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Research Article

Melatonin-induced brassinosteroid biosynthesis enhances seed size, senescence tolerance, and salt stress susceptibility in transgenic rice plants overexpressing an archaeal serotonin *N*-acetyltransferase

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Running Title: Melatonin increases BR biosynthesis

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ABSTRACT

Serotonin *N*-acetyltransferase (SNAT) is the penultimate enzyme catalyzing serotonin into *N*-acetylserotonin in the melatonin biosynthetic pathway. Recently, an archaeal *SNAT* from *Thermoplasma volcanium* (TvSNAT) was cloned and rice lines with its ectopic overexpression (TvSNAT-OE) exhibited higher melatonin levels and generated larger seeds than those of the wild type (WT). In this study, we hypothesized that the increased seed size in TvSNAT-OE rice line might be linked to enhanced brassinosteroid (BR) synthesis since melatonin level is positively associated with BR production. In rice seedlings, roots were shorter and the lamina angle was larger in TvSNAT-OE lines than those in the WT. Also, the second leaves of seedlings were longer in the TvSNAT-OE lines than that in the WT, supporting BR elevation in the TvSNAT-OE lines. In parallel with these phenotypic features, BR levels were higher in the TvSNAT-OE lines than that in the WT. Increased BR levels were associated with enhanced expression of *DWARF4* and *BZR1*, the key genes for BR biosynthesis and signaling, in the TvSNAT-OE lines compared with the WT. Consequently, the TvSNAT-OE lines showed delayed senescence, but were more susceptible to salt stress than the WT due to enhanced BR levels.

Key words: Archaea, brassinosteroid, lamina angle, melatonin, serotonin *N*-acetyltransferase (SNAT), transgenic rice

1. INTRODUCTION

Melatonin is a common molecule found in almost all living organisms, including bacteria, archaea, animals, and plants (1–3). Antioxidant activity is a primary function of melatonin among organisms and other functions of melatonin vary across species. For example, melatonin behaves as a neurohormone, influencing circadian rhythms and seasonal reproduction, in animals (4), whereas it functions as a master regulator orchestrating a diverse array of physiological functions that regulate protein quality control (5, 6) and defense response against various environmental stresses (7-12), in plants.

Melatonin biosynthesis is tightly regulated at the step of *N*-acetylserotonin (NAS) synthesis in plants. NAS is catalyzed by serotonin *N*-acetyltransferase (SNAT), and the catalytic efficiency (V_{max}/K_m) of SNAT is dramatically lower than that of two enzymes involved in serotonin biosynthesis, the tryptophan decarboxylase (TDC) and tryptamine 5-hydroxylase (T5H) (2). The enzyme NAS deacetylase reverses NAS synthesis to produce serotonin (13), and both of these processes limit melatonin synthesis in plants. The final enzyme in the melatonin synthesis pathway is *N*-acetylserotonin *O*-methyltransferase (ASMT), which converts NAS into melatonin. ASMT and ASMT-like enzymes including caffeic acid *O*-methyltransferase (COMT) also have lower catalytic efficiency than that of TDC and T5H, which reduces melatonin synthesis in plants. SNAT is the penultimate enzyme for melatonin biosynthesis, and plays a key role in melatonin synthesis in plants and animals (1, 14, 15).

Recently, the novel gene *SNAT* was cloned from the archaeon *Thermoplasma volcanium*. *TvSNAT* overexpression in rice led to increased seed size accompanied with higher melatonin levels, suggesting that melatonin is involved in seed size regulation (3). Although transgenic rice plants overexpressing various genes involved in melatonin biosynthesis, including rice *TDC* (16), sheep *SNAT* (17), rice *SNAT1* (18), and rice *SNAT2* (19) have been produced in recent studies, their effects on rice seed size increase have not been reported. By contrast, transgenic rice plants with downregulation of rice *SNAT2* (19) produced decreased seed size whereas transgenic rice plants overexpressing rice *COMT* (20) exhibited increased seed size due to the enhanced melatonin levels. These findings suggest that melatonin biosynthesis genes play a role in the control of seed size in a gene-specific manner. Correspondingly, Huangfu *et al*. (20) reported that larger seeds were produced under enhanced cytokinin signaling and synthesis, whereas Hwang and Back (19) suggested that low level of brassinosteroid (BR) was the causative factor for decreased rice seed size since BR levels were decreased in the *SNAT2* suppression rice plants.

