The melatonin receptor CAND2 is involved in the regulation of photosynthesis and chloroplast gene expression in *Arabidopsis thaliana* under photooxidative stress

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Abstract

Melatonin is a well-known bioactive molecule able to mitigate photooxidative damage caused by excess light. Here we have shown that mutant *Arabidopsis* lines with disrupted genes for melatonin putative receptor *CAND2/PMTR1* and *GPA1* encoding the α-subunit of heterotrimeric G-protein were partially insensitive to melatonin treatment under high light stress. They exhibited a higher degree of photodamage due to a significantly decreased photosynthetic activity and diminished expression of chloroplast and nuclear-encoded genes and the corresponding proteins. A possible mechanism for melatonin-dependent regulation of chloroplast genes is associated with a change in the activity of the genes for chloroplast RNA polymerases. We conclude that under high light stress, melatonin may act as a hormone-like signaling molecule *via* the CAND2/PMTR1-mediated signaling pathway.

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), an important bioactive molecule with regulatory and antioxidant functions, was first discovered in plants in 1995 (Reiter *et al.* 2015, Sharif *et al.* 2018, Sun *et al.* 2021). As a universal regulator, melatonin participates in a variety of processes including plant growth and development, root morphology and floral transition, leaf senescence, and fruit ripening (Sun *et al.* 2021). According to transcriptomic studies, melatonin, in both small and high doses, regulates the expression of a large number of genes associated with stress response, antioxidant functions, intracellular signaling mechanisms, and hormonal regulation (Weeda *et al.* 2014, Wan *et al.* 2018).

Melatonin is also involved in photosynthesis, especially when plants are challenged with abiotic stresses. In particular, melatonin treatment reduced the inhibitory effect of osmotic stress in soybean and increased PSI and PSII-related gene expression (Zhang *et al.* 2019). In tomato seedlings, melatonin increased PSII activity and photochemical quenching coefficient and facilitated

Highlights

- Melatonin (MT) may act *via* the CAND2/PMTR1 signaling pathway under high light stress
- CAND2 participates in the regulation of photosynthesis and chloroplast gene expression
- The protective antioxidant function of exogenous MT did not depend on the CAND2 pathway

the repair of PSII by maintaining the availability of D1 protein, otherwise reduced by salinity stress (Zhou *et al.* 2016). Exogenous melatonin also improved maize photosynthetic capacity and alleviated damage caused by salinity stress (Wang *et al.* 2021a). A *snat* knock-out mutant of *Arabidopsis thaliana* with the decreased endogenous melatonin content accumulated much less starch than the wild type and exhibited lower LHCB1, LHCB4, and RbcL protein contents suggesting the involvement of this regulator in chloroplast protein quality control (Lee and Back 2021). Melatonin is synthesized from tryptophan through several intermediates including serotonin. At the last stage, three enzymes are involved in the synthesis – serotonin *N*-acetyltransferase (SNAT), *N*-acetylserotonin methyltransferase (ASMT), and caffeic acid *O*-methyltransferase (COMT) (Back *et al.* 2016). Knock-outs for any of the genes encoding these proteins have reduced vital indexes and increased susceptibility to stressors.

The original function of melatonin, as a direct free radical scavenger, suggests its location close to the sites of ROS formation which is essential for antioxidant activity. However, to expand its functional repertoire, it was necessary to evolve specific binding sites/receptors and associated signaling transduction (Zhao *et al.* 2019). In animal cells, many of the known activities of mela-tonin are mediated by membrane G-protein coupled receptors. In 2018, the first potential phytomelatonin receptor CAND2/PMTR1 (Candidate G-protein Coupled Receptor 2) was discovered (Wei *et al.* 2018). The membrane-bound receptor was shown to be involved in the stomatal closure through the α-subunit of heterotrimeric G-protein GPA1 regulated by H_2O_2 and Ca^{2+} signaling molecules. The *pmtr1* mutants lost diurnal stomatal closure, with stomata remaining open during daytime and night, and exhibited more water loss and drought sensitivity when compared with the wild type plants (Li *et al.* 2020). The receptor was also shown to play a key role in the melatonin-mediated plant response to osmotic stress. However, these observations were challenged by Lee and Back (2020) who argued that CAND2 is neither a phytomelatonin receptor localized in the plasma membrane nor is it involved in the melatonin-mediated defense signaling pathway *via* G protein components. Nevertheless, they did not rule out that CAND2 may be a melatonin-binding protein and that this binding may result in a decrease of free melatonin contents in plants.

Melatonin is closely associated with high-light (HL) stress tolerance in plants. Protecting the photosynthetic apparatus from photodamage is critical for plant survival since it induces the production of reactive oxygen species (ROS) (Gruszecki 2010). *SNAT1* overexpressing plants exhibited increased tolerance to HL stress (Lee and Back 2018). At the same time, in *snat1* mutant highly susceptible to HL stress, melatonin treatment reduced superoxide production and increased the expression of various ROS-responsive genes.

All the essential processes of photosynthesis are carried out in chloroplasts which are the main targets of HL stress.

Chloroplasts contain their genome including about 100 genes, some of which encode proteins directly involved in photosynthesis (Kusnetsov 2018). Transcription of chloroplast genes is carried out by two types of RNA polymerases: bacterial type chloroplast-encoded RNA polymerase (PEP), consisting of four subunits (α, β, β', β'') and a single-subunit nuclear-encoded RNA polymerase of the phage-type (NEP). The latter is represented by two enzymes: RPOTp which is targeted into chloroplasts and RPOTmp which occurs only in dicots and is imported both into chloroplasts and mitochondria (Pfannschmidt *et al.* 2015).

In previous work, we showed that exogenous melatonin increases the expression of the genes encoding chloroplast transcription machinery under mild photooxidative stress in *Arabidopsis* detached leaves (Bychkov *et al.* 2019). This work is aimed to elucidate whether exogenous melatonin regulates chloroplast gene expression and photosynthesis in young intact *Arabidopsis* plants under severe HL stress and to unravel how melatonin deficiency and disruption of its putative signaling pathway affect molecular processes in chloroplasts.

