Melatonin Regulates Chloroplast Protein Quality Control via a Mitogen-Activated Protein Kinase Signaling Pathway

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Abstract: Serotonin N-acetyltransferase 1 (SNAT1), the penultimate enzyme for melatonin biosynthesis has shown N-acetyltransferase activity toward multiple substrates, including histones, serotonin, and plastid proteins. Under two different light conditions such as 50 or 100 µmol m⁻² s⁻¹, a SNAT1-knockout (snat1) mutant of Arabidopsis thaliana ecotype Columbia (Col-0) exhibited small size phenotypes relative over wild-type (WT) Arabidopsis Col-0. Of note, the small phenotype is stronger when growing at the 50 µmol m⁻² s⁻¹, exhibiting a dwarfism phenotype and delayed flowering. The snat1 Arabidopsis Col-0 accumulated less starch than the WT Col-0. Moreover, snat1 exhibited lower Lhcb1, Lhcb4, and RBCL protein levels, compared with the WT Col-0, but no changes in the corresponding transcripts, suggesting the involvement of melatonin in chloroplast protein quality control (CPQC). Accordingly, caseinolytic protease (Clp) and chloroplast heat shock proteins (CpHSPs), two key proteins involved in CPQC, as well as ROS defense were suppressed in snat1. In contrast, exogenous melatonin treatment induced expression of Clp, CpHSP, APX1, and GST, but not other growth-related genes such as DWF4, KS, and IAA1. Finally, the induction of ClpR1, APX1, and GST1 in response to melatonin was inhibited in the mitogen-activated protein kinase (MAPK) knockdown Arabidopsis (mpk3/6), suggesting that melatonin-mediated CPQC was mediated, in part, by the MAPK signaling cascade. These results suggest that melatonin is involved in CPQC, which plays a pivotal role in starch synthesis in plants.

Keywords: melatonin; starch synthesis; MAPK kinase pathway; Clp protease; ROS defense

1. Introduction

Serotonin N-acetyltransferase (SNAT1) protein was first identified in Arabidopsis that interacts with the nuclear shuttle protein (NSP) of geminivirus [1]. SNAT1 belongs to the GCN5-like N-acetyltransferase (GNAT) family and is responsible for acetylation of H2A and H3 histones and the geminivirus coat protein, but not NSP [1]. Thus, SNAT1 was first called by the name of nuclear shuttle interacting (NSI) protein and reported to be involved in viral infection and pathogenicity by regulating nuclear export of the viral genome via interactions with NSP [2]. In contrast to the initial nuclear protein acetylation activity of SNAT1, the cDNA expression analyses of 31 rice SNAT genes in Escherichia coli revealed that SNAT1 also possesses serotonin N-acetyltransferase (SNAT) activity, catalyzing serotonin into N-acetylserotonin the penultimate substrate for melatonin biosynthesis [3,4]. SNAT1 exhibited $K_m$ and $V_{max}$ values for serotonin of 309 µM and 1.4 nmol/min/mg protein, respectively, and was localized to chloroplasts but in a different manner to previous nuclear localization results for NSI [1,4].

Recently, it was reported that SNAT1 was also discovered to catalyze the N-acetylation of chloroplast proteins, such as Lhcb1.4, which resulted in an impaired state transition in a SNAT1-knockout Arabidopsis (snat1) [5]. In addition to the state transition changes, many other biochemical phenotypes have been observed in the snat1 Arabidopsis, including enhanced pathogen susceptibility [6], reduced high-light stress tolerance [7], salinity...
hypersensitivity [8], and increased endoplasmic reticulum stress susceptibility [9]. These many defects in \textit{snat1} Arabidopsis have been complemented by exogenous supplementation of melatonin, suggesting that melatonin is predominantly responsible for the many phenotypes induced by \textit{SNAT1} knockout rather than the acetylation of chloroplast proteins. Correspondingly, the \textit{snat1} decreased melatonin by 25\% compared to the wild type (5), and its overexpression line (OE) increased melatonin two-fold relative to the wild type (7).