In this study, we examined the causative factor of seed size in transgenic rice plants overexpressing archaeon *TvSNAT*. We focused on BR because endogenous melatonin levels are closely associated with BR levels (21–23). This study is the first to reveal a novel functional role for endogenous melatonin in BR synthesis increment in rice plants.

MATERIALS AND METHODS

2.1. Lamina angle measurements.

The rice plant used as the wild type (WT) in this study was the Korean *japonica* cultivar “Dongjin” (*Oryza sativa* cv. Dongjin). We obtained T_2 homozygous transgenic rice seeds overexpressing *SNAT* from the archaeon *Thermoplasma volcanium* (*TvSNAT*-OE) from a previous study (3). WT and TvSNAT-OE transgenic rice seeds were soaked in distilled water, and germinated seeds were transferred into pots filled with soil mixtures designed for rice seedling growth (Nongwoobio, Suwon, Korea). Seedlings were grown in a plant growth room at 28 ^{o}C and 70% humidity in a 12/12-h light/dark cycle at a photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} for 2 weeks. The second-leaf lamina joint angles of 14-day-old rice seedlings were measured.

2.2. RNA extraction and real-time polymerase chain reaction (qPCR) analysis.

Total RNA from rice seedlings was isolated using a NucleoSpin RNA plant kit (Macherey-Nagel, Düren, Germany). First-strand cDNA was synthesized from 2 μg total RNA using EcoDry Premix (Takara Bio Inc., Mountain View, CA, USA). Real-time polymerase chain reaction (qPCR) was performed in a Mic qPCR Cycler system (Biomolecular Systems, Queensland, VIC, Australia) as described previously (24). The primer set for gene expression analysis was as follows; *DWARF4* forward 5´-GGA GAA GAA CAT GGA ATC AC-3´, *DWARF4* reverse 5´-GTA ATC TTG AAC GCG GAT ATG-3´, *DWARF11* forward 5´-TGA GGC ACT GAG ATG TGG-3´, *DWARF11* reverse 5´-AAG GTG ATG GAG GAA GAA-3´, *BRI1* forward 5´-CAG CTA CTT GGC TAT CTT GAA GCT CAG C-3´, *BRI1* reverse 5´-CCA TTC TTG TTG AAG GTG TAC TCC GTG C-3´, *BZR1* forward 5´-ATG ACG GCC ATT ATT GCC GAG CA -3´, *BZR1* reverse 5´-TCG CCC AAA TCG CAG CAT-3´, *UBQ5* forward 5'-CCG ACT ACA ACA TCC AGA AGG AG-3', and *UBQ5* reverse 5'-AAC AGG AGC CTA CGC CTA AGC -3'.

2.3. Quantification of bioactive brassinosteroids.

Bioactive BR was analyzed using an enzyme-linked immunosorbent assay (ELISA; MyBioSource, San Diego, CA, USA) according to the manufacturer’s instructions. Briefly, rice seedlings were ground with a mortar and pestle with 10 ml 80% methanol extraction solution containing 1 mM butylated hydroxytoluene, followed by centrifugation for 10 min at 3,500 × *g*. The supernatants were dried with N_2 and dissolved in 2 ml phosphate-buffered saline buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5). Further procedures were performed as described previously (24).

2.4. Stress treatments and biochemical analyses.

Rice leaves were detached from 3-week-old rice plants grown as described above. The detached leaves were transferred to 50-ml conical polypropylene tubes containing 25 ml water without nutrients and incubated for 7 days at 28 ^{o}C under a 12/12-h light/dark cycle. For salt stress treatment, 11-day-old seedlings grown in pots were irrigated with 200 mM NaCl for 6 days. Rice leaves were frozen in liquid nitrogen and stored at -80 ^{o}C until analysis. Malondialdehyde (MDA) and chlorophyll content analyses were performed using frozen rice leaf samples (50 mg) ground to powder in liquid nitrogen using a TissueLyser II sample disruptor (Qiagen, Tokyo, Japan), followed by extraction and spectrophotometer analysis as described previously (18).