Materials and methods

Growth conditions and experimental design: *Arabidopsis thaliana* plants, ecotype Columbia-0, and insertion knockout mutant lines *asmt* (NASC680911), *cand2* (NASC678658), and *gpa1* (NASC6534) were used in the experiments. Molecular characterization of the mutants is presented in supplementary data (Fig. 1S, *supplement*).

The seeds were planted in Petri dishes on half-strength Murashige and Skoog (MS) medium with 1% sucrose and 0.5% agar. After stratification for 48 h at 4°C in the dark, they were grown in a growth chamber at 23°C with at a 16-h photoperiod, and PPFD of 60 µmol m^{-2} s^{-1}. At the age of two weeks, the seedlings were transferred for 72 h to paper filters moistened with a liquid MS medium supplemented with 50 µM of melatonin or an equal aliquot of solvent. The optimal concentration of melatonin was determined in preliminary experiments. After that, the plants were exposed to HL stress [600 µmol(photon) m^{-2} s^{-1}] for 24 h or kept under 60 µmol(photon) m^{-2} s^{-1} (control). At the end of the exposure, measurements were directly taken, or the samples were frozen in liquid nitrogen and stored at –80°C.

Pigment content and fluorimetry: Chlorophyll (Chl) and carotenoids were extracted and determined as described by Lichtenthaler (1987). Chl *a* fluorescence parameters [the maximum quantum yield (F_v/F_m), the effective quantum yield of PSII (Φ_{PSII}), and nonphotochemical quenching (NPQ)] were measured using a *DUAL-PAM-100* (*Walz*, Germany) following Kozuleva *et al.* (2017). The following parameters were determined: measuring light – 460 nm, 9 µmol(photon) m^{-2} s^{-1}; saturating pulses – 500 ms, 635 nm, 4,000 µmol(photon) m^{-2} s^{-1}; actinic light – 635 nm, 37 µmol(photon) m^{-2} s^{-1}. The dark incubation time for measurements was 10 min.

RNA isolation and quantitative Real-Time (RT-qPCR): Relative transcript levels of nuclear and chloroplast genes were evaluated using RT-qPCR according to Danilova *et al.* (2018) in a *LightCycler 96* (*Roche*, Switzerland). The following standard thermal profile was used for all PCR reactions: 95°C for 5 min, 40 cycles of 95°C for 15 s, 58°C 15 s, and 72°C for 25 s. The nucleotide sequences of primers for RT-qPCR analysis are presented in Table 1S (*supplement*). All data were normalized to the amount of the transcript levels of the nuclear-encoded polyubiquitin *UBQ10* gene which was used as the internal control.

Protein isolation and Western blot analysis: Protein extraction and Western blot analysis were performed according to our previous study (Bychkov *et al.* 2019). In brief, the samples were blotted to PVDF membrane after SDS-PAGE electrophoresis and blots were incubated with anti-ELIP1 (Heddad *et al.* 2006), anti-PsbD (PSII; AS06 146), anti-RbcL (Rubisco; AS03 037), anti-AtpB (ATP synthase; AS05 085), and anti-PsaB (PSI; AS10 695) primary antibodies (*Agrisera*, Sweden) overnight at 4°C followed by the secondary antibody (anti-rabbit IgG horse-radish peroxidases conjugated from *Agrisera*, AS09 602) for 1 h at room temperature according to the manufacturer's instructions. Signals from immunoblotting were detected using the ECL method (*ECL Western blotting detection Kit*, *Bio-Rad*) by the *Invitrogen iBright Imaging Systems* (*Thermo Fisher Scientific*, USA).

Endogenous melatonin (ELISA): Melatonin measurement was performed as described by Lee and Back (2018). Leaves (0.2 g) were ground in 2 ml of 100% chloroform. After centrifugation for 15 min at $12,000 \times g$, the supernatant was transferred into new tubes and placed in a vacuum evaporator until the liquid was completely removed. The precipitate was then dissolved in distilled water and vortexed for 3 h. To determine the melatonin content, *ELISA Kit CEA908GE* (*Cloud-Clone Corp.*, USA) was applied according to the manufacturer's instructions. Optical measurement was performed at 450 nm using a Multiskan MS Microplate Reader *LabSystems 352* (*Thermo/LabSystems*, USA).

Statistical data processing: All experiments were performed in three biological replicates. The data were analyzed by the online calculator (https://astatsa.com/ OneWay_Anova_with_TukeyHSD/) using a one-way analysis of variance (*ANOVA*) followed by *Tukey*'s method. All data are presented as means ± their standard errors (SE).

Results

Antioxidant function, melatonin content, and genes for melatonin synthesis and signaling: HL induced oxidative damage which was manifested in an increase of lipid peroxidation, electrolyte leakage, and hydrogen peroxide contents in all genotypes (Table 2S, *supplement*). In parallel, protective mechanisms were reinforced as evidenced by elevated proline accumulation and SOD activity. Melatonin treatment reduced the rates of oxidative damage in the wild type and *asmt* but had a slightly lesser effect in *cand2* and *gpa1*.

Exogenously applied melatonin changed its endogenous concentration (Fig. 1). In the wild type, under normal light conditions, treatment with melatonin increased the endogenous content by more than 1.5-fold. HL stress caused a twofold decrease in the content by the end of the experiment in plants without any treatment. However, there was almost no decrease in melatonin contents in treated plants under HL, as compared to treated plants under moderate light. The results were similar for the *asmt* mutant, which showed a reduced melatonin content and a corresponding decrease under HL. In contrast, HL promoted a greater decrease of melatonin concentration in *cand2* and *gpa1* mutants supplemented with the exogenous regulator under HL, although initial contents were comparable to those of the wild type.