The major genes responsible for melatonin biosynthesis are tryptophan decarboxylase, which catalyzes tryptophan into tryptamine, and tryptamine 5-hydroxylase, which converts tryptamine into serotonin. SNAT plays a pivotal role in synthesizing \textit{N}-acetylserotonin, the key substrate for melatonin biosynthesis, and \textit{N}-acetylserotonin is then converted into melatonin by \textit{N}-acetylserotonin O-methyltransferase [10]. Although \textit{SNAT} exists as multiple isogenes in plants and belongs to the GNAT family, these isogenes share a very low amino acid identity, except for within the acetyltransferase domain. \textit{SNAT1} is derived from cyanobacteria, since cyanobacteria also harbor a \textit{SNAT1} orthologous gene, and it shares a 58\% amino acid identity with that of rice \textit{SNAT1} [11]. Furthermore, the \textit{SNAT1} ortholog of red algae resides in the chloroplast genome, whereas those of plants and green algae reside in the nuclear genome [12]. This suggests that \textit{SNAT1} was transferred from the chloroplast to the nuclear genome during plant evolution, and it may have specific roles in chloroplast functionality, such as the state transition in photosynthesis [5]. Overall, \textit{SNAT1} plays a pivotal role in melatonin biosynthesis in many plant species, including rice. Its overexpression (OE) leads to increased melatonin production, whereas its suppression results in decreased melatonin production and the corresponding physiological and biochemical phenotypes described above [13–16].

Melatonin had long been recognized as an animal pineal hormone that regulates many physiological activities such as sleep, the circadian rhythm, innate immunity, and cellular oxidative status [17]. However, melatonin has also been identified in various plants [18,19], where it has pleiotropic biological roles in plant growth and development, and in plant defense systems against biotic and abiotic stresses [20]. The representative roles of melatonin in plant growth and development include promoting seedling growth [13], early flowering [21,22], enhanced seed germination and viability [23,24], delayed senescence [25], diurnal stomatal closure [26], and increased secondary metabolites [27], etc. [20,28,29]. Moreover, melatonin has a profound effect on plant defenses against a vast array of adverse environmental stresses, helping plants to survive and thrive [30]. For example, melatonin confers tolerance in response to virus and pathogen attacks [31,32], and to many abiotic stresses, including cold, heat, salinity, drought, heavy metals, herbicides, and tunicamycin [9,20,33], via either its potent antioxidant role or melatonin signaling cascade through its receptor [34,35].

Although the multiple roles of melatonin against various stresses have been investigated extensively in plants, its role in chloroplast function during normal plant growth and development has not been examined in detail. In this report, we used a \textit{snat1} Arabidopsis to show that changes in melatonin biosynthesis result in the \textit{snat1} phenotype, i.e., dwarfism, reduced starch synthesis, and delayed flowering in conjunction with an impaired chloroplast quality control by way of MAPK cascade.

**2. Materials and Methods**

**2.1. Plant Material and Growth Conditions**

All \textit{Arabidopsis thaliana} lines used in this study were in the ecotype Columbia (Col-0) background. \textit{SNAT1} (At1g32070) knockout Arabidopsis line containing T-DNA insert in SALK_020577 (\textit{snat1}) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA), as described previously [7]. Transgenic lines overexpressing \textit{SNAT1} (OE) and double suppression lines of \textit{MPK3} and \textit{MPK6} (\textit{mpk3/6}) have been previously described [7,36]. Plants were grown in plastic pots containing a commercial horticultural substrate (coco peat (47\%): peat moss (35): vermiculite (10): zeolite (7\%) (Farmhannong, Seoul, Korea)): perlite (3:1) (SJ Company, Ulsan, Korea). The
soil mixture was washed out by two-times irrigation with tap water to exclude possible interference of artificial nutrition. Controlled environmental conditions were provided in a growth room at 23 °C and relative humidity of 50% under 12-h light/12-h dark photoperiod with white light illumination (50 µmol m⁻² s⁻¹). Fluorescent light from OSRAM (Seoul, Korea) with 50:50 of 6500K (865 FPL36EX-D) and 4000K (840 FPL36EX-W) was used for the light source.

2.2. Flowering Time, Leaf Area, and Weight Measurements

Flowering times were determined by counting the number of rosette or cauline leaves on the main shoot when the plants had the first flower at about seven weeks after seeding. At the same time, rosette leaf area was measured using Fiji ImageJ, as described previously [37]. Photographs of rosette leaves were taken next to a ruler that was used as a reference to convert pixels to the corresponding metric unit. Data were processed using Microsoft Excel 2010. The fresh weight of aerial parts was measured at indicated growth stages. At least five plants per genotype were used for each measurement, and average and standard deviation were calculated accordingly.

