Research Article

Melatonin plays a pivotal role in conferring tolerance against endoplasmic reticulum stress via mitogen-activated protein kinases and bZIP60 in *Arabidopsis thaliana*

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ABSTRACT

Melatonin has diverse roles as a signaling molecule that activates a number of downstream defense systems against various biotic and abiotic stresses in plants. However, there have been no reports regarding a direct protective role of melatonin against endoplasmic reticulum (ER) stress. Here, we report that exogenous melatonin treatment attenuated ER stress damage by preserving ER structure and enhancing secretory protein folding capacity in response to tunicamycin treatment. Further transgenic experiments indicated that melatonin-deficient *snat1* mutant was hypersensitive to ER stress, whereas melatonin-proficient *SNAT1* overexpression (OE) was tolerant to ER stress, as evidenced by reduced ion leakage and higher transcript levels of ER chaperones, including luminal binding protein (BIP) 2, BIP3, and CNX1, compared to wild-type controls. Moreover, this melatonin-mediated ER stress tolerance was dependent on the bZIP60 transcription factor and mitogen-activated protein kinase. Our data suggest that melatonin is actively involved in maintaining homeostasis of the ER during normal plant growth, and also has a protective effect against many environmental stressors that induce ER stress.

Keywords: ER chaperones, melatonin, mitogen-activated protein kinase, protein folding capacity, unfolded protein response

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1. INTRODUCTION

Melatonin, which is believed to have evolved with the appearance of photosynthetic bacteria, plays a multifunctional role in all living organisms (1, 2). In addition to its common function as an apex antioxidant in both animals and plants, melatonin is involved in a plethora of functions in plants, including growth and development (3–5) as well as defense against biotic and abiotic stresses (6–8). Chief among these is the defensive role of melatonin against a wide range of abiotic stresses, including salt (9, 10), high temperature (11, 12), cold (13, 14), drought (15, 16), heavy metals (4, 17), and high light (18). Enhanced tolerance to these abiotic stresses has been suggested to be due to melatonin being a potent antioxidant that induces the expression of various antioxidant enzymes (15, 17, 19).

There have been several recent reports regarding novel functions of melatonin in plants, including roles in stomata closure (20) and brassinosteroid (BR) biosynthesis (5). In

Arabidopsis, melatonin is known to induce stomatal closure via melatonin receptor (Cand2)-mediated H_2O_2 and Ca^{2+} signaling cascades. Cand2 belongs to the G protein-coupled receptor (GPCR) family, which contains seven transmembrane domains that interact with GTP-binding proteins (G proteins). Moreover, Cand2 has been shown to bind melatonin with an apparent K_d of 0.73 nM, indicating that the pleiotropic effects of melatonin are attributable to the presence of melatonin receptors in plants, as in animals (21). On the other hand, melatonin has been suggested to have auxinic activity due to their common biosynthetic precursor, tryptophan; thus, alteration of the melatonin level may affect the auxin level, although there is now some controversy regarding this hypothesis (22). Another recent report indicated that melatonin regulates BR biosynthesis by regulating the gene expression of *DWARF4*, a key BR biosynthetic gene, and its transcription factor, *RAVL1* (5). Endogenous suppression of melatonin in transgenic rice decreased BR levels, resulting in a semi-dwarf with erect-leaf phenotype. Moreover, expression of the key melatonin biosynthesis gene, *SNAT2*, is closely associated with that of *DWARF4* during the day/night cycle, suggesting mutual regulation. These data are consistent with a previous report showing that G proteins are related to BR-induced stomatal closure in *Arabidopsis* (23). Taken together, it is hypothesized that GPCR (or Cand2) may be coupled to BR regulation for stomatal closure, although this remains to be confirmed.

Although melatonin confers resistance against various stresses through its potent antioxidant activity and enhancement of the expression of antioxidant enzymes, many reports have shown that melatonin induction is only marginal, in parallel with slight induction of antioxidant enzymes, in the presence of various stresses (10, 18). For example, exogenous melatonin treatment enhanced salt stress in maize, but the melatonin level was increased only very slightly by salt treatment in parallel with marginal increases in the levels of ascorbate peroxidase (APX) and catalase (CAT) (10). Thus, the combination of an only marginal melatonin increase and slightly increased antioxidant enzyme levels cannot explain how melatonin efficiently overcomes various stresses in plants. Therefore, there may be other defense mechanisms orchestrated by melatonin to cope with abiotic stresses. Based on a few previous studies in which melatonin was shown to induce autophagy in animals (24, 25) and plants (26), it is highly likely that melatonin is involved in endoplasmic reticulum (ER) regulation because autophagy is activated by ER stress (27). Unfortunately, there have been no reports regarding the possible role of melatonin in protecting against ER stress.

ER is the organelle responsible for sorting secretory proteins by precisely folding polypeptides. Inappropriate protein folding in the ER gives rise to misfolded proteins that are sensed by the ER quality control system, resulting in ER stress. ER stress can be induced by a number of biotic and abiotic stresses, including pathogen attack, drought, and heat (28). Upon ER stress, plants operate the ER defense system, called the unfolded protein response (UPR), to facilitate proper folding and degradation of misfolded proteins by upregulating UPR-related genes (known as chaperones), such as luminal binding protein (*BIP*) and calnexin (*CNX*).

In this study, to determine whether melatonin confers protection against ER stress, melatonin-deficient and -proficient *Arabidopsis* plants were challenged with tunicamycin (Tm), an ER stress inducer that inhibits N-linked glycosylation of glycoproteins. Here, we report that the melatonin-deficient knockout *snat1* mutant was highly sensitive to Tm treatment compared to wild-type controls, and that melatonin treatment ameliorated changes in ER structure and protein folding capacity in response to Tm by upregulating UPR-related genes, such as *BIP2*, *BIP3*, and *CNX1*, through enhancing expression of the *bZIP60* transcription factor via the mitogen-activated protein kinase (MAPK) signaling pathway.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions.