The decrease in melatonin content under HL was accompanied by downregulation in the expression of genes for melatonin synthesis (*SNAT1*, *ASMT*, *COMT*) in the wild type plants (Table 1). Under normal conditions, the addition of exogenous melatonin slightly reduced the expression of these genes. On the other hand, during stress, melatonin increased their expression to almost
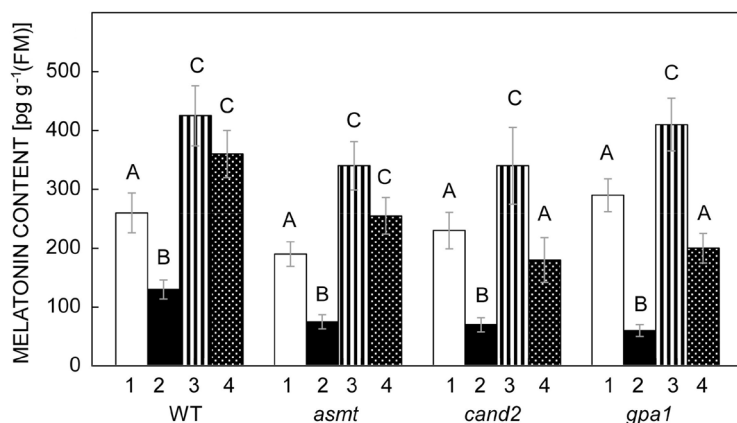

Fig. 1. Melatonin accumulation in *Arabidopsis* wild plants and mutants for melatonin synthesis and signaling in response to HL stress. 1 – moderate light MS; 2 – HL stress MS; 3 – moderate light MS + melatonin; 4 – HL stress MS + melatonin. Two-week-old plants were shifted for 72 h to paper filters moistened with a liquid MS medium supplemented or not with 50 μM of melatonin and exposed to HL stress (PPFD of 600 μmol m^{-2} s^{-1}) for 24 h. Control plants were grown under moderate light (PPFD of 60 μmol m^{-2} s^{-1}). The data presented in the figure are the mean values ($n \geq 3$). Error bars represent SEs. *Different letters* denote statistically significant differences at $p<0.05$ (*ANOVA* with post hoc *Tukey*'s multiple-comparison test).

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initial values. In the *asmt* mutant, HL inhibited the expression of the *SNAT1* to a lesser extent and did not affect the level of *COMT* transcripts. In the *cand2* and *gpa1* mutants, stress decreased gene expression to a lesser extent (*SNAT1*, *COMT*) as compared to the wild type or did not significantly change it (*ASMT*).

The transcript levels of the putative participants of melatonin-signaling chain *CAND2* and *GPA1* were significantly downregulated in the wild type plants and *asmt* under HL conditions and turned to initial levels (*CAND2*) or were upregulated (*GPA1*) following HL+melatonin treatment (Fig. 2). However, the *cand2*

mutant, in which the levels of *CAND2* mRNA were comparable to those in the wild type under HL stress, displayed no significant changes of *CAND2* mRNA following HL+melatonin treatment. In parallel, *gpa1* mutant did not show changes of *CAND2* mRNA at all, and stress and melatonin-mediated changes of the expression levels of *GPA1* were absent in the *cand2*.

We can conclude that long-term HL oxidative stress was accompanied by activation of the components of the scavenging system and a drop in endogenous melatonin contents. Melatonin treatment under HL contributed to maintaining higher levels of expression of genes for

Table 1. Effect of HL stress and melatonin treatment on the expression of the genes for melatonin synthesis. Two-week-old plants were shifted for 72 h to paper filters moistened with a liquid MS medium supplemented or not with 50 μM of melatonin and exposed to HL stress (PPFD of 600 μmol m^{-2} s^{-1}) for 24 h. Control plants were grown under moderate light (PPFD of 60 μmol m^{-2} s^{-1}). RNAs were analyzed by RT-qPCR using *UBQ10* as internal standard. The data presented in the table are the mean values ± SE ($n \geq 3$). *Different letters* denote statistically significant differences at $p < 0.05$ (*ANOVA* with post hoc *Tukey*'s multiple-comparison test).

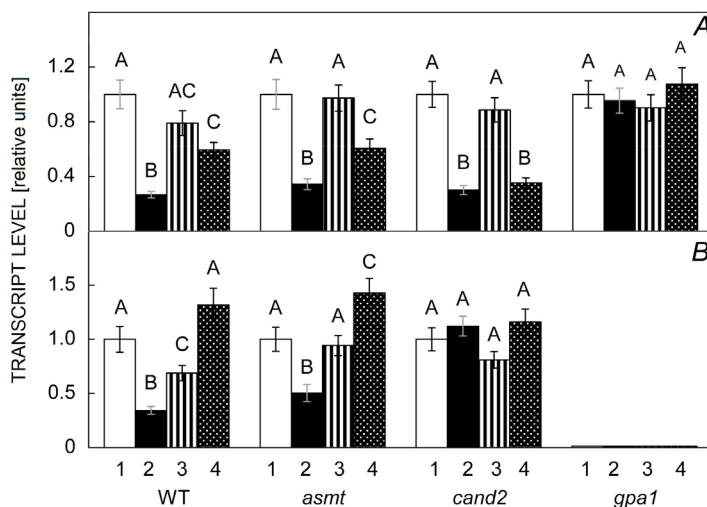| Genotype	Moderate light MS	High light stress MS	Moderate light MS + melatonin	High light stress MS + melatonin
SNAT				
Wild type	1.000 ± 0.121[a]	0.081 ± 0.008[b]	0.678 ± 0.098[c]	0.480 ± 0.059[c]
asmt	1.000 ± 0.099[a]	0.182 ± 0.020[b]	1.097 ± 0.113[a]	0.310 ± 0.033[c]
cand2	1.000 ± 0.105[a]	0.336 ± 0.051[b]	0.962 ± 0.063[a]	0.457 ± 0.050[b]
gpa1	1.000 ± 0.102[a]	0.678 ± 0.079[b]	0.990 ± 0.081[a]	0.705 ± 0.045[b]
ASMT				
Wild type	1.000 ± 0.089[a]	0.269 ± 0.031[b]	0.697 ± 0.057[c]	0.577 ± 0.061[c]
asmt	-	-	-	-
cand2	1.000 ± 0.106[a]	0.794 ± 0.086[a]	1.154 ± 0.111[a]	1.063 ± 0.106[a]
gpa1	1.000 ± 0.100[a]	0.890 ± 0.081[a]	0.974 ± 0.084[a]	0.735 ± 0.055[a]
COMT				
Wild type	1.000 ± 0.121[a]	0.193 ± 0.020[b]	0.799 ± 0.071[a]	0.791 ± 0.069[a]
asmt	1.000 ± 0.102[a]	0.986 ± 0.082[a]	1.324 ± 0.121[a]	1.136 ± 0.089[a]
cand2	1.000 ± 0.106[a]	0.639 ± 0.065[bc]	0.855 ± 0.069[ac]	0.543 ± 0.066[b]
gpa1	1.000 ± 0.092[a]	0.675 ± 0.074[b]	0.950 ± 0.090[a]	0.673 ± 0.045[b]