2.5. Statistical analysis.

All data were analyzed using the IBM SPSS Statistics 25 software (IBM Corp. Armonk, NY, USA). Means were compared using analysis of variance, at a significance level of $P < 0.05$ according to Tukey's post-hoc honestly significant difference test. Data are presented as means ± standard deviations.

3. RESULTS

3.1. Seed weight and root length in TvSNAT-overexpressing transgenic rice lines.

Transgenic TvSNAT-OE rice plants exhibited larger, heavier seed phenotypes than did the WT (Figure 1A–C). On average, WT and TvSNAT-OE seeds weighed approximately 29 and 32 mg, respectively; thus, TvSNAT-OE seeds were approximately 10% larger by weight than WT seeds. In rice, increasing and decreasing endogenous BR levels tend to increase and decrease seed size, respectively (25, 26). By contrast, exogenous BR treatment inhibits rice seedling growth in a dose-dependent manner (27). To determine whether the increased seed size observed in TvSNAT-OE plants was associated with BR, we investigated seedling phenotypes in the TvSNAT-OE rice lines. WT and TvSNAT-OE seeds were imbibed and grown in water for 6 days. Overall shoot lengths in the TvSNAT-OE lines were comparable to those of the WT, whereas roots were shorter in the TvSNAT-OE lines than in the WT, although line 5 exhibited non-significant root growth inhibition (Figure 1D–E). These results suggest that reduced root length was a result of increased BR production through increased melatonin biosynthesis in the TvSNAT-OE lines (3).

3.2. Lamina inclination measurement in TvSNAT-OE transgenic rice seedlings.

To determine whether melatonin-mediated seed enlargement in the TvSNAT-OE lines was associated directly with BR, we measured lamina angles in rice seedlings, as BR application increases lamina inclination and coleoptile length (27). WT and TvSNAT-OE seedlings had average leaf angles of approximately 29° and 37°, respectively, indicating a 28% increase in the TvSNAT-OE lines (Figure 2A, B). Second leaves were longer in the TvSNAT-OE lines than in the WT (Figure 2C). To verify that these increases in leaf angle and length were associated with BR, we investigated the expression patterns of several BR-related genes, including two BR biosynthetic genes, *DWARF4* and *DWARF11*, as well as the BR receptor *BRI1* and transcription factor *BZR1*. *DWARF4* expression was significantly enhanced in the TvSNAT-OE lines compared with the WT, whereas *DWARF11* expression was comparable to that of the WT (Figure 2D). The expression levels of *BRI1* did not differ between the WT and TvSNAT-OE lines, but *BZR1* transcription factor was enhanced in the TvSNAT-OE lines relative over that of wild type. These findings indicate that melatonin positively regulates *DWARF4* and *BZR1* expression, and are highly consistent with those of a previous study, in which *DWARF4* suppression resulted in reduced melatonin synthesis in rice

(22). In summary, the endogenous increase in melatonin by ectopic *TvSNAT* overexpression led to enhanced expression of the BR biosynthetic and signaling genes, resulting in increased seed size and second-leaf lamina angle.