2.3. Starch Staining

Iodine-stained visualization of starch was conducted as described [38]. Briefly, whole Arabidopsis rosettes of four- or six-week-old plants were collected and decolorized using 90% (v/v) of hot ethanol. Ethanol was removed by rinsing in water before staining Lugol’s iodine Reagent (Sigma-Aldrich, Saint-Louis, MO, USA). Over 6-h iodine staining, plants were destained in water until optimal visualization of the amylose–iodine complex in starch was achieved.

2.4. Melatonin Treatment

All transcripts of target genes studied here have been positively regulated during daylight. To abstain from the daylight induction of these genes, four-week-old Arabidopsis plants grown under the standard condition (50 µmol m⁻² s⁻¹) were infiltrated with 1 µM melatonin (in 2 mM MgCl₂) by needless 1 mL syringe at ZT0 and transferred to the dim light condition (7 µmol m⁻² s⁻¹), followed by sample harvest at various time intervals. Melatonin was infiltrated into the abaxial sides of four-week-old rosette leaves of Arabidopsis.

2.5. Protein Extraction and Protein Gel Blotting Analysis

Protein extracts were prepared with 40 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Triton X-100, and 1x Roche Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA) and then centrifuged at 10,000×g for 10 min at 4 °C. Aliquots of the supernatant were mixed with sample buffer (Tris-HCL, pH 6.8, 10% SDS, 10 mM DTT, 20% glycerol, and 0.05% bromophenol blue). Then, samples were boiled and loaded onto SDS–PAGE gels. After electroblotting on a nitrocellulose membrane, protein gel blot analysis was performed using antibodies against Lhcb1, Lhcb4, RBCL, RBCS and ClpR1 (Agrisera AB, Vannas, Sweden). Secondary rabbit antibody conjugated with Horseradish Peroxidase (Roche) was incubated with the membrane for at least 1 h. Proteins were detected using the ECL system (RPN2132; Amersham Biosciences, Piscataway, NJ, USA).

2.6. RNA Analysis

Total RNA was extracted from Arabidopsis plants using a Nucleospin RNA Plant Kit (Macherey-Nagel, Duren, Germany). Reverse transcription was performed using a Stratagene Reverse Transcription Kit (Stratagene, La Jolla, CA, USA) with 500 ng of oligo (dT)18 or random octamer primer for RBCL and ClpP1 (CancerROP, Seoul, Korea). The PCR reaction was conducted as following conditions initial denaturation 95 °C (3 min), denaturation 95 °C (30 s), annealing 56 °C (30 s), and extension 72 °C (1 min) with 30 µL of master mix. qRT-PCR was performed on a Mic qPCR Cycler System (Bio Molecular Systems, Queens-
land, Australia) using 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 are shown in Table S1. EF-1α (EF1ALPHA; AT5G60390) was used for signal normalization. The data were analyzed by analysis of variance using IBM SPSS Statistics 25 software (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. All data are presented as mean \( \pm \) standard deviation.

3. Results

3.1. Phenotypic Features of the SNAT1-Knockout Arabidopsis Mutant

The SNAT1 knockout Arabidopsis (snat1) showed slight dwarfism when grown under an ambient light regime (100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) but exhibited a stronger dwarf phenotype under a decreased light regime (50 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) (Figure S1). A light intensity of 50 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) was sufficient for normal growth and development of Arabidopsis thaliana ecotype Col-0, as indicated below. Under a light intensity of 50 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), WT Arabidopsis Col-0 showed healthy growth (Figure 1), whereas the snat1 showed the dwarfism phenotype and delayed flowering (Figure 1A–C). In contrast, SNAT1 OE resulted in faster growth and earlier flowering compared with the WT, but at nine weeks, the total biomass was lower than that of the WT (Figure 1A–C). Compared with the WT, the snat1 had more leaves, whereas OE resulted in fewer leaves at the seven-week flowering stage (Figure 1D,F). The snat1 had lower biomass and smaller leaf area compared with the WT (Figure 1E,G). OE resulted in higher numbers of cauline leaves compared with the WT, but the underlying mechanism for this is unclear (Figure 1D). Collectively, these growth parameters indicated that the snat1 exhibits retarded growth and development phenotypes compared with the WT, suggesting that SNAT1 has physiological roles in growth, which were observed previously in the SNAT1 RNAi rice seedlings [13].