Fig. 2. Effect of HL stress and melatonin treatment on the expression of melatonin-signaling genes: A – *CAND2*; B – *GPA1*. 1 – moderate light MS; 2 – HL stress MS; 3 – moderate light MS + melatonin; 4 – HL stress MS + melatonin. Two-week-old plants were shifted for 72 h to paper filters moistened with a liquid MS medium supplemented or not with 50 μM of melatonin and exposed to HL stress (PPFD of 600 μmol m^{-2} s^{-1}) for 24 h. Control plants were grown under moderate light (PPFD of 60 μmol m^{-2} s^{-1}). RNAs were analyzed by RT-qPCR using *UBQ10* as internal standard. The data presented in the figure are the mean values ($n \geq 3$). Error bars represent SEs. *Different letters* denote statistically significant differences at $p < 0.05$ (*ANOVA* with post hoc *Tukey*'s multiple-comparison test).

melatonin synthesis and signaling in WT and *asmt* but practically did not affect the expression of these genes in *cand2* and *gpa1*.

Photosynthetic activity and expression of chloroplast genes: The plants exposed to HL stress, along with oxidative stress, exhibited a moderate drop in the total Chl content (Table 2), the steady-state levels of which were approximately the same in all genotypes. In wild type and *asmt* mutant exposed to HL, exogenous melatonin maintained the Chl amount at a higher level as compared to untreated samples but had no effect in signaling mutants *cand2* and *gpa1*. Similar results were revealed for the total carotenoid content; however, the *gpa1* mutant was not susceptible to its decrease under HL.

The protective effect of melatonin was also shown for the functional activity of the reaction centers of PSII. HL treatment triggered a strong reduction of the maximum quantum yield (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}) while melatonin treatment mitigated photoinhibition. The exceptions were *cand2* and *gpa1*. Light stress also increased the nonphotochemical quenching (NPQ) but this response was almost independent of melatonin in all the genotypes studied.

Next, we analyzed whether melatonin affects the expression of chloroplast genes under photooxidative stress. HL exposure increased the level of *ELIP1* (EARLY LIGHT-INDUCIBLE PROTEIN 1) transcripts 20-fold in the wild type and more than 40–50-fold in mutants (Table 3). *ELIP1* is often used as an indicator of light stress and encodes proteins with photoprotective functions which are directly involved in the synthesis and assembly of specific photosynthetic complexes. Melatonin treatment reduced the expression of *ELIP1* by about 1.5- and 2-fold in the wild type and *asm*t, respectively, under HL but did not have a reliable effect in the *cand2* and *gpa1*.

In all lines, HL caused a decrease in the transcript accumulation of the nuclear-encoded *LHCB2* (*light-*

Table 2. Effect of HL stress and melatonin treatment on photosynthetic pigment content and chlorophyll fluorescence parameters. Two-week-old plants were shifted for 72 h to paper filters moistened with a liquid MS medium supplemented or not with 50 μM of melatonin and exposed to HL stress (PPFD of 600 μmol m^{-2} s^{-1}) for 24 h. Control plants were grown under moderate light (PPFD of 60 μmol m^{-2} s^{-1}). Pigment measurements were performed according to Lichtenthaler (1987). The parameters of activity of PSII were obtained using PAM fluorimetry. The data presented in the table are the mean values ± SE ($n \geq 3$). *Different letters* denote statistically significant differences at $p<0.05$ (*ANOVA* with post hoc *Tukey*'s multiple-comparison test).