Arabidopsis thaliana Columbia (Col-0), *SNAT1*-overexpressing line (OE), *snat1* knockout mutant plants (18), and mapk3/6 silencing line (29) were grown at 22°C with a 16-hour photoperiod under a photon flux density of 50 µmol/m^2/s using 14-W LED bulbs (Phillips, Amsterdam, The Netherlands) providing a full spectrum of light. Mature leaves of 5-week-old *Arabidopsis* plants were used for analysis of ion leakage, gene expression induction, and superoxide levels. *Arabidopsis* leaves were syringe-infiltrated with 1 µM melatonin (in H_2O) or 1 µg/mL Tm (in 0.05% dimethyl sulfoxide [DMSO]).

2.2 Seedling assay under ER stress conditions.

Arabidopsis seedlings were surface-sterilized with 1% sodium hypochlorite for 10 minutes with shaking and washed five times with sterilized water. The seeds were stratified for 2 days at 4°C in the dark. Stratified seeds were germinated on half-strength Murashige and Skoog (MS) medium containing 0.1 µg/mL Tm in vertically oriented square dishes (SPL Life Sciences, Pocheon-Si, Korea). The growth conditions were 16 hours of light at 22°C/8 hours of dark at 18°C at a photon flux density of 50 µmol /m^2/s. After 7 days of growth, seedlings were transferred to square plates containing MS medium (without 0.1 µg/mL Tm) for an additional 10 days. The 17-day-old plants grown in MS medium (recovery medium) were weighed using a precision balance. About six seedlings were used per replicate, with three replicates used for each assay.

2.3 Measurement of electrolyte leakage.

Arabidopsis leaves treated with 1 µg/mL Tm (in 0.05% DMSO) were collected using a razor blade and immersed in distilled H_2O for 30 s to eliminate signals from wounded cells. The resulting leaves were placed in 50-mL tubes containing 15 mL of distilled H_2O and gently agitated for 10 hours, and then the solution conductivity was measured using a conductivity meter (Cole-Parmer Instrument Co., Vernon Hills, IL, USA).

2.4 Superoxide staining.

Detection of superoxide was carried out by staining leaves with nitroblue tetrazolium (NBT) as described previously (18*). Arabidopsis* leaves treated with 1 µg/mL Tm (in 0.05% DMSO) were detached and placed in a solution containing 0.1% NBT (in 10 mM MES, pH 6.8) for 4 hours. Thereafter, the leaves were destained and stored in 96% ethanol. All experiments were repeated three times (biological repeats), and a total of nine rosette leaves collected from multiple plants (5 weeks old) were analyzed in each experiment.

2.5 Confocal analysis.

The binary vector plasmids pBIN61-HDEL-CFP (an ER fluorescence marker) and pER8-BRI1-mCherry (or FLS2-mCherry; a plasma membrane [PM] fluorescence marker) were kind gifts from Dr. HG Kang (Texas State University, San Marcos, TX, USA) and Dr. Sorina C. Popescu (Mississippi State University, Starkville, MS, USA), respectively. These plasmids were transformed into the *Agrobacterium tumefaciens* GV2260 strain using the freeze-thaw method. Three-week-old *Nicotiana benthamiana* leaves were syringe-infiltrated with GV2260 carrying pBIN61-HDEL-CFP or pER8-BRI1-mCherry (or pER8-FLS2-mCherry), and then incubated in the growth room for 2 days. In the case of pER8 constructs containing the

estrogen-inducible XVE promoter, treatment was performed with 10 µM *β*-estradiol (Sigma-Aldrich, St. Louis, MO, USA) for 12 hours before confocal analysis. A Leica TCS-SP5 confocal microscope (×63 oil immersion objective; Leica, Wetzlar, Germany) was used. CFP was excited with a mercury arc lamp at 435 nm and emitted light was collected at 480 nm. mCherry was excited with the yellow-green laser at 587 nm and emitted light was collected at 610 nm.

2.6 Gene expression analysis.

Total RNA was isolated from frozen leaf tissue with a NucleoSpin RNA Plant Kit (Macherey-Nagel, Duren, Germany). Aliquots of 1 µg of DNA-free total RNA were reverse-transcribed in accordance with the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Quantitative RT-PCR (qRT-PCR) was performed with a Mic qPCR Cycler system (Bio Molecular Systems, Queensland, Australia) using a SYBR Green RT-PCR Reagent Kit (Luna® Universal qPCR Master Mix; NEB, Hitchin, UK) in accordance with the manufacturer's protocol. The primer sequences for RNA analysis were as follows: BIP2 (forward, 5′-GCA GGA GGA GAA TCA TCG AC-3′; reverse, 5′-AAA GAG AAC GTC CAG GGA GA-3′); BIP3 (forward, 5′-GGA GAA GCT TGC GAA GAA GA-3′; reverse, 5′-ATA ACC GGG TCA CAA ACC AA-3′); CNX1 (forward, 5′-TGG TTT TGG GTT GAA TGT TG-3′; reverse 5′-CTG AAG GGG ATG AAA AAG AAA-3′); bZIP60 (forward, 5′-GAA GGA GAC GAT GAT GCT GTG GCT-3′; Runspl 5′-GCA GGG ATT CCA ACA AGA GCA CAG-3′; Rspl 5′-AGC AGG GAA CCC AAC AGC AGA CT-3′); bZIP28 (forward, 5′-TTT CAC TTG TGG CGT CAG AG-3′; reverse 5′-TGG AAA TGC CTT TAA AAC AAG G-3′); APX1 (forward, 5′-GTG TGT GTC TCC CCG AGA GT-3′; reverse 5′-CTA AGC AGC AAA AGC GCA AC-3′); CAT1 (forward, 5′-ATC CGC AGC ATT TGG ATC T-3′; reverse 5′-CTA AGC AGC AAA AGC GCA A-3′); GST1 (forward, 5′-GGA CTC ACC AAG CCT GTG TT-3′; reverse 5′- TGA ATC GCA TGA GTT TGA CC-3′), and EF-1a (EF1ALPHA; At5G60390) was used for signal normalization (forward, 5′-TGG TGA CGC TGG TAT GGT TA-3′; reverse 5′-CAT CAT TTG GCA CCC TTC TT-3′). The bZIP60 primers were adopted from Sagor et al. (30).