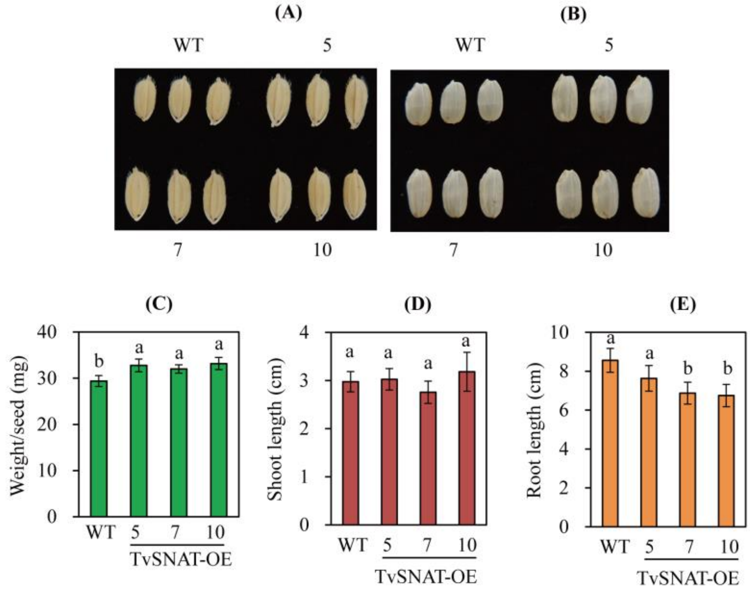

Fig. 1. Seed and seedling phenotypes of *TvSNAT*-overexpressing (TvSNAT-OE) transgenic rice plants.

Phenotypes of (A) rough rice and (B) brown rice seeds. (C) Seed weight of rough rice seeds. (D) Shoot length. (E) Root length. Rough rice seeds were grown in water for 6 days at 25 °C to monitor seedling growth. Different letters indicate significant differences from the wild type (WT) (Tukey's honestly significant difference test, P < 0.05).

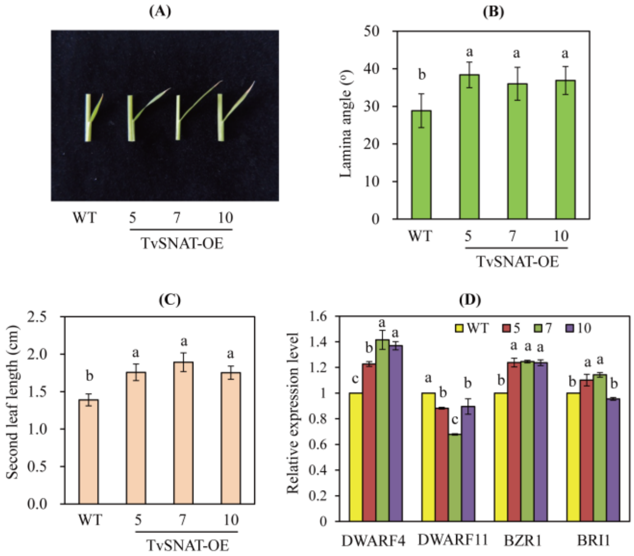

Fig. 2. Second leaf morphology and BR-related gene expression of TvSNAT-OE seedlings.

*(**A**) Photograph of the second leaf angle. (**B**) Second leaf angle measurement. (**C**) Second leaf length. (**D**) Real-time polymerase chain reaction analysis of brassinosteroid (BR)-related gene expression. Rice seeds were imbibed for 2 days in water and sown in pots filled with soil. The sheath and blade of the second leaves were collected at 17 days after imbibition for leaf angle measurement and total RNA isolation. Different letters indicate significant differences from the wild type (WT; Tukey's honestly significant difference test, P < 0.05). GenBank accession numbers: DWARF4, AB206579; DWARF11, AK106528; BRI1, AK101085; BZR1, Os07g39220; UBQ5, Os03g13170.*

3.3. Enhanced BR levels and stress responses in the TvSNAT-OE lines.

To obtain direct evidence of the melatonin-induced BR increase, we measured BR contents in the lamina joints of the TvSNAT-OE lines and WT using ELISA. The WT produced BR at a rate of 3 ng/g fresh weight (FW), whereas the TvSNAT-OE lines produced 3.6 ng/g FW on average, which was 20% greater than that of the WT (Figure 3A). Next, we applied salt and senescence stress, with which BR is associated negatively and positively, respectively (19, 28–31). To determine whether melatonin-induced BR increases in the TvSNAT-OE plants changed in response to abiotic stress, 11-day-old rice seedlings were challenged with 200 mM NaCl for 6 days. The TvSNAT-OE lines exhibited greater salt susceptibility than did the WT, according to MDA content (Figure 3).