Genotype	Moderate light MS	High light stress MS	Moderate light MS + melatonin	High light stress MS + melatonin
Chlorophyll (*a+b*) content [mg g^{-1}(FM)]				
Wild type	0.946 ± 0.041[a]	0.636 ± 0.030[b]	0.857 ± 0.065[a]	0.745 ± 0.024[c]
asmt	0.838 ± 0.040[a]	0.537 ± 0.027[b]	0.835 ± 0.042[a]	0.699 ± 0.031[c]
cand2	0.935 ± 0.042[a]	0.510 ± 0.031[b]	0.836 ± 0.056[a]	0.452 ± 0.039[b]
gpa1	0.898 ± 0.039[a]	0.650 ± 0.034[b]	0.881 ± 0.050[a]	0.704 ± 0.037[b]
Carotenoid content [mg g^{-1}(FM)]				
Wild type	0.160 ± 0.008[a]	0.135 ± 0.009[b]	0.154 ± 0.010[a]	0.148 ± 0.004[a]
asmt	0.157 ± 0.004[a]	0.139 ± 0.007[b]	0.159 ± 0.008[a]	0.160 ± 0.006[a]
cand2	0.176 ± 0.012[a]	0.109 ± 0.005[b]	0.162 ± 0.007[a]	0.104 ± 0.005[b]
gpa1	0.151 ± 0.008[a]	0.136 ± 0.007[a]	0.145 ± 0.008[a]	0.146 ± 0.010[a]
F_v/F_m				
Wild type	0.816 ± 0.014[a]	0.456 ± 0.016[b]	0.791 ± 0.013[a]	0.564 ± 0.018[c]
asmt	0.785 ± 0.006[a]	0.402 ± 0.011[b]	0.779 ± 0.014[a]	0.615 ± 0.023[c]
cand2	0.792 ± 0.020[a]	0.469 ± 0.016[b]	0.766 ± 0.015[a]	0.504 ± 0.026[b]
gpa1	0.812 ± 0.013[a]	0.467 ± 0.019[b]	0.802 ± 0.006	0.437 ± 0.020[b]
Φ_{PSII}				
Wild type	0.661 ± 0.016[a]	0.319 ± 0.019[b]	0.646 ± 0.018[a]	0.385 ± 0.008[c]
asmt	0.644 ± 0.011[a]	0.294 ± 0.014[b]	0.630 ± 0.017[a]	0.418 ± 0.015[c]
cand2	0.646 ± 0.018[a]	0.407 ± 0.021[b]	0.621 ± 0.025[a]	0.431 ± 0.021[b]
gpa1	0.673 ± 0.012[a]	0.357 ± 0.017[b]	0.658 ± 0.016[a]	0.319 ± 0.036[b]
NPQ				
Wild type	0.208 ± 0.013[a]	0.306 ± 0.012[b]	0.211 ± 0.013[a]	0.267 ± 0.011[c]
asmt	0.199 ± 0.006[a]	0.259 ± 0.008[b]	0.194 ± 0.007[a]	0.252 ± 0.015[b]
cand2	0.233 ± 0.018[a]	0.299 ± 0.012[b]	0.247 ± 0.005[a]	0.285 ± 0.021[b]
gpa1	0.204 ± 0.010[a]	0.287 ± 0.011[b]	0.235 ± 0.022[ac]	0.277 ± 0.024[bc]

Table 3. Effect of HL stress and melatonin treatment on the expression chloroplast and nuclear genes. Two-week-old plants were shifted for 72 h to paper filters moistened with a liquid MS medium supplemented or not with 50 μM of melatonin and exposed to HL stress (PPFD of 600 μmol m^{-2} s^{-1}) for 24 h. Control plants were grown under moderate light (PPFD of 60 μmol m^{-2} s^{-1}). RNAs were analyzed by RT-qPCR using *UBQ10* as internal standard. The data presented in the table are the mean values ± SE ($n \geq 3$). *Different letters* denote statistically significant differences at $p<0.05$ (*ANOVA* with post hoc *Tukey*'s multiple-comparison test).

Genotype	Moderate light MS	High light stress MS	Moderate light MS + melatonin	High light stress MS + melatonin
ELIP1				
Wild type	1.000 ± 0.101[a]	23.546 ± 2.999[b]	0.798 ± 0.088[a]	13.802 ± 1.560[c]
asmt	1.000 ± 0.111[a]	58.340 ± 6.291[b]	1.654 ± 0.356[a]	30.472 ± 3.510[c]
cand2	1.000 ± 0.103[a]	62.257 ± 7.998[b]	1.226 ± 0.111[a]	49.206 ± 5.213[b]
gpa1	1.000 ± 0.108[a]	39.279 ± 4.409[b]	0.945 ± 0.106[a]	33.449 ± 3.721[b]
LHCB2				
Wild type	1.000 ± 0.123[a]	0.023 ± 0.003[b]	0.918 ± 0.100[a]	0.087 ± 0.015[c]
asmt	1.000 ± 0.098[a]	0.015 ± 0.002[b]	1.015 ± 0.111[a]	0.112 ± 0.016[c]
cand2	1.000 ± 0.101[a]	0.044 ± 0.005[b]	0.920 ± 0.060[a]	0.048 ± 0.060[b]
gpa1	1.000 ± 0.112[a]	0.029 ± 0.030[b]	1.114 ± 0.097[a]	0.021 ± 0.040[b]
psbD				
Wild type	1.000 ± 0.117[a]	0.395 ± 0.039[b]	0.955 ± 0.119[a]	0.706 ± 0.086[c]
asmt	1.000 ± 0.113[a]	0.233 ± 0.039[b]	0.898 ± 0.099[a]	0.538 ± 0.061[c]
cand2	1.000 ± 0.103[a]	0.481 ± 0.044[b]	0.905 ± 0.093[a]	0.521 ± 0.057[a]
gpa1	1.000 ± 0.113[a]	0.564 ± 0.060[b]	1.122 ± 0.121[a]	0.517 ± 0.052[a]
trnE				
Wild type	1.000 ± 0.102[a]	0.494 ± 0.045[b]	0.974 ± 0.098[a]	0.508 ± 0.056[b]
asmt	1.000 ± 0.103[a]	0.531 ± 0.051[b]	1.320 ± 0.129[a]	0.554 ± 0.054[b]
cand2	1.000 ± 0.101[a]	0.516 ± 0.049[b]	1.189 ± 0.109[a]	0.612 ± 0.066[b]
gpa1	1.000 ± 0.099[a]	0.683 ± 0.071[b]	1.042 ± 0.111[a]	0.657 ± 0.069[b]

harvesting antenna protein of PSII), and a set of selected chloroplast-encoded genes transcribed by PEP (*rbcL*, *psbA*, *psbD*, *psaA*, *trnE*), NEP (*accD*), or both polymerases (*atpB*) (Table 3; Table 3S, *supplement*). *LHCB2* transcript accumulation decreased very significantly and was the most vulnerable to photodamage. The expression of the genes for structural proteins of PSI (*psaA*), PSII (*psbA*, *psbD*), ATP synthase (*atpB*), and acetyl-CoA carboxylase subunit (*accD*) engaged in the fatty acid cycle was downregulated 3–5-fold. It should be noted that the steady-state levels of the above-mentioned gene transcripts were practically the same for all genotypes and did not change when treated with melatonin under moderate light. Melatonin maintained the expression of these genes at a higher level in the stressed wild type, and especially in the *asmt* mutant, as compared to untreated plants, but had almost no effect in *cand2* and *gpa1*. The only exception was *trnE* whose expression was not regulated by melatonin under stress.

