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Research article

# Melatonin modifies the expression of the genes for nuclear- and plastid-encoded chloroplast proteins in detached Arabidopsis leaves exposed to photooxidative stress

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ABSTRACT