2.7 Melatonin measurement.

Measurement of melatonin was conducted using a melatonin ELISA kit (Arigo Biolaboratories, Hsinchu, Taiwan). The ELISA data were further confirmed by LC-MS as described previously (31). Briefly, *Arabidopsis* leaves (0.1g) were ground in liquid nitrogen and homogenized in 2 mL of chloroform (100%). After centrifugation at 12,000 × *g* for 10 minutes, the supernatant was evaporated in fresh 2 mL tubes using a vacuum concentrator, and the melatonin was allowed to dissolve in 1 mL of distilled H_2O for over 3 hours to increase recovery. The resulting samples were subjected to melatonin analysis in accordance with the manufacturer's protocol.

2.8. Protein analysis.

Ground leaf tissues were mixed with EB buffer (40 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, and complete protease inhibitor cocktail; Roche, Basel, Switzerland), and centrifuged at 15,000 × *g* for 10 minutes at 4°C. Total protein was quantified and mixed with 5× sample buffer. pER8-BRI1-mCherry expressed in *N. benthamiana* leaves was visualized by immunoblotting analysis using anti-mCherry antibody (Abcam, Cambridge, UK).

2.9. Statistical analysis.

Student's *t*-test was used for statistical evaluation with SigmaPlot (ver. 10.0; Systat Software, Point Richmond, CA, USA). In all analyses, $P<0.05$ was taken to indicate statistical significance.

3. RESULTS AND DISCUSSION

3.1. Loss of *SNAT1* shows hypersensitivity upon Tm treatment.

To determine whether melatonin is involved in the ER stress response, we first performed Tm recovery assay by seeding *Arabidopsis* seeds in MS medium containing Tm for 7 days. These seedlings were subsequently transferred into medium without Tm for 10 days. SNAT is the penultimate enzyme in the melatonin biosynthetic pathway involved in catalyzing serotonin to *N*-acetylserotonin. Thus, the *snat1* knockout mutant produces less melatonin than the wild-type control (6). As shown in Figure 1, the *snat1* mutant showed a significant decline in biomass compared to the wild-type. The complementation line (CP) expressing a genomic fragment of the *SNAT1* gene in the *snat1* mutant exhibited comparable biomass weight to the wild-type control. In contrast, the biomass weight of *SNAT1*-overexpression line (OE) was reduced compared with wild-type.

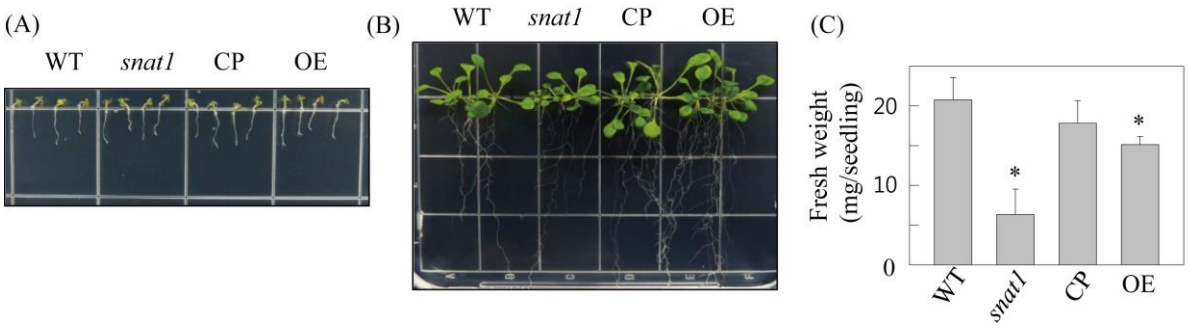

Fig. 1. *Arabidopsis snat1* knockout mutant showed hypersensitivity under conditions of endoplasmic reticulum (ER) stress.

(A) Representative photograph of snat1 knockout mutant, SNAT1-overexpressing line (OE), complementation of snat1 mutant expressing genomic SNAT1 (CP), and wild-type Col-0 (WT) control seedlings grown for 7 days on Murashige and Skoog (MS) agar plates containing 0.1 μg/mL tunicamycin (Tm) under standard long-day conditions (22°C, 16/8 hours light/dark). (B) Picture of seedlings transferred to normal MS medium without Tm for an additional 10 days of growth. (C) Fresh weight of 17-day-old plants shown in Fig. 1B. Asterisks (*) indicate significant differences compared to wild-type (WT) (Student's t-test; P < 0.05).

The reason for biomass reduction in the OE line remains to be determined. These data clearly indicated that melatonin deficiency causes hypersensitivity in response to ER stress. We measured Tm response by ion leakage analysis; this analysis involved direct syringe infiltration of various concentrations of Tm into *Arabidopsis* leaves followed by incubation in water to measure ion leakage. Tm increased ion leakage levels in wild-type control plants in a dose-dependent manner (Fig. 2A), suggesting that ion leakage analysis is a good means of detecting Tm severity in *Arabidopsis*. Consistent with these assumptions, the *snat1* mutant showed 1.6-fold higher ion leakage than wild-type, whereas the OE line exhibited 3-fold less ion leakage than wild-type (Fig. 2B), indicating that ion leakage analysis is more sensitive than biomass analysis.

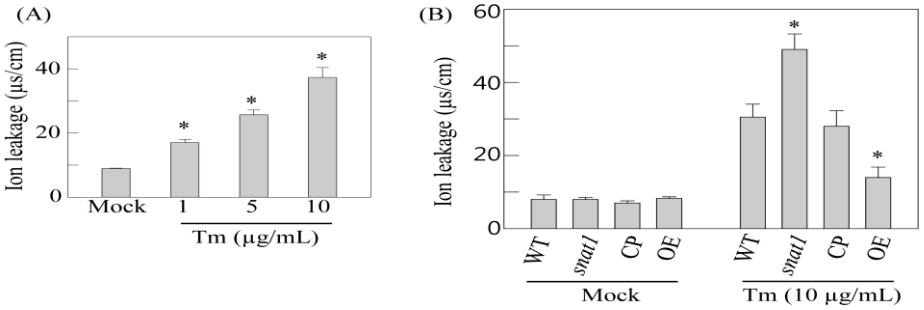

Fig. 2. Measurement of ion leakage in response to Tm treatment.