Thus, HL significantly suppressed the photosynthetic parameters and the expression of the selected chloroplast genes, while melatonin treatment alleviated this effect. However, mutants for melatonin signaling were compromised for melatonin protective function under photo-oxidative stress.

The expression of chloroplast RNA polymerase genes: Adjustment of chloroplast gene expression can occur through the regulation of the transcription machinery of chloroplasts. In our experiment, HL stress and exogenous melatonin affected the gene expression of both RNA polymerases (Table 4). However, there were some peculiarities in regulation.

The genes for chloroplast-encoded multi-subunit RNA polymerase of the bacterial type were strongly suppressed by HL in the wild type and *asmt*, and melatonin treatment maintained their transcript levels at almost initial values. In signaling mutants, melatonin had no effect, with stress downregulating *rpo* expression to a lesser extent.

The expression of the two genes for nuclear-encoded RNA polymerases differed significantly (Table 4). In the wild type plants, the activity of the chloroplast targeted *RPOTp* was downregulated 2-fold by HL and recovered by melatonin to the initial levels. In *asmt*, HL had a similar effect, but melatonin-induced upregulation was higher. In *cand2* and *gpa1*, *RPOTp* transcript accumulation did not change under any of these effectors. On the other hand, the expression of *RPOTmp* targeted into both chloroplasts and mitochondria was strongly upregulated under photooxidative stress, and melatonin even enhanced

Table 4. Effect of HL stress and melatonin treatment on the expression of chloroplast RNA polymerase genes. Two-week-old plants were shifted for 72 h to paper filters moistened with a liquid MS medium supplemented or not with 50 µM of melatonin and exposed to HL stress (PPFD of 600 µmol m^{-2} s^{-1}) for 24 h. Control plants were grown under moderate light (PPFD of 60 µmol m^{-2} s^{-1}). RNAs were analyzed by RT-qPCR using *UBQ10* as internal standard. The data presented in the table are the mean values ± SE ($n \geq 3$). *Different letters* denote statistically significant differences at $p < 0.05$ (*ANOVA* with post hoc *Tukey*'s multiple-comparison test).

Genotype	Moderate light MS	High light stress MS	Moderate light MS + melatonin	High light stress MS + melatonin
rpoA				
Wild type	1.000 ± 0.101[a]	0.235 ± 0.029[b]	0.865 ± 0.094[ac]	0.605 ± 0.056[c]
asmt	1.000 ± 0.109[ac]	0.176 ± 0.020[b]	1.245 ± 0.139[a]	0.704 ± 0.072[c]
cand2	1.000 ± 0.099[a]	0.386 ± 0.041[b]	0.957 ± 0.089[a]	0.312 ± 0.035[b]
gpa1	1.000 ± 0.104[a]	0.528 ± 0.066[b]	0.977 ± 0.098[a]	0.478 ± 0.055[b]
rpoB				
Wild type	1.000 ± 0.102[a]	0.112 ± 0.028[b]	0.821 ± 0.084[a]	1.301 ± 0.139[a]
asmt	1.000 ± 0.113[a]	0.433 ± 0.047[b]	1.086 ± 0.107[a]	0.917 ± 0.095[a]
cand2	1.000 ± 0.107[a]	0.589 ± 0.061[b]	0.814 ± 0.088[a]	0.509 ± 0.051[b]
gpa1	1.000 ± 0.100[a]	0.730 ± 0.064[bc]	0.892 ± 0.091[ab]	0.560 ± 0.057[c]
RPOTp				
Wild type	1.000 ± 0.086[a]	0.583 ± 0.048[b]	0.869 ± 0.085[a]	0.676 ± 0.069[ab]
asmt	1.000 ± 0.067[a]	0.556 ± 0.053[b]	1.031 ± 0.120[a]	1.766 ± 0.208[c]
cand2	1.000 ± 0.093[a]	0.879 ± 0.080[a]	0.862 ± 0.094[a]	0.883 ± 0.106[a]
gpa1	1.000 ± 0.095[a]	1.352 ± 0.198[a]	0.987 ± 0.091[a]	1.079 ± 0.246[a]
RPOTmp				
Wild type	1.000 ± 0.089[a]	1.464 ± 0.128[a]	0.925 ± 0.088[a]	2.006 ± 0.179[b]
asmt	1.000 ± 0.082[a]	2.188 ± 0.208[b]	0.963 ± 0.097[a]	4.346 ± 0.389[c]
cand2	1.000 ± 0.093[a]	1.963 ± 0.199[b]	0.830 ± 0.081[a]	2.004 ± 0.209[b]
gpa1	1.000 ± 0.118[a]	2.972 ± 0.314[b]	0.674 ± 0.088[c]	2.365 ± 0.286[b]

this effect, but only in the wild type and the mutant for melatonin synthesis.

These data demonstrate that melatonin altered the expression of genes for chloroplast RNA polymerases, in genotypes sensitive to this regulator. At the same time, there were variations in the response of these genes to HL irradiation.

The contents of photosynthetic proteins: To investigate whether the changes of the mRNA profiles correspond to shifts in protein contents, we used immunodetection analysis. A dramatic rise in the accumulation of the marker protein ELIP1 under HL stress was mitigated by melatonin treatment in the wild type and *asmt* plants (Fig. 3). However, in *cand2* and *gpa1* mutants with impaired melatonin perception, elevated contents of ELIP1 were retained after incubation with melatonin.