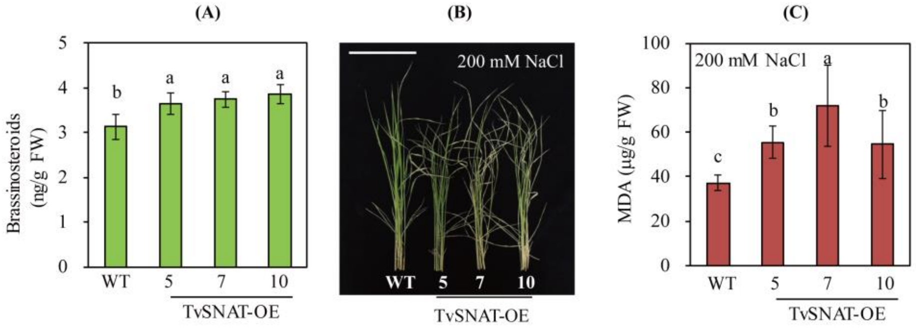

Fig. 3. Measurement of the brassinosteroid (BR) level and salt response in TvSNAT-OE plants.

*(**A**) BR levels in the second leaf and sheath, measured by enzyme-linked immunosorbent assay. (**B**) Photographs of harvested leaves from rice seedlings after salt treatment. (**C**) Measurement of malondialdehyde (MDA) levels after salt treatment. Eleven-day-old rice seedlings grown in soil were incubated in salt solution (200 mM NaCl) for 6 days at 28 °C/25 °C (day/night). Values are means ± standard deviations (n = 3). Different letters indicate significant differences from the wild type (WT; Tukey's honestly significant difference test, P < 0.05). Bar = 10 cm.*

This result suggests that increased BR, rather than melatonin, played a predominant role in the salt response of TvSNAT-OE rice seedlings, as BR is associated negatively (19, 29) and melatonin is associated positively [32, 33] with salt tolerance. By contrast, both BR and melatonin are associated positively with senescence. Therefore, we challenged detached rice leaves with a senescence treatment. TvSNAT-OE leaves had lower MDA and higher chlorophyll content than did the WT, indicating enhanced tolerance against senescence (Figure 4).

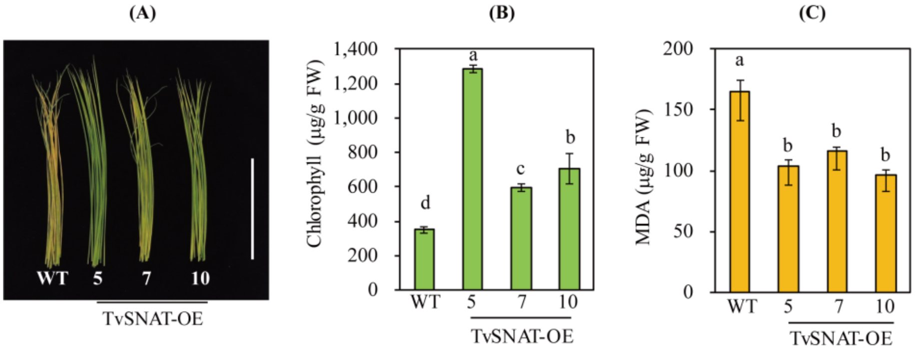

Fig. 4. Delayed senescence in TvSNAT-OE plants.

*(**A**) Photograph of detached rice leaves after senescence treatment. Malondialdehyde (MDA; **B**) and chlorophyll (**C**) content after senescence treatment. Detached leaves from 21-day-old rice seedlings were incubated in water for 7 days at 28 °C/25 °C (day/night). Values are means ± standard deviations (n = 3). Different letters indicate significant differences from the wild type (WT; Tukey's honestly significant difference test, P < 0.05). Bar = 10 cm.*

4. DISCUSSION

The first report of melatonin to increase yield of crops showed that hydroprimed seeds of corn, mung bean, and cucumber with melatonin had higher yields than that of controls (34). Tomato seeds soaked in melatonin also had high yields accompanied with increased ascorbic acid and lycopene content compared to control (35). Many other crops treated with melatonin also showed the improved yields, suggesting a compelling role of melatonin in crop production (36, 37). Consistent with the results of exogenous melatonin treatment, endogenously enriched melatonin by overexpressing melatonin biosynthetic genes in plants also improved their yield (37). For example, ectopic overexpression of rice *SNAT1* increased seed yield with the increased number of panicles per plant (18), whereas silencing rice *SNAT1* or rice *ASMT1* decreased seed yield (38). Although the increased endogenous melatonin is positively associated with seed yield in rice, the seed size of transgenic rice is similar to that of the wild type. In contrast, the declined yield was observed in rice overexpressing the sheep *SNAT*, suggesting that the increased endogenous melatonin is not necessary positively to associate with seed increase, particularly in rice (17). These results suggest that the subcellular location of SNAT proteins may play the different roles to regulate the crop yield. For example, the sheep SNAT is expressed in the cytoplasm (39), but rice SNAT1 is expressed in the chloroplast (40).