(A) Effects of different Tm concentrations on ion leakage in 5-week-old mature WT plants. (B) Differences in ion leakage among WT, OE, and snat1 mutant treated with 10 μg/mL Tm. Mock (0.05% dimethyl sulfoxide [DMSO]) or Tm was syringe-infiltrated into Arabidopsis leaves and incubated for 5 hours, and the leaves were then collected and gently agitated with 20 mL of water for an additional 10 hours for ion leakage measurement. Asterisks (*) indicate significant differences compared to Mock or WT (Student's t-test; $P < 0.05$).

To confirm the active effect of melatonin against ER stress, we quantified melatonin levels in response to various concentrations of Tm. As shown in Figure 3, melatonin levels increased at 1 μg/mL Tm, reaching a maximum at 5 μg/mL Tm with a 2-fold increase relative to untreated mock controls. However, 10 μg/mL Tm did not further induce melatonin production at 6 or 24 hours after Tm treatment (Fig. 3 A, B). Analogous to the ion leakage results (Fig. 2B), the *snat1* mutant produced less melatonin than wild-type upon Tm treatment. In contrast, no melatonin induction was observed in the OE, while the melatonin level was higher in the absence of Tm than wild-type (Fig. 3C). Accordingly, exogenous melatonin also reduced Tm-induced ion leakage (Fig. 3D). These data combined with the results of biomass analysis clearly demonstrated that melatonin is closely associated with ER stress tolerance.

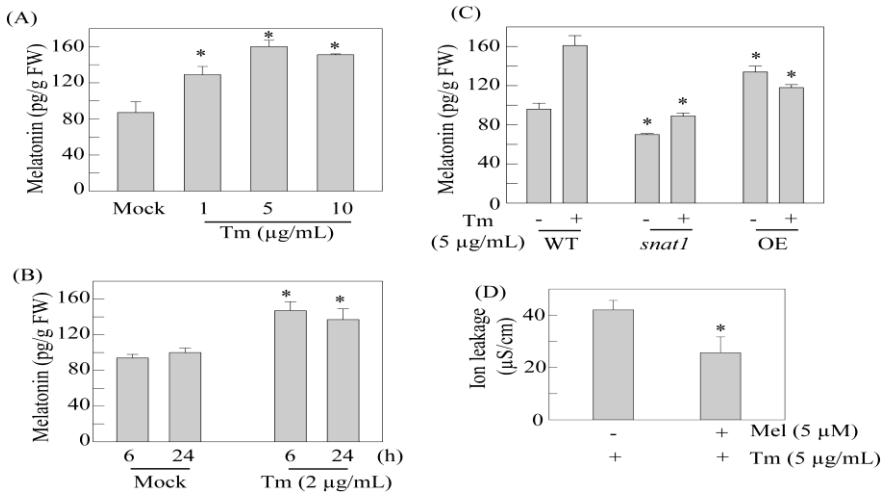

Fig. 3. Melatonin contents in response to Tm stress.

(A) Melatonin levels in WT upon treatment with various concentrations of TM. (B) Time course analysis of melatonin levels in WT in response to 2 μg/mL Tm. (C) Melatonin contents in WT, snat1 mutant, and OE line at 6 hours after 5 μg/mL Tm treatment. (D) Effect of melatonin on ion leakage upon Tm treatment. Arabidopsis leaves were syringe-infiltrated with solution containing 5 μg/mL Tm with or without 5 μM melatonin (Mel), and incubated for 6

hours (or 24 hours) followed by ion leakage analysis. Mock solution was 0.05% DMSO. Asterisks (*) indicate significant differences compared to Mock or WT (Student's t-test; P < 0.05).

3.2. Melatonin protects ER structure and enhances protein folding capacity.

To obtain greater insight into the functional role of melatonin against ER stress, we performed confocal microscopy to examine the ER structure and protein folding capacity in response to ER stress. First, to examine the ER network structure, we performed syringe infiltration with *Agrobacterium* harboring the pBIN61-HDEL:CFP (cyan fluorescent ER protein marker), driven by the constitutively active 35S promoter, into tobacco leaves. Tm treatment at a concentration of 10 µg/mL disrupted the tubular ER network, whereas the ER network was normal in leaves subjected to mock control (0.05% DMSO) or melatonin treatment alone (Fig. 4A–C). Surprisingly, melatonin plus Tm treatment completely recovered the damaged ER network caused by Tm treatment (Fig. 4D), indicating a potent protective role of melatonin against ER stress from the viewpoint of ER network structure.