The reaction center protein D2, the gene product of *psbD*, exhibited a severe decline following HL stress in all genotypes and especially in *asmt*. However, it displayed almost initial values in wild-type plants and *asmt* mutant treated with melatonin. In contrast, decreased contents of D2 after HL exposure remained unaltered in *cand2* and *gpa1* following melatonin treatment. These results indicate that the mitigating capacity was attenuated in the mutants

with the impaired melatonin perception and that signaling proteins CAND2 and GPA1 are necessary for mediating the melatonin-dependent light-stress response.

The protein contents of RbcL, AtpB, and PsaB were approximately identical in wild type and mutants under all modes of treatment even though corresponding transcript levels were strongly regulated by HL and melatonin. The unaltered contents of these proteins suggest that the synthesis of these proteins may not be impaired. In addition, the behavior of mRNA levels and translational activity may differ due to reduced protein degradation, as a potential regulatory mechanism (Krantz *et al.* 2021).

Discussion

The primary function of melatonin as a scavenger and a broad-spectrum antioxidant is thought to be receptor-independent (Wei *et al.* 2018). However, its hormone-like signaling function, which is performed through gene expression regulation, was evolved during the subsequent evolution. At high concentrations, melatonin acts primarily as an antioxidant molecule engaged in defense responses while at low concentrations it performs hormone-like functions, though a survey of the relevant literature does not confirm this unambiguously (Arnao and Hernández-Ruiz 2015).
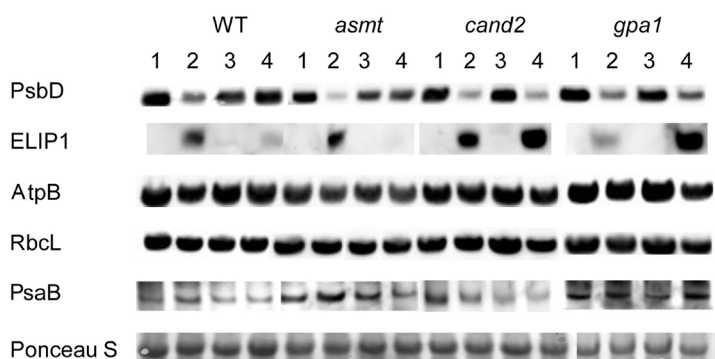
Fig. 3. Western blots of chloroplast-encoded proteins and ELIP1 under HL stress and melatonin treatment. Pouncea S staining was used as a loading control. 1 – moderate light MS; 2 – HL stress MS; 3 – moderate light MS + melatonin; 4 – HL stress MS + melatonin. Two-week-old plants were shifted for 72 h to paper filters moistened with a liquid MS medium supplemented or not with 50 μM of melatonin and exposed to HL stress (PPFD 600 μmol m^{-2} s^{-1}) for 24 h. Control plants were grown under moderate light (PPFD 60 μmol m^{-2} s^{-1}). Proteins were visualized by immunoblotting using antibodies specific for RbcL, PsaB, PsbD, AtpB, and ELIP1 proteins.

A lack of melatonin reduces the vital parameters of plants and reduces resistance to various stressors, as it has been shown in mutants with impaired melatonin biosynthesis genes *SNAT*, *ASMT*, *COMT* (Back 2021). To assess the possible consequences of the shortage of endogenous melatonin under HL stress, we used a single *asmt* mutant with the disrupted gene for *N*-acetylserotonin methyltransferase catalyzing the last step of melatonin biosynthesis. Under normal conditions, despite a slight decrease in endogenous melatonin content (Fig. 1), the mutant was indistinguishable from the wild type, probably due to compensatory effects of other biosynthetic enzymes. However, high irradiance caused greater damage in the mutant as could be judged by physiological parameters thereby confirming the beneficial impact of endogenous melatonin. In addition, *asmt* showed increased sensitivity to exogenous melatonin which was manifested under moderate light and especially under HL stress.

Exogenously applied melatonin increased endogenous concentrations and in addition, activated the expression of genes for melatonin biosynthesis in wild type and *asmt*. Melatonin is considered to have an amphiphilic nature (Cipolla-Neto and Amaral 2018), which implies a relatively low penetrating ability. Sufficiently strong compartmentalization of the plant cell suggests a complex relationship between an externally penetrating regulator that reduces oxidative stress and a newly synthesized endogenous melatonin targeting regulatory processes. Hence, exogenous melatonin might be a stimulus, which could activate the expression of genes associated with endogenous phytomelatonin biosynthesis and apparently with signaling.

The first putative phytomelatonin receptor CAND2/PMTR1 with a confirmed specific L-melatonin binding was shown to be engaged in signaling transduction cascade regulating stomatal closure in *Arabidopsis thaliana* (Wei *et al.* 2018). However, later Lee and Back (2020) showed that CAND2 protein is not functionally associated with melatonin-mediated stress tolerance in response to tunicamycin treatment, which is dependent on MAPK activation. More recently, the role of CAND2/PMTR1 as a potential receptor was corroborated by studies by Wang *et al.* (2021b) which revealed that CAND2 plays a key role in melatonin-mediated plant response to osmotic stress *via* modulating the ROS-scavenging ability in *Arabidopsis* and regulation of *SOD* and *CAT* gene expression. In

the presented work, we demonstrated that exogenous melatonin can markedly rescue the decreased HL stress tolerance of wild type plants and *asmt* mutant line, but these effects of melatonin were largely compromised in T-DNA insertion lines *cand2/pmtr1* and *gpa1*.

It is necessary to mention that *gpa1* and *asmt* were the *bona fide* knock-out mutants and exhibited no mRNA expression. At the same time, *cand2-1* (Salk_071302), which was used in this study, as well as in the works of Wei *et al.* (2018) and Wang *et al.* (2021b), has T-DNA inserted in the promoter region and under moderate light, the expression levels of *CAND2* mRNA in the *cand2-1* mutant were comparable to those in the wild type. Nonetheless, the mutant was more sensitive to HL stress (Fig. 1) and exhibited decreased melatonin accumulation in conjunction with the enhanced expression of the genes for melatonin biosynthesis. In addition, melatonin-mediated changes of the expression levels of *CAND2* and *GPA1* were abolished in the *cand2*. This data suggest that T-DNA insert in the promoter may result in impairment of transcription regulation under adverse conditions.