Figure 1. (A) Rosette or (B) flowering, (C) phenotype of wild type (WT) (Col-0), snat1, and SNAT1 overexpression (OE) Arabidopsis lines. Plants were grown for four (A), nine (B), or seven weeks (C) under 50 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) light conditions. (D) The flowering times of Arabidopsis were denoted as the total number of rosette and cauline leaves averaged over five independent plants. (E) Fresh weights of the WT, snat1, and OE lines 4–9 weeks after planting. (F) Representative rosette leaves from the WT, snat1, and OE lines after the OE line had started to bolt. (G) Rosette leaf areas of each leaf position in the WT, snat1, and OE lines, measured at the same time as in F. Scale bar: 1 cm (A,C,F) and 3 cm (B). Different letters indicate significant differences between groups (small letters or small letters with an apostrophe) (Tukey’s post hoc HSD test; \( p < 0.05 \)). SNAT1 encodes the gene with At1g32070.
3.2. Defective Starch Accumulation in the Snat1 Mutant

We deduced that the growth retardation of snat1 Arabidopsis was likely associated with the growth rate-related starch synthesis. To evaluate starch biosynthetic capacity, we measured starch levels during the day, which were increased by photosynthesis [39]. Lugol staining of plants showed undetectable starch levels at Zeitgeber time (ZT) 2 (after 2 h of light exposure) in the WT, snat1, and OE lines (Figure 2A). Starch accumulation was clearly evident at ZT9 in the WT but was much less evident in snat1. In particular, higher levels of starch accumulated in the OE than WT lines. Changes in starch accumulation during the day were equally observed in both four- and six-week-old Arabidopsis plants among WT, snat1, OE, providing strong evidence for the role of SNAT1 in photosynthesis.

To ascertain whether the changes in starch accumulation were associated with changes in starch synthesis and degradation [40], we quantified the mRNA levels of phosphoglucomutase (PGM1) and phosphoglucomutase (SEX1). As shown in Figure 2B, the diel expression patterns of these two genes were not significantly different among the WT, snat1, and OE plants. These data indicated that the decrease in starch synthesis in snat1 was not due to transcript changes involved in starch synthesis and degradation.

![Figure 2. (A) Comparison of leaf starch contents. WT (Col-0), snat1, and SNAT1 OE Arabidopsis lines grown under 50 μmol m\(^{-2}\) s\(^{-1}\) light conditions for four or six weeks were decolorized and stained with iodine solution, washed with water, and photographed. Plants were collected at Zeitgeber time (ZT)2 or ZT9. (B) qRT-PCR analysis showing induced expression of starch metabolism-related genes in WT (Col-0), snat1, and OE Arabidopsis plants. Leaf samples were collected at various ZT intervals. The target transcript levels were normalized to those of the αEF1α endogenous control. ZT, Zeitgeber time; ZT0 represents dawn. Values are means ± standard deviation of three independent experiments. Different letters indicate significant differences (Tukey’s post hoc HSD test; p < 0.05).](image-url)

3.3. Suppression of Lhcb1, Lhcb4, and RBCL Proteins in the snat1 Mutant

Light-harvesting antenna proteins such as Lhcb1 play important roles in photosynthetic electron transport. For example, a decrease in the Lhcb1 level reduced chlorophyll levels and state transition in Arabidopsis [41]. We performed Western blot analyses to evaluate Lhcb protein levels in snat1. As shown in Figure 3, the protein levels of Lhcb1 and Lhcb4 were greatly reduced in snat1, compared with the WT. The ribulose-1,5-bisphosphate carboxylase/oxygenase (RBC) large subunit (RBCL) was also slightly reduced in snat1,
whereas the RBC small subunit (RBCL) was not affected, compared with the WT. The OE line of Arabidopsis also exhibited a decrease in the Lhcb1 protein level, but not to the same extent as in snat1. In marked contrast to the protein levels, the mRNA levels of these proteins, except for RBCL, were not affected, suggesting that the reduced levels of Lhcb1 and Lhcb4 in snat1 resulted from altered protein stability in the chloroplasts rather than from altered protein synthesis in the cytoplasm.

Figure 3. (A) Western blot analysis using anti-Lhcb1, -Lhcb4, -RBCL, and -RBCS antibodies in the WT, snat1, and OE Arabidopsis lines at ZT8. Total leaf protein extracts (1, 2, 4, and 8 µg) were subjected to 14% SDS–PAGE. The immunoblot was probed with specific antibodies, as indicated on the right. Molecular weights are shown on the left. The bottom panel shows a loading control stained with Ponceau S solution (PS). (B) qRT-PCR analysis of the corresponding transcripts at ZT8. EF1α was used as a loading control. Numbers in parentheses indicate the number of PCR cycles.