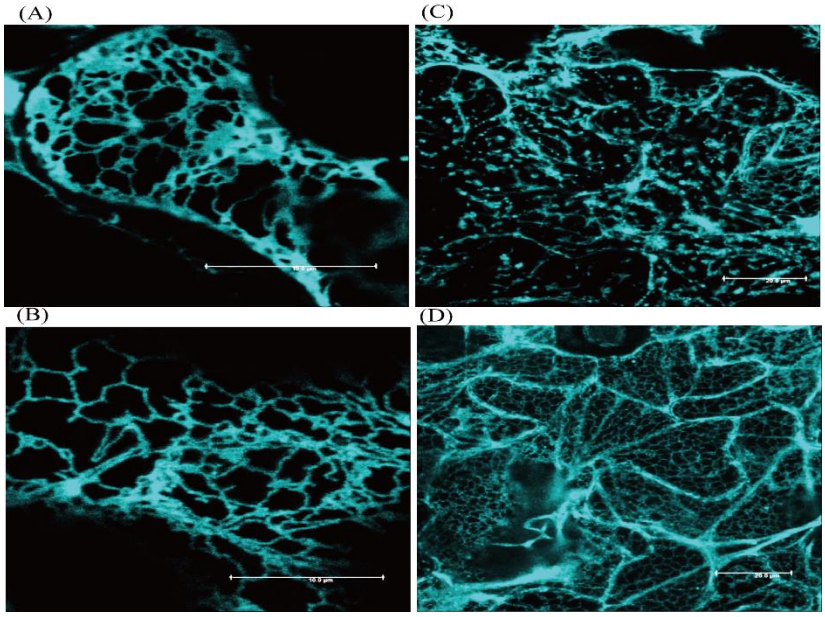

Fig. 4. ER structures in response to Tm treatment determined by confocal microscopy.
(A) Mock treatment (0.05% DMSO). (B) Treatment with 5 µM melatonin. (C) Treatment with 10 µg/mL Tm. (D) Co-treatment with 10 µg/mL Tm plus 5 µM melatonin. N. benthamiana leaves were syringe-infiltrated with Tm, with or without melatonin, in conjunction with Agrobacterium-expressing pBIN61-HDEL-CFP plasmid (an ER fluorescence marker) and incubated for an additional 10 hours before confocal microscopy. Bars indicate 10 µm (A, B) and 20 µm (C, D).

To obtain more detailed physiological evidence of melatonin in terms of ER protein sorting capacity, two ER-processed secretory proteins, BRI1 and FLS2, were monitored in response to Tm (32, 33). BRI1 is a well-known BR PM receptor (32) and FLS2 is a flagellin-binding receptor, the activation of which is the first step in initiation of the plant innate immune response to bacterial pathogen attack (34). Secretion of these two receptors into the PM requires proper folding in the ER by ER-resident chaperones. The intracellular distributions of XVE-

inducible BRI1-mCherry or FLS2-mCherry were examined in response to Tm treatment with and without 5 μM melatonin in *N. benthamiana* leaves. Both BRI1 and FLS2 fluorescence exhibited perfect PM localization (Fig. 5A, D), as reported previously (32, 33). Treatment of *N. benthamiana* leaves with 0.5 μg/mL Tm resulted in abnormal aggregation in their fluorescence to the PM (Fig. 5 B, E), indicating that both receptors were misfolded by Tm. However, on co-treatment with Tm and melatonin, both PM receptors showed a normal distribution of fluorescence in the PM (Fig. 5 C, F). In addition, the accumulation of misfolded protein in the ER induces ER-associated degradation (ERAD) through a proteasome-dependent pathway, which therefore reduces the protein load. To examine whether melatonin overcomes Tm-induced ERAD, BRI1 protein levels were monitored by immunoblotting analysis. As shown in Figure 5H, the total amount of BRI1 protein was reduced upon Tm treatment, but this decrease was fully restored by the addition of melatonin in the presence of Tm. These results clearly indicated that melatonin is required for protein folding in the ER stress response.

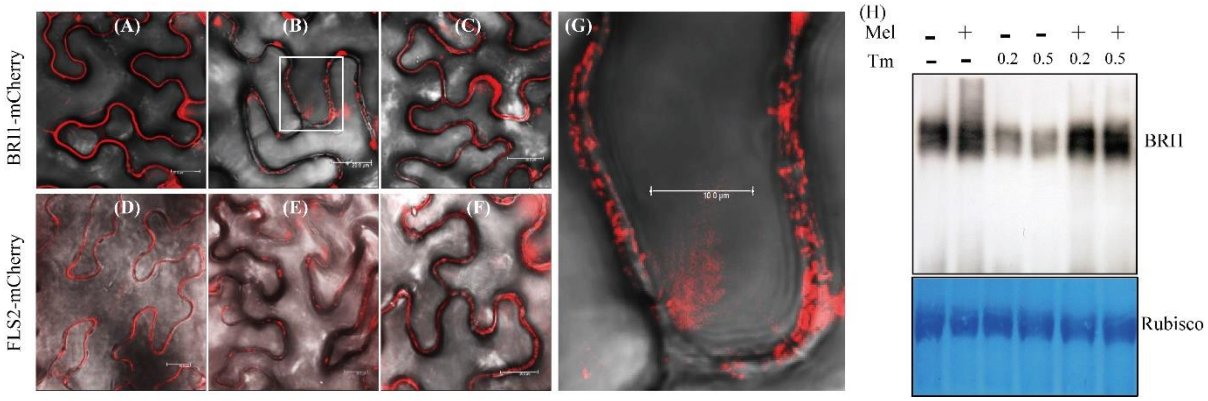

Fig. 5. Effects of melatonin on plasma membrane (PM) accumulation of BRI1 and FLS2 under conditions of ER stress.

(A) Mock treatment BRI1-mCherry. (B) Tm treatment BRI1-mCherry. (C) Tm plus melatonin treatment BRI1-mCherry. (D) Mock treatment FLS2-mCherry. (E) Tm treatment FLS2-mCherry. (F) Tm plus melatonin treatment FLS2-mCHerry. (G) Enlarged view of B. (H) Immunoblotting analysis of BRI1. The blot was stained with Coomassie blue; Rubisco is shown as an equal loading control. Agrobacterium tumefaciens GV2260 harboring pER8-BRI1-mCherry or pER8-FLS2-mCherry, which localize to the PM, was infiltrated into N. benthamiana in conjunction with Tm, or with Tm (0.5 μg/mL) plus melatonin (5 μM). Mel, melatonin.