Receptor-dependent effects of melatonin treatment were mainly associated with the changes of photosynthetic activity (pigment content and fluorescence parameters) and the expression of the chloroplast genome. At the same time, the scavenging properties of melatonin, judging from H_2O_2 contents and SOD activity, proved to be receptor-independent.

To evaluate the possible outcome of melatonin treatment on the expression of the chloroplast genome, we studied the transcript levels of selected chloroplast-encoded genes belonging to different operons and functional complexes and transcribed by various RNA polymerases. Under moderate light, exogenous melatonin did not trigger up- or downregulation of chloroplast gene expression. Interestingly, according to transcriptome data, treatment with melatonin even inhibited the expression of several genes encoding the subunits of PSI and PSII (Wan *et al.* 2018). However, in our experiments, melatonin was particularly effective in maintaining elevated levels of chloroplast transcripts and the corresponding photosynthetic proteins under HL, as compared with transcript levels of untreated stressed seedlings. Nevertheless, except for *rpoB* encoding β-subunit of PEP, none of them exceeded the control values, probably due to severe injury caused by prolonged HL exposition (24 h). It should

be emphasized that in contrast to the results of Lee and Back (2018), which showed that melatonin content and the expression of its biosynthesis genes were transiently induced under less prolonged HL stress (3–6 h), in our experimental setting, endogenous melatonin concentration decreased, as well as the expression of biosynthesis genes (Fig. 1, Table 1).

The activity of chloroplast genes, at least in part, may be maintained due to the elevated expression of genes for chloroplast RNA polymerases. Previously, we showed that under mild photooxidative stress melatonin induces the expression of genes for almost all elements of the chloroplast transcription machinery, including NEP, PEP, sigma factors, and PEP-associated proteins in detached *Arabidopsis* leaves (Bychkov *et al.* 2019). In the described experimental design, melatonin rather maintained the activity of PEP and RPOTp under increased photodamage, although in the highly sensitive *asmt* mutant, it even induced *RPOTp* expression in stress conditions. The elevated transcript level of chloroplast RNA polymerases probably provides additional opportunities for better recovery of photosynthetic processes and accelerated adaptation to changing conditions (Table 4).

The putative role of chloroplast RNA polymerases in managing melatonin-dependent upregulation of chloroplast genes is confirmed by the fact that transcript levels of *trnE* encoding tRNA-Glu did not respond to melatonin treatment. tRNA-Glu, in addition to its well-known fundamental functions in translation and 5-aminolevulinate synthesis, functions as an inhibitor of RPOTp-dependent transcription. Hence, the induction of *trnE* under deleterious conditions could result in downregulation of *RPOTp* gene expression, thereby reducing chloroplast transcription and HL tolerance.

In contrast to *RPOT*, *RPOTmp* expression was upregulated by HL stress, and melatonin augmented this reaction in a receptor-dependent manner. Since RPOTmp is active mainly in mitochondria where it modulates the expression of the genes belonging to respiratory complexes I and IV (Kühn *et al.* 2009, Tarasenko *et al.* 2016), its induction upon HL and melatonin treatment suggests the role in the melatonin-dependent expression of mitochondrial genes. Indeed, under photooxidative stress generating intensive ROS production, exogenously applied melatonin induced mitochondrial RNA-polymerase gene *RPOTm* and maintained the expression of mitochondrial encoded genes, operating through the CAND2 and GPA. Unlike wild-type plants, *cand2* and *gpa1* did not show a decrease in the alternative pathway of leaf respiration, as well as the activity of an alternative oxidase, and the expression of the *AOX1a* gene when treated with melatonin under excessive light (Bychkov *et al.*, unpublished results).

Mitochondria are known to be one of the sites of melatonin synthesis (Wang *et al.* 2017). Moreover, in addition to chloroplasts, mitochondria are hypothesized to be the original sites of melatonin synthesis in the early stage of endosymbiotic organisms (Tan *et al.* 2013). These data imply that melatonin may act as an essential regulatory element of organelle gene expression. Hence, a challenge for future research is to elucidate the molecular chain linking melatonin to organelle gene expression and possible outcomes for inter-organelle and organelle to nucleus communication.

CAND2 and GPA1 with their predicted localization in chloroplasts and mitochondria (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi) may represent the input points of melatonin-mediated signaling pathway which is involved in HL stress responses of plant organelles. However, components and the hierarchical structure of signaling pathways directing melatonin signals to the nucleus, as well as targets of downstream transcription factors affecting organelle genome expression, remain unclear. It might be that *WRKY54* whose expression is perturbed in *cand2* and *gpa1*, is involved in melatonin-mediated signaling network associated with defense responses. However, direct interaction between CAND2 and GPA1, on one hand, and *WRKY54*, on the other, remains to be shown. Transcriptome analysis revealed a total of 29 genes for transcription factors that were upregulated by melatonin treatment (Weeda *et al.* 2014). These findings, together with the data on phytomelatonin receptors, provide a starting point for unraveling the molecular mechanisms that link melatonin perception and function in various physiological responses.

In conclusion, we have shown for the first time that putative melatonin receptor CAND2/PMTR1 which is associated with G-protein GPA1 is an essential factor for protection under HL stress. Mutants with the impaired function of this signaling circuit were partially insensitive to melatonin treatment upon HL. The higher degree of photodamage could be due to a significantly decreased photosynthetic activity as measured by maximum quantum yield and effective quantum yield of PSII and diminished expression of chloroplast genes, both of nuclear and chloroplast coding. Along with data on the regulation of stomatal closure (Wei *et al.* 2018) and osmotic stress tolerance in *Arabidopsis* (Wang *et al.* 2021b), our results confirmed the participation of CAND2/PMTR1 and GPA1 in melatonin-mediated signaling transduction and their essential role in photosynthesis and related processes under stress conditions which is at least partially due to the control of chloroplast gene expression.

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