3.4. Downregulation of ClpR1, a Chloroplast Molecular Chaperone, in the snat1 Mutant

For optimal functionality, the majority of chloroplast proteins are imported from the cytoplasm. In the chloroplast, these proteins undergo precise targeting and folding via the chloroplast protein quality control (CPQC) system, which is controlled by plastid chaperones and heat shock proteins (CpHSPs) [42,43]. Caseinolytic protease (Clp) is an ATP-dependent protease with multiple isoforms that functions as a molecular chaperone. The ClpR1 subunit of Clp plays an important role in chloroplast development by controlling Lhcb2 protein levels, and the ClpR1-knockout mutant had dwarfism and virescent phenotype [44]. Interestingly, snat1 exhibited decreased ClpR1 protein in conjunction with decreased ClpR1 mRNA levels, compared with the WT (Figure 4A). Other Clp subunits, such as ClpR4 and ClpP1, were also downregulated in snat1 compared with the WT at ZT4 and ZT8. The OE line showed a slight decrease in the ClpR1 protein level with a transit increase in the levels of ClpR1, ClpR4, and ClpP1 mRNA at ZT2, compared with the WT. Additionally, mRNA levels of the chloroplast heat shock proteins CpHSP70.1 and CpHSP70.2 were significantly downregulated in snat1, compared with the WT (Figure 4B). The suppression of CpHSP70.1 and CpHSP70.2 in the OE line was unexpected, but their suppression was partly coupled with suppression of the ClpR1 protein (Figure 4A). These data suggest that suppression of the Lhcb and RBCL proteins in snat1 is attributed to the downregulation of chloroplast chaperones such as Clp and CpHSP.
Chloroplasts are the major organelles responsible for the production of reactive oxygen species (ROS) such as O$_2^-$ and H$_2$O$_2$ during photosynthesis. Although ROS can cause oxidative damage, they also act as retrograde signals in chloroplasts to induce a series of nuclear-encoded genes responsible for photosynthesis-related genes such as Lhcb and RBCL, chaperone genes such as CpHSP and Clp, and antioxidant genes such as ascorbate peroxidase 1 (APX1) and glutathione S-transferase (GST1) [45]. Since melatonin is a potent antioxidant believed to be enriched in chloroplasts [46,47], it is highly likely that melatonin plays a specific role in ROS balance in plants. Thus, we investigated whether ROS-responsive genes are also modulated in the snat1 mutant by measuring the mRNA expression levels of various APX and GST isoenzymes during the day. As shown in Figure 5, APX1, encoding a cytoplasmic APX with a central role in the chloroplast H$_2$O$_2$-scavenging system of Arabidopsis [48], was induced at ZT8 in the WT, but its induction was greatly inhibited in the snat1 mutant. In contrast, two other APX isogenes—sAPX (stomatal and mitochondrial APX) and tAPX (thylakoid APX)—were not different between snat1 and WT lines at ZT8. Interestingly, all three GST genes were induced during the day in the WT, but not in snat1. In contrast to the results for snat1, the OE line exhibited much higher induction of the APX1, sAPX, and all three GST genes compared with the WT at ZT8. This enhanced expression of APX and GST genes by OE possibly contributed to the enhanced growth and increased starch synthesis during the early growth stages (Figures 1 and 2). However, a slight reduction in Lhcb1, Lhcb4, and ClpR1 protein levels in the OE line eventually led to a reduction in biomass relative to the WT at nine weeks (Figure 1). Taken together, these data suggest that SNAT1 plays a pivotal role in regulating antioxidant genes, such as APX1, which predominantly acts as a retrograde signal of chloroplast ROS [45]. In analogy, it was reported that singlet oxygen in chloroplasts also induced SNAT1 and melatonin synthesis, followed by an increase in APX1 in the flu knockout mutant of Arabidopsis [7].
Figure 5. Temporal qRT-PCR analysis of various Arabidopsis genes involved in the reactive oxygen species (ROS) defense and scavenging systems at various ZT intervals. Total RNA was isolated, and transcript levels in WT, snat1, and OE Arabidopsis lines were measured by qRT-PCR. The relative fold expression values are normalized to EF1α expression. Error bars show the standard deviation of three biological replicates. Different letters indicate significant differences (Tukey’s post hoc HSD test; p < 0.05).