3.3. Melatonin confers ER stress tolerance by increasing ER chaperone rather than antioxidant defense.

Melatonin has been shown to be a potent antioxidant, as well as a signal activator of antioxidant defense systems (7). It is intriguing to examine whether ER defense or the antioxidant defense system is dominantly coupled to melatonin-mediated ER stress tolerance. To examine this, we measured the expression levels of their corresponding genes when *Arabidopsis* leaves were challenged with Tm stress. The *snat1* mutant showed higher accumulation of O^{2-} than wild-type, whereas the OE line had a comparable O^{2-} level to wild-type against Tm (Fig. 6A). However, the OE line always exhibited a slightly lower O^{2-} level than wild-type. These results were further corroborated by measuring the expression levels of various defense gene transcripts responsible for either ER stress tolerance or antioxidant

defense. Representative transcripts of the ER-resident chaperones, *BIP* and *CNX*, were highly induced rather than representative antioxidant genes, i.e., *APX*, *CAT*, and glutathione S-transferase (*GST*), in wild-type controls upon Tm stress (Fig. 6B, C). Consistent with this result, *BIP2*, *BIP3*, and *CNX1* expression were markedly suppressed in the *sant1* mutant and highly promoted in the OE line in response to Tm. In contrast, only *APX1* was suppressed in the *snat1* mutant and a marginal increase in *CAT1* was observed in the OE line upon Tm treatment (Fig. 6B, C). There were no marked differences between the OE line and wild-type in the levels of O^{2-} or antioxidant enzymes, but the ER chaperones were markedly elevated in the OE line compared to the wild-type controls upon ER stress, suggesting that melatonin was of more relevance to ER chaperones than antioxidant enzymes. However, we could not exclude the possibility that melatonin may act as an antioxidant factor for scavenging reactive oxygen species (ROS), including O^{2-}, produced by ER stress. These data strongly suggest that melatonin regulates ER-resident chaperones, resulting in ER stress tolerance.

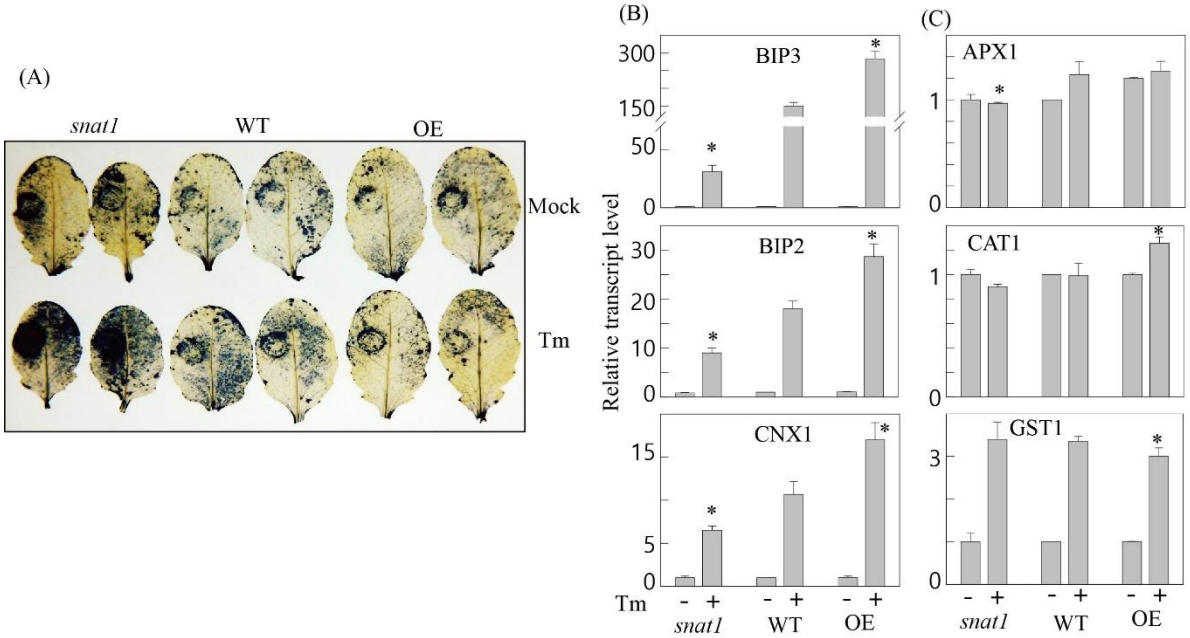

Fig. 6. Superoxide level and quantitative RT-PCR (qRT-PCR) analysis of *Arabidopsis* in response to Tm treatment.

(*A) Superoxide level in response to Tm (1 μg/mL). (B) Relative ER chaperone gene expression levels. (C) Relative antioxidant gene expression levels. The expression level of each gene was normalized to the expression of EF-1a as an internal control. Five-week-old Arabidopsis leaves were syringe-infiltrated with a solution containing 1 μg/mL Tm or 0.05% DMSO (-) (Mock) and incubated for 5 hours before staining with nitrotetrazolium blue (NBT) or RNA analysis. Asterisks (*) indicate significant differences compared to Mock or WT (Student's t-test; P < 0.05). Tm, tunicamycin.*

3.4. Melatonin confers ER stress tolerance via bzip60 transcription factor.

Plants have two ER stress sensors (27, 28), i.e., inositol-requiring enzyme 1 (IRE1) and bZIP28 (also known as ATF6). IRE1 is activated upon ER stress and splices ER membrane-associated basic leucine zipper (bZIP) transcription factor mRNAs, such as bZIP60, leading to translocation into the nucleus to induce the expression of chaperone genes, such as *BIP*. Upon ER stress, bZIP28 is activated by proteolytic cleavage in the Golgi and translocated to the

nucleus to induce chaperone gene expression. To assess which of these ER sensors is required for melatonin-mediated ER stress tolerance, two knockout mutants, i.e., *bzip60* and *bzip28*, were challenged with Tm or melatonin and chaperone gene expression was monitored. The ER stress inducer Tm strongly induced ER chaperones in wild-type controls, but significantly lower levels of ER chaperone expression were observed in the *bzip60* mutant by Tm treatment (Fig. 7A). Although melatonin was not as potent an inducer as Tm, we found marked transcriptional induction of *BIP2*, *BIP3*, and *CNX1* upon melatonin treatment (Fig. 7B). Interestingly, none of the ER chaperone genes showed obvious differences between wild-type controls and the *bzip28* mutant line (Fig. 7C). Therefore, it is highly likely that melatonin-mediated ER stress tolerance is transduced by the IRE1 sensor via the bZIP60 pathway.