The first evidence showing that endogenous melatonin levels are related to seed size come from a report on *SNAT2* downregulated transgenic rice with (19). These *SNAT2* RNAi rice seeds had the reduced grain length and width compared to the wild type, resulting in a decrease in weight per 1,000 grains. However, *SNAT2* overexpressing rice seeds showed a similar phenotype to the wild type. Interestingly, the smaller leaf angle phenotypes and shorter grain lengths exhibited in *SNAT2* RNAi rice plants are similar to the phenotypes of mutants with reduced brassinosteroid (BR) content or reduced BR signaling (24, 41). In addition, the *SNAT2* RNAi rice produced less BR as well as, had the reduced expression of the key BR biosynthetic gene *DWARF4* than that of wild type, while levels of gibberellin (GA) and indole acetic acid (IAA) were similar to wild type (19, 24). However, *SNAT2* overexpressing rice plants did not further increase BR levels compared to wild type indicating the optimal BR level already being present in the wild type. The results revealed a close relationship between melatonin and BR levels in plants.

Generally, seed size is determined by the interaction of several plant hormones such as cytokinin, auxin, GA, and BR (42). For example, *BIG GRAIN 1*, encoding a positive regulator of auxin response and transport, has a positive effect to improve grain size in both rice and *Arabidopsis* due to an increase in cell number (43). In rice, increasing cytokinin by downregulating *cytokinin oxidase 2* expression was shown to improve 1000-grain weight by 5–15% compared to wild type (44). In the case of GA, GW6, encoding a GA regulatory family of proteins, promotes cell expansion in the spikelet hull, actively regulating grain width and weight (45). BR has long been recognized as a key factor in regulating seed size as well as many other functions, such as enlarging leaf angle, delaying leaf senescence, and promoting seed germination in plants (46). Overexpression of the BR biosynthetic gene *DWARF4* increased seed yield along with improved seed length and width in *Arabidopsis* (47). Short grain length was observed in other BR biosynthesis mutants such as *DWARF11* and *ebisu dwarf* (*d2*), whereas overexpression of BR-responsive transcription factors produced long grains in rice, indicating that BR is an important hormone that regulates grain shape. (48-50). A smaller seed size phenotype has been reported in melatonin-deficient rice lines (14), but a larger seed size phenotype has not been reported in melatonin-rich transgenic or melatonin-treated plants. Recently, melatonin-rich transgenic rice plants overexpressing rice *COMT* exhibited heavier seed weights with increased seed length and width (20). The main reason for the increase in seed size in *COMT*-overexpressing rice is the improved photosynthetic efficiency and increased cytokinin content. Similar to this report, the TvSNAT-OE line also displayed a greater seed phenotype than the wild type through increased melatonin biosynthesis (3). We also found that BR and BR-related genes were upregulated in the TvSNAT-OE line, although we did not measure cytokinin content (Figures 2 and 3). The reason for focusing on BR in terms of seed shape is that melatonin is known to be involved in skotomorphogenesis or etiolated growth (24), which is regulated only by BR (51). In melatonin-rich rice plants, the major phytohormones causing seed shape enlargement appear to be cytokinins or BRs, depending on their target genes. However, recently, unlike BR, which increases leaf angle in rice (24), cytokinin was reported to decrease leaf angle (52). Additionally, BR and GA act as inducers of melatonin synthesis in rice, indicating that melatonin interacts with these hormones rather than with cytokines (22, 53). Based on the above observations, we believe that the increased endogenous melatonin is coupled with increased seed size through enhanced BR biosynthesis, although other plant hormones such as cytokinins cannot be excluded.