3.6. Induction of Genes Responsible for the CPQC and ROS Defense Systems by Exogenous Melatonin Treatment

To elucidate the direct relationship between SNAT1 and melatonin in response to the CPQC and ROS defense systems, melatonin (1 μM) was infiltrated onto Arabidopsis leaves and incubated for 6 h. This was followed by qRT-PCR analysis to investigate the induction patterns of the related genes. As shown in Figure 6, there were no increases in four Lhcb isogenes, two RBCS isogenes, or RBCL after melatonin treatment. However, there was a two-fold increase in the expression of three Clp genes, such as ClpR1, ClpR4, and ClpP1, and two CphSP genes, such as CphSP70.1 and CphSP70.2. The highest induction was observed in APX1 followed by GSTF6 (GST1) and GST8, whereas tAPX was not induced. Other genes that affect Arabidopsis growth were also evaluated for their possible involvement in the snat1 dwarfism phenotype. These genes were associated with brassinosteroids (DWF4, BZR1, and CDC2b), gibberellin biosynthesis (KS), and auxin responsiveness (IAA1 and EXP1). These hormone-related genes were not enhanced in response to melatonin treatment. Together with the results observed in snat1, SNAT1-catalyzed melatonin deficiency appears responsible for the dwarfism phenotype via the regulation of genes or proteins involved in the CPQC and ROS defense systems rather than various hormonal genes related to growth.
Effects of exogenous melatonin treatment on various genes related to photosynthesis, protein quality control, ROS defense, and growth. WT Arabidopsis (four weeks old) leaves were infiltrated with 1 μM melatonin at ZT0 and transferred to dim-light conditions (7 μmol m⁻² s⁻¹) to rule out potential light induction of various target genes. The samples were harvested at ZT6 for total RNA isolation. The genes evaluated were Lhcb1.1 (At1g29910), Lhcb1.3 (At1g29930), Lhcb1.4 (At2g34430), Lhcb4.1 (At5g01530), RBCL (At1g02930), RBCS1A (At1g67090), RBCS3B (At5g38410), ClpR1 (At1g49970), ClpR4 (At4g17040), ClpP1 (At1g69530), ClpP1 (Atcg00670), ClpHSP70.1 (At4g24280), ClpHSP70.2 (At5g49910), APX1 (At1g07890), GSTF6 (GST1; At1g02930), GSTF8 (At2g47730), GSTF9 (At2g30860), sAPX (At1g08390), iAPX (At1g77490), DWF4 (At4g50660), BZR1 (At1g75080), CDC2b (At3g54180), KS (At1g79460), IAA1 (At4g14560), and EXP1 (At1g69530). The relative fold expression values are normalized to EF1α expression, and the expression level after each mock treatment (2 mM MgCl₂) was set at a relative level of 1. Different letters indicate significant differences (Tukey’s post hoc HSD test; p < 0.05).

3.7. Induction of ClpR1 Protein by Melatonin via the Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

Melatonin-mediated defense responses against pathogens and endoplasmic reticulum (ER) stress require MAPK signaling [9,36]. ROS-mediated retrograde signaling also depends on MAPK signaling [45,49]. We used an MPK3/MPK6-double knockdown Arabidopsis line (mpk3/6) to confirm whether the MAPK pathway is involved in the CPQC and ROS defense systems [36]. The mpk3/6 exhibited a dwarfism phenotype and a defect in starch accumulation at ZT8, comparable with that in snat1 (Figure 7A,B). Both the ClpR1 and Lhcb1 protein levels were significantly downregulated in mpk3/6, whereas the Lhcb4 and RBCS protein levels were similar to those in the WT. In contrast, the RBCL level was higher in mpk3/6 than in the WT. The different protein expression patterns in snat1 and mpk3/6 indicate that MPK3/6 does not function exclusively in melatonin signaling. Meanwhile, the expression of ClpR1, APX1, and GSTF6 (GST1) transcripts was lower in mpk3/6 than in the WT, suggesting defects in chloroplast chaperone activity and the ROS defense system in mpk3/6 (Figure 7D). Finally, to ascertain whether melatonin-induced ClpR1 was dependent on the MAPK signaling pathway, the leaves of WT and mpk3/6 were challenged with melatonin, and the levels of ClpR1 protein and related transcripts were measured. As shown in Figure 8, ClpR1 was induced in response to 1 μM melatonin treatment in the WT but was significantly inhibited in the mpk3/6 mutant. Moreover, induction of the ClpR1 transcript together with APX1 and GST1 was suppressed in mpk3/6, compared with the WT. These data suggest that melatonin is involved in the induction of ClpR1 via the MAPK signaling cascade, followed by enhanced and stable expression of Lhcb1 protein and related transcripts such as APX1, and GSTF6, which are important regulators of growth and photosynthesis in Arabidopsis (Figure 8C).
**Figure 7.** (A) Rosette phenotype of WT (Col-0) and mpk3/6-double knockdown plants. Plants were grown for four weeks under a light intensity of 50 μmol m$^{-2}$ s$^{-1}$. (B) Leaf starch contents of the WT (Col-0) and mpk3/6 lines. Plants were collected at ZT2 or ZT8. (C) Western blot analysis using anti-ClpR1, -Lhcb1, -Lhcb4, -RBCS, and -RBCL antibodies, as described in Figure 3. Plants were collected at ZT6 or ZT8. (D) Diurnal expression of ClpR1, APX1, and GST1 at ZT2, ZT4, and ZT6. Scale bar: 1 cm. Different letters indicate significant differences (Tukey’s post hoc HSD test; p < 0.05).