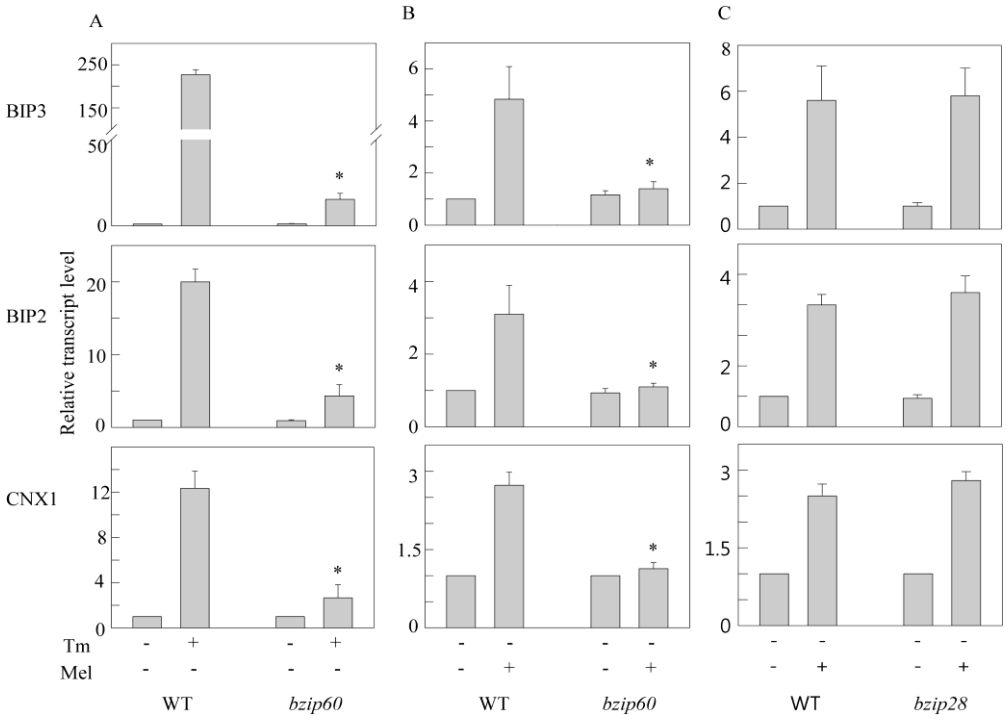

Fig. 7. Relative ER chaperone gene expression levels in *bzip60* and *bzip28* knockout mutants in response to Tm treatment.

(A) Relative transcript levels in the bzip60 knockout mutant upon Tm (1 μg/mL) treatment. (B) Relative transcript levels in the bzip60 knockout mutant upon melatonin (2 μM) treatment. (C) Relative transcript levels in the bzip28 knockout mutant upon melatonin treatment. Leaves were collected 3 hours after infiltration of solutions. Total RNA was analyzed by qRT-PCR. The expression level of each gene was normalized to the expression of EF-1a as an internal control. Asterisks (*) indicate significant differences compared to WT (Student's t-test; P < 0.05). Mel, melatonin; Tm, tunicamycin.

To verify the activation of bZIP60 by melatonin, *Arabidopsis* leaves were treated with either Tm or melatonin, and the levels of active *bZIP60* transcript were determined. The full-length form of *bZIP60* transcript localized to the ER is spliced upon ER stress to synthesize active *bZIP60* (23-base intron deleted) by the RNase activity of IRE1. In response to ER stress, the active mRNA of *bZIP60* (spliced) was markedly induced in wild-type controls (Fig. 8A). In addition, melatonin treatment induced the production of active *bZIP60* (spliced), although the level of induction was far lower than that seen with Tm treatment (Fig. 8A). Moreover, the levels of the spliced form of *bZIP60* transcript were far lower in the *snat1* mutant, but higher

in the OE line than in the wild-type controls (Fig. 8B). In contrast to *bZIP60*, *bZIP28* levels were not altered in either the *snat1* mutant or OE line upon Tm treatment. These results confirmed that not only exogenously applied melatonin, but also endogenous melatonin regulate the ER chaperones through activation of *bZIP60*.

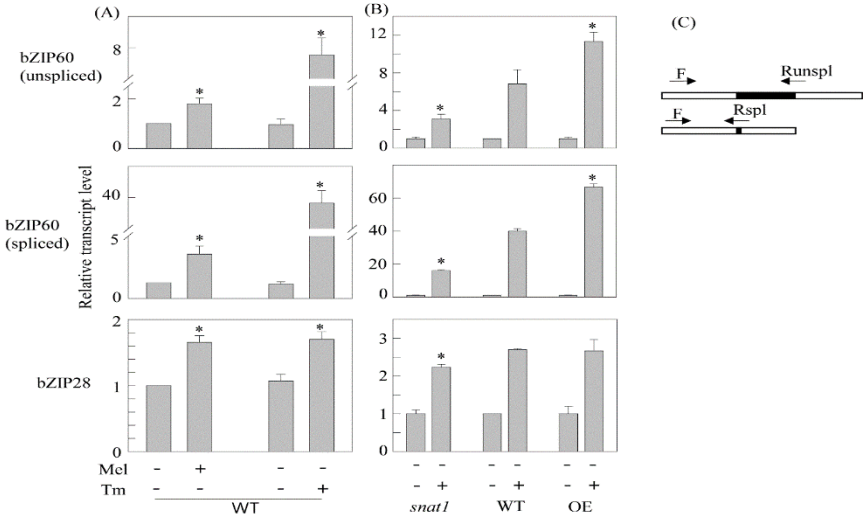

Fig. 8. Relative gene expression levels of *bZIP60* and *bZIP28*.

(A) Relative transcript levels of unspliced bZIP60, spliced bZIP60, and bZIP28 in response to either melatonin or Tm in WT. (B) Relative transcript levels of unspliced bZIP60, spliced bZIP60, and bZIP28 in response to either melatonin or Tm in the snat1 mutant and OE line. Arabidopsis leaves were treated with 0.05% DMSO (−) (Mock),1 μM melatonin, or 1 μg/ml Tm for 3 hours and then RNA analysis was performed for bZIP60 (unspliced or spliced transcript) and bZIP28. (C) Schematic representation of primer locations. The expression of each gene was normalized to that of EF-1a as an internal control. Asterisks (*) indicate significant differences compared to Mock (A) and WT (B) (Student's t-test; P < 0.05). Mel, melatonin; Tm, tunicamycin.

