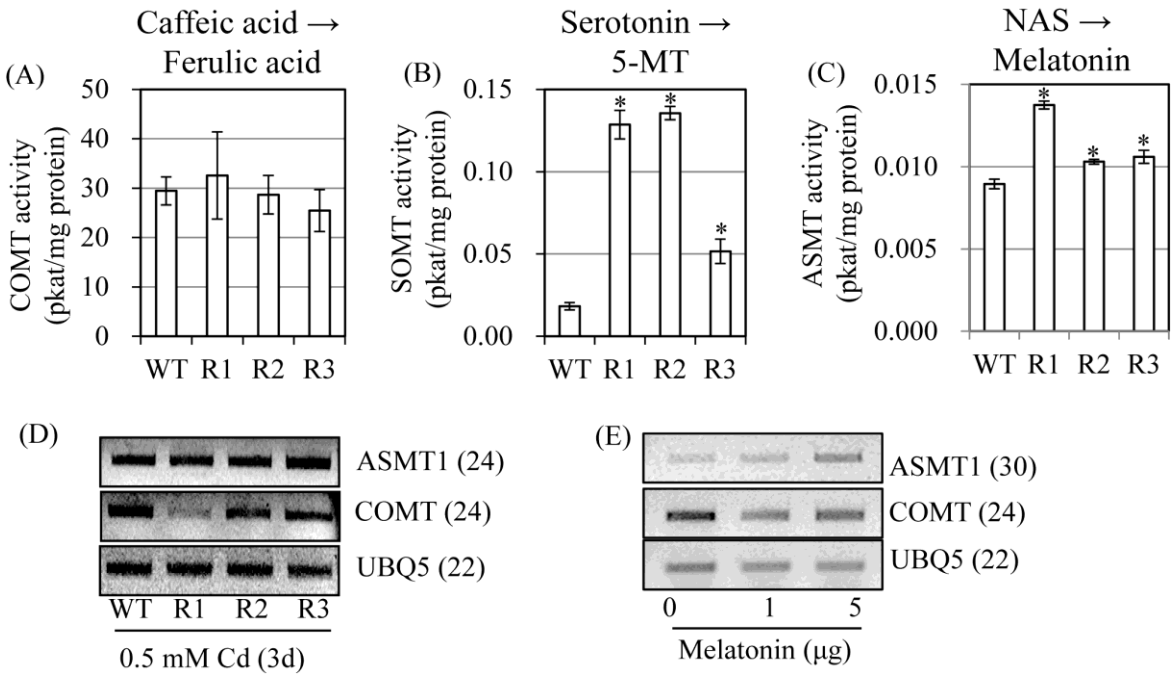

Fig. 4. Activity of various O-methyltransferase enzymes in the WT and RNAi lines.

(A) Caffeic acid O-methyltransferase (COMT) enzyme activity. (B) Serotonin O-methyltransferase (SOMT) activity. (C) N-Acetylserotonin O-methyltransferase (ASMT) activity. (D) RT-PCR analysis of ASMT1 and COMT. (E) Expression of ASMT1 and COMT transcripts following melatonin treatment. Samples (A–D) were identical to those shown in Figure 3. WT, wild type; R1–R3, RNAi lines; GenBank accession numbers are AK072740 for ASMT1 and AK064768 for COMT. For sample E, 1 μL ethanol containing 0, 1, or 5 μg melatonin (Sigma-Aldrich, St. Louis, MO, USA) was spotted on the lamina regions of leaves from rice seedlings grown for 6 d. These samples were then used for RNA extraction after 18 h as described previously (14).

3.3. Generation of *ASDAC*-overexpressed transgenic rice plants.

To gain further insight into the role of *ASDAC* in melatonin biosynthesis, we generated transgenic rice plants overexpressing the *ASDAC* gene under the control of the maize ubiquitin promoter (Figure 5A). Five independent T_1 transgenic rice lines were screened on half-strength MS medium containing 50 μg hygromycin per mL. Hygromycin-resistant 7-day-old seedlings were examined to determine whether the *ASDAC* transcript was overexpressed. *ASDAC* was overexpressed in all transgenic lines except line 5 (Figure 5B, C). Rice leaves detached from 6-week-old plants grown in pots were treated with 0.2 mM cadmium for melatonin induction for 3 d. The melatonin level of the WT was about 85 ng/g FW, whereas that of the *ASDAC* overexpression lines averaged 30 ng/g FW (Figure 5D). However, transgenic line 5 was devoid of *ASDAC* overexpression and thus produced melatonin similar to the WT. These *ASDAC* gene loss-of-function and gain-of-function analysis results clearly indicate that ASDAC is functionally linked to melatonin biosynthesis in rice plants. Further in-depth studies should be performed to determine the role of *ASDAC* in rice growth, development, and agronomic traits such as yield.