**Figure 8.** (A) Induction of ClpR1 in the WT and mpk3/6-double knockdown plants in response to melatonin. To eliminate light interference in target gene induction, Arabidopsis leaves were infiltrated with 1 μM melatonin at ZT0 and transferred to dim light (7 μmol m$^{-2}$ s$^{-1}$) conditions for 3 h (ZT3) and 5 h (ZT5) before sample harvest. Ponceau S stained blots were used as the loading control. (B) Gene expression analysis of ClpR1, APX1, and GST1 after treatment with 1 μM melatonin in the WT and mpk3/6 lines at ZT5. (C) Proposed model of melatonin-mediated CPQC in Arabidopsis. Asterisks denote significant differences as determined by post hoc Tukey’s HSD test at p < 0.05.
4. Discussion

4.1. Melatonin and Photosynthesis

SNAT1 appears to have originated from cyanobacteria according to the presence of a plant SNAT1 ortholog in cyanobacteria [11]. Of note, SNAT1 is present in the plastid genome of the red alga Pyropia yezoensis, whereas other SNAT genes from green algae and higher plants are present in their nuclear genome, suggesting possible endosymbiosis of cyanobacteria [12,50]. SNAT1 was first identified as an NSI with histone acetyltransferase activity [1]. Thereafter, it was found that SNAT1 displays both serotonin N-acetyltransferase activity in melatonin synthesis and protein lysine N-acetyltransferase activity [3,5]. SNAT1 plays a pivotal role in melatonin biosynthesis in various plant species. Suppression of SNAT1 led to a decrease in melatonin, followed by enhanced pathogen susceptibility [6], salinity hypersensitivity [8], and susceptibility to high-light stress [7]. SNAT1 OE increased melatonin levels, resulting in cadmium tolerance [51], delayed senescence [14], lateral root promotion [52], salt tolerance [53], and thermotolerance [16]. In addition to the genetic evidence, exogenous melatonin treatment conferred stress tolerance to a wide range of biotic and abiotic stressors, including viral attack [8,54,55]. In addition to being involved in defense responses, melatonin is also involved in plant growth in conjunction with photosynthesis when plants were challenged with abiotic stresses [20,56]. For example, melatonin treatment enhanced soybean growth by increasing PSI- and PSII-related genes and increased the rate of photosynthesis in Chinese hickory and grape seedlings upon stresses [57–59]. Melatonin treatment in tomato seedlings increased PSII activity and the photochemical quenching coefficient against salt stress [60]. Based on these findings, it is clear that melatonin is implicated in photosynthesis and growth, but its exact role in photosynthesis during normal growth and development conditions remains unknown thus far [61,62]. Based on our new data from the snat1 mutant, we found for the first time that melatonin catalyzed by SNAT1 plays an important role in improving starch synthesis via the regulation of CPQC in plants.

4.2. Melatonin Signaling Pathway in CPQC

SNAT1 uses various substrates including histones, proteins, and serotonin. The snat1 mutant grown under the light intensity of 50 µmol m⁻² s⁻¹ exhibited a severe defect in leaf size relative to that of 100 µmol m⁻² s⁻¹ light intensity (Figure 1 and Figure S1). Light intensity at 50 µmol m⁻² s⁻¹ did not induce low-light stress in Arabidopsis, whereas that below 30 µmol m⁻² s⁻¹ induced low-light stress [63]. The light-dependent dwarfism phenotype was also observed in transgenic SNAT2-knockout rice under low light (30 µmol m⁻² s⁻¹), whereas no such dwarfism was observed under high light (600 µmol m⁻² s⁻¹) [64]. The major cause of the snat1 dwarfism phenotype in Arabidopsis was decreased starch accumulation, compared with the WT. This reduced starch accumulation in snat1 was independent of the regulation of starch synthesis or degradation (Figure 2).