In rice root growth, both cytokinin and BR treatments inhibited root growth, but root inhibition was more pronounced in cytokinin-treated rice than in BR-treated rice (27, 54). In this report, the differential inhibition of root growth in all three TvSNAT-OE lines suggests the possibility that increased BR rather than increased cytokinin is responsible. Unlike the root growth, two hormones react differently in response to senescence and abiotic stresses such as salinity. Cytokinins are well known to delay leaf senescence by inhibiting chlorophyll degradation and redistributing necessary nutrients to other parts of the plant (55). Cytokinins also provide negative or positive effects on salt stress, depending on the plant species. For example, *OsCKX2* knockout rice mutants have higher cytokinin levels and are more salt tolerant than the wild type (56). However, the increased cytokinins reduced the tolerance of radish and tobacco to salt (57). Likewise, exogenous BR treatment confers tolerance in response to many stresses, including cold, salt and heat (58). Conversely, BR plays a role in promoting leaf senescence in *Arabidopsis*, mung bean and wheat (41), while BR delays senescence in papaya (59). Unlike cytokinin and BR, melatonin plays a positive role in conferring abiotic stress tolerance, such as senescence and salt stress, in all plants tested (7). As a result, it stands to reason that the main reason for senescence tolerance in TvSNAT-OE transgenic rice plants, which produce higher melatonin and BR, is due to increased melatonin rather than increased BR because BR induces senescence. However, since BR may have a negative effect on salt stress, the enhanced sensitivity of TvSNAT-OE to salt stress may be explained by increased BR.

It has been reported that melatonin does not directly regulate leaf angle when melatonin is administered to rice lamina joints, indicating an indirect effect of melatonin (24). The wider leaf angle, increased seed size, and salinity sensitivity of TvSNAT-OE rice plants can be explained by elevated BR synthesis through enhanced carbon assimilation by high melatonin content (5, 60). Conversely, the senescence tolerance of TvSNAT-OE is due to enhanced melatonin synthesis since BR induces leaf senescence (Figure 5).

Further experiments using transgenic plants or hormone-treated plants overproducing endogenous melatonin by way of either the overexpression of a diverse array of archaeal *SNAT* orthologous genes such as *human Naa50* (61), *Escherichia coli RimI* (62), rice *SNAT3* (63) and ciliate *Stylonychia lemane SNAT* (64) or the exogenous treatment of plant hormones such as BR (22) and gibberellin (40) will shed light on the functional roles of melatonin in plants in great detail (3, 20, 22, 23, 65-68).

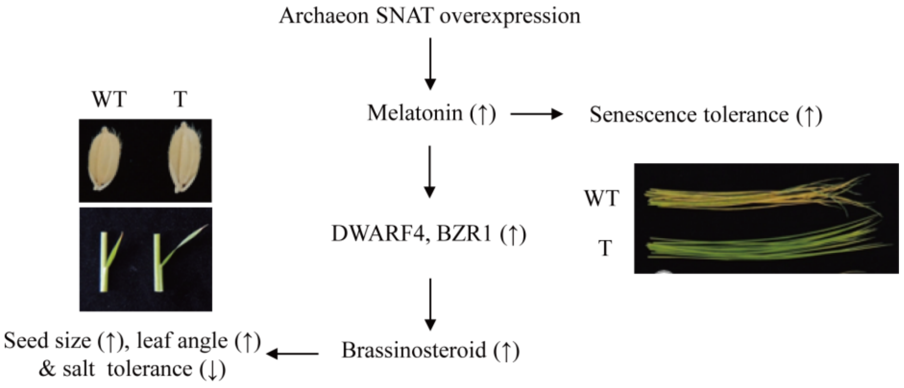

Fig. 5. A model for melatonin-induced seed size increase and abiotic stress response.

Transgenic rice plants overexpressing archaeal SNAT induced senescence tolerance by enhancing melatonin content. Endogenous melatonin increases enhanced BR synthesis, resulting in increased seed size, leaf angle, and salinity sensitivity. WT, wild type; T, transgenic line. Upward arrow indicate enhancement, whereas downward arrows denote suppression.

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AUTHORSHIPS

KL performed the experiment. KB designed, wrote, and revised the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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