3.5. Melatonin-mediated ER stress tolerance requires MAPK signaling pathway.

Previously, we reported that specific MAPKKK, MAPKK, and MAPK3/6 cascades are involved in the melatonin-mediated pathogen defense signaling pathways in *Arabidopsis* (29, 31). Therefore, we examined whether the MAPK pathway is involved in the melatonin-mediated ER stress response by comparing the levels of chaperone gene transcripts between wild-type and a *mapk3/6*-silenced line. The induced levels of ER chaperone and sensor gene transcripts in *mpk3/6*-silenced plants were significantly reduced compared to those in wild-type controls in response to melatonin treatment (Fig. 9). These observations indicated that the MAPK signaling cascade is required for the induction of active *bZIP60* and its chaperone genes.

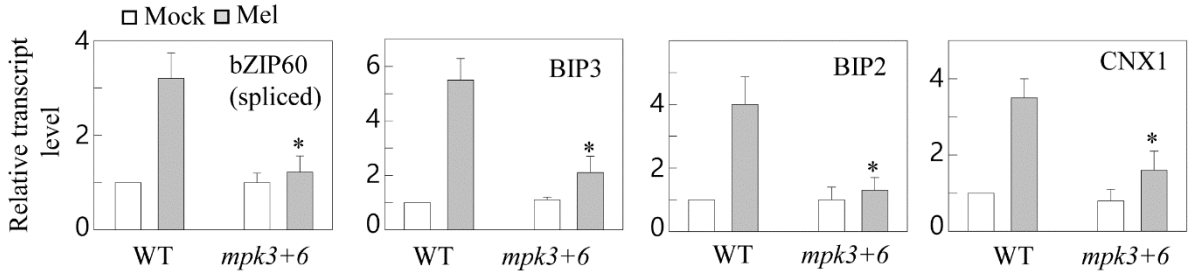

Fig. 9. Relative gene expression levels of *bZIP60* and ER chaperone genes in *mapk3+6* mutant in response to melatonin.

Five-week-old WT and mpk3+6 knockdown Arabidopsis leaves were subjected to mock (H_2O) or melatonin (2 μM) treatment and collected at 3 hours for infiltration. Asterisks (*) indicate significant differences compared to WT (Student's t-test; $P < 0.05$). Mel, melatonin.

4. CONCLUSION

As sessile organisms, plants are frequently exposed to adverse environmental conditions, including high light, heat, water deficit, high salt, and pathogens. All of these stimuli can trigger ER stress, and even normal healthy plants are exposed to ER stress (as evidenced by the basal levels of autophagy and housekeeping ER defense processes) (28). Although there have been many studies in animals (24, 25), there has been only one report regarding the possible involvement of melatonin in autophagy in plants (26). Here, using molecular genetics and pharmacological approaches, we demonstrated that melatonin is involved in the regulation of ER stress via the *bZIP60* activation pathway. In addition, we further discovered that melatonin-mediated ER stress regulation is transduced by the MAPK signaling cascade, similar to the melatonin-mediated regulation of pathogen resistance. Our observations suggest that both melatonin-induced pathogen and ER resistance are initiated by melatonin receptors (Fig. 10). In particular, two findings suggested that the homeostatic melatonin level may participate in ER stress regulation rather than other antioxidant factors. First, plants have very low levels of melatonin (10, 35) compared to other plant antioxidants (36). Even under conditions of stress, plants do not show high levels of melatonin synthesis (36). Second, melatonin deficiency in plants causes greater susceptibility to ER stress than other biotic and abiotic stresses (7). All of these types of stress induce ER stress. However, we cannot underestimate the role of melatonin as a potent antioxidant in plants, as it is in animals, in response to various stress stimuli (38).

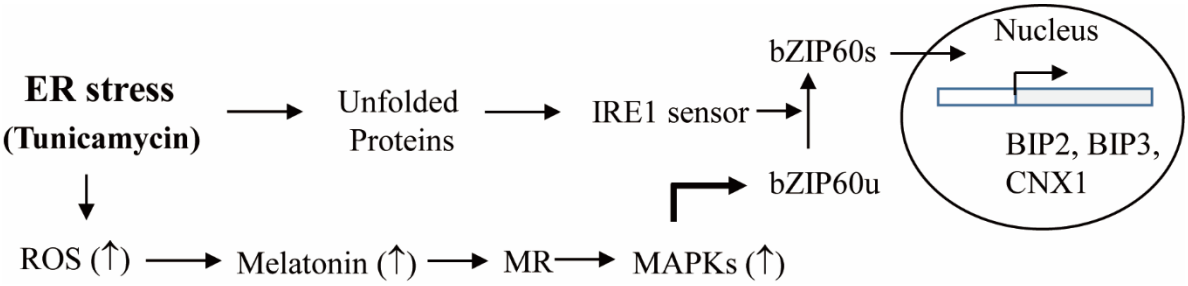

Fig. 10. Proposed model of the melatonin-mediated ER stress resistance signaling pathway.

Under ER stress conditions, such as tunicamycin treatment, the unfolded proteins are accumulated and activate the IRE1 RNase function, which in turn splices bZIP60. The resulting spliced bZIP60 (bZIP60s) is translated into the active transcription factor bZIP60, which translocates into the nucleus leading to transcriptional upregulation of various ER chaperone genes, such as luminal binding protein (BIP) and calnexin (CNX) (28). Upon ER stress, melatonin is induced due to oxidative stress (39) and may bind to melatonin receptors (MR or Cand2). The binding of melatonin to MR enhances the mitogen-activated protein kinase (MAPK) cascade, which in turn activates the IRE1-bZIP60-mediated ER chaperone pathway resulting in melatonin-mediated ER stress resistance. MR, melatonin receptor. Cand2 was reported to be a melatonin receptor which is involved in stomatal closure via the H_2O_2 and calcium signaling cascade (20).

AUTHORSHIP

HY Lee designed and carried out the experiments. K Back advised and wrote the article.

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CONFLICT INTREST

No potential conflicts of interest were disclosed.

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