To achieve optimal photosynthesis or plastid biogenesis, chloroplasts require the CPQC system, consisting of CpHSPs and Clp, for correct folding of the many proteins imported from the cytoplasm [43,65–67]. Clp family members are ATP-dependent serine proteases that include many subunits, such as ClpR and ClpP. Among these Clp subunits, ClpR1 plays an important role in the accumulation of many chloroplast-localized proteins, including Lhcb2, RBCL, RBCS, Cpn60, and several PSI subunits [44]. Interestingly, ClpR1 knockout Arabidopsis (clpR1) caused a marked decrease in the RBCL protein level but an increase in the RBCL mRNA level compared with the WT, suggestive of a pivotal role of ClpR1 for CPQC. Comparable with clpR1, snat1 showed a significant decrease in the levels of ClpR1 protein and other chloroplast-localized proteins, including Lhcb1, Lhcb4, and RBCL. However, snat1 showed a dramatic increase in the RBCL mRNA level, similar to that in clpR1, suggesting that SNAT1 is involved in CPQC.

Chloroplasts possess chaperone proteins, including various CpHSPs and Cnp60, which promote the correct folding and assembly of chloroplast-localized proteins. Among the chloroplast chaperones, two plastid HSP70 proteins, CpHSP70.1 and CpHSP70.2, have
been well investigated in Arabidopsis [68]. Knockout of CpHSP70.1 resulted in dwarfism, but knockout of CpHSP70.2 resulted in a comparable phenotype with that of the WT. Mutants defective in both CpHSP70.1 and CpHSP70.2 are lethal, suggesting the crucial role of CphSPs in CPQC [69]. In this study, the mRNA level of only two chaperones, CpHSP70.1 and CpHSP70.2, were decreased in snat1. These results further indicate that SNAT1 is involved in the regulation of chloroplast HSP chaperones.

The correct balance between ROS production and scavenging in chloroplasts is essential for photosynthesis and plant growth; otherwise, oxidative damage can occur, leading to plant cell death [48]. Many antioxidants and antioxidant enzymes have roles in orchestrating ROS balance in chloroplasts. Of these, APX1 is a central player in chloroplast ROS regulation, as suggested by a defective chloroplast ROS-scavenging system, together with a late-flowering phenotype and stunted growth, in an APX1-knockout Arabidopsis mutant [48]. GST superfamily genes also participate in regulating ROS level balance and protecting plants from various oxidative stresses [70]. Suppression of the expression of APX1 and GST genes was observed in snat1, suggesting the involvement of the ROS defense system of SNAT1.

Melatonin interacts directly with a variety of ROS, functions as a potent radical scavenger, and induces a series of antioxidant enzymes, including APX, superoxide dismutase, and catalase, in plants [17,71]. The radical scavenging and antioxidant enzyme induction functions of melatonin are believed to be mediated by a plant melatonin receptor (PMTR), but the integrity of a previously proposed PMTR is controversial [34,35]. In contrast to the PMTR, it is clear that melatonin-mediated defense responses against pathogen attack and ER stress require the MAPK cascade signaling, especially MPK3 and MPK6 [9,36].

5. Conclusions

In this study, we showed that suppression of starch accumulation in snat1 was mediated by SNAT1-catalyzed melatonin decrease, which triggers the decreased accumulation of Lhcb1, Lhcb4, and RBCL proteins. The suppression of these photosynthesis-related proteins was ascribed to the combined effects of both CPQC (CpHSP70s and ClpR1) and ROS defense system (APX1 and GSTF6) (Figure 8C). Of note, the expression of ClpR1, APX1, and GSTF6 was partly dependent on the MAPK pathway.

Based on our data, we can conclude that defective SNAT1 expression gives rise to dwarfism and a delayed flowering phenotype, and the causative factor of these defects is melatonin, which is catalyzed by SNAT1.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3921/10/4/511/s1, Figure S1: Phenotypic difference depending on light intensity in Arabidopsis wild type, snat1, and OE at four weeks after planting, Table S1: Sequences of primers in quantitative real-time RT-PCR.

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