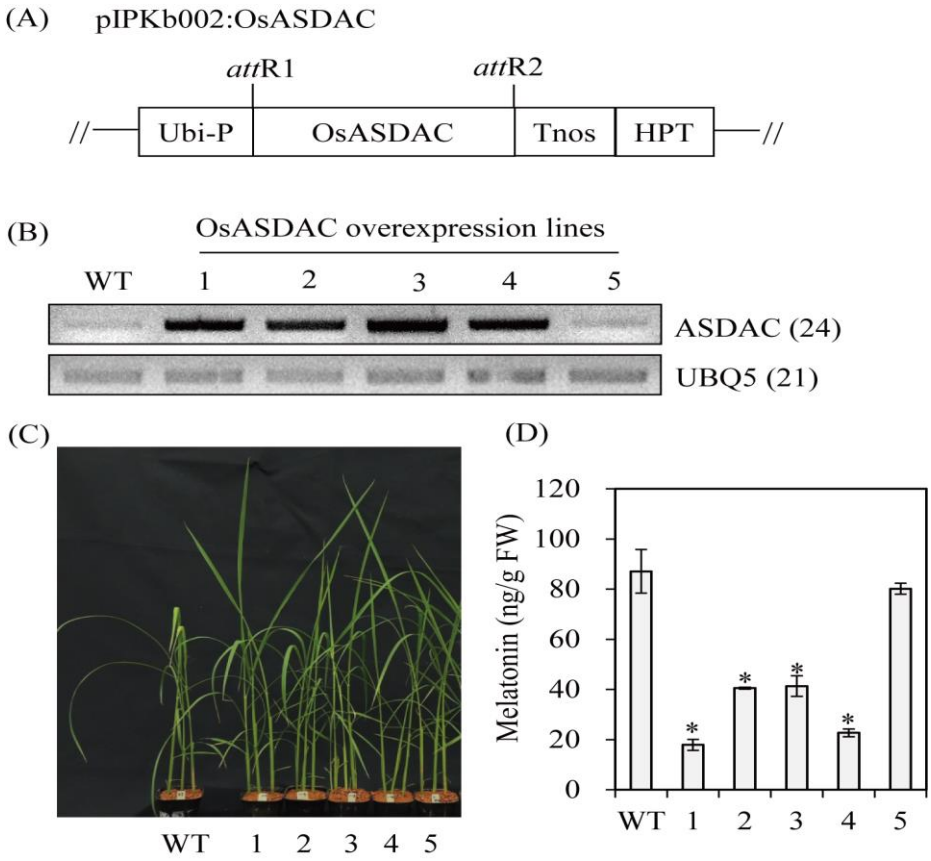

Fig. 5. Schematic diagram of overexpression binary vector, RT-PCR analysis, and melatonin content in transgenic rice

(A) Binary vector used for ASDAC overexpression. (B) RT-PCR analysis of ASDAC. (C) Phenotypes of T_1 transgenic and WT lines grown for 5 weeks in a growth room. (D) Melatonin content. OsASDAC, rice N-acetylserotonin deacetylase; Ubi-P, maize ubiquitin promoter; HPT, hygromycin phosphotransferase; WT, wild type; UBQ5, rice ubiquitin 5 gene; 1–5, ASDAC-overexpression line. GenBank accession numbers of ASDAC and UBQ5 are AK072557 and Os03g13170, respectively. Leaves detached from 5-week-old rice plants were subjected to 0.2 mM cadmium treatment for 3 days. Asterisks indicate significant differences from the WT (Tukey's HSD; $P < 0.05$).

4. CONCLUSION

The last two enzymes for melatonin biosynthesis are SNAT and ASMT in both plants and animals (Figure 6). The melatonin reverse pathway is also present in animals, in which $CP_{450}1B1$ catalyzes melatonin into NAS in mitochondria. $CP_{450}1B1$ deficiency has been shown to reduce the anticancer effect of melatonin in conjunction with low NAS levels (39). Because the last enzyme, ASMT, is also present in animal mitochondria, an equilibrium mechanism may exist between NAS and melatonin, which in turn orchestrates the potential antitumor role of melatonin in tumor cells (37). Plants possess a similar reverse melatonin pathway, in which ASDAC catalyzes NAS into serotonin. Both ASDAC and SNAT are localized in the chloroplast; therefore, an equilibrium mechanism may exist between serotonin and NAS in chloroplasts. To date, a *bona fide* regulatory role for *ASDAC* in melatonin biosynthesis or homeostasis remains unknown, as we do not have a knockout *ASDAC* mutant for plants. Given the various key roles played by melatonin in plants (2), it is likely that *ASDAC* plays a regulatory role in orchestrating melatonin levels in specific organelles

or tissues for melatonin actions such as stomatal movement (40). Further development of *ASDAC* knockout mutant rice using CRISPR techniques will shed light on the specific role of *ASDAC* from the perspective of melatonin levels or a balance between serotonin and NAS.

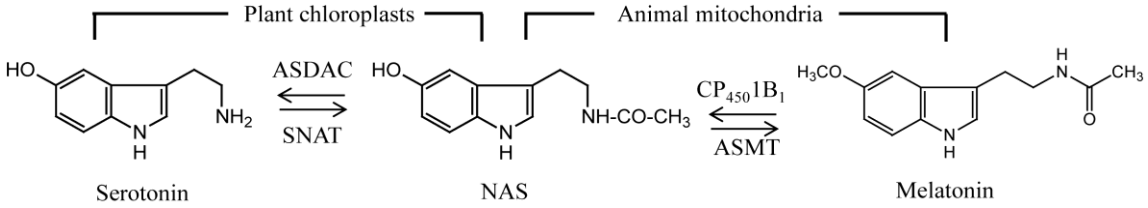

Fig. 6. The proposed equilibrium of melatonin and its precursors in animals and plants.

Plants and animals possess reverse melatonin pathways; plants have an equilibrium mechanism between serotonin and NAS in chloroplasts, whereas animals harbor a potential equilibrium mechanism between NAS and melatonin in mitochondria (37). This similarity suggests that the ratio of serotonin and NAS plays a regulatory role in the mode of action of melatonin in plants, and the ratio of NAS and melatonin plays a similar role in animals. ASDAC, N-acetylserotonin deacetylase; ASMT, N-acetylserotonin O-methyltransferase; NAS, N-acetylserotonin; CP_{450}, cytochrome P_{450} enzyme.

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AUTHORSHIP

K. Lee and O. Hwang carried out the experiments and K. Back designed, advised and wrote the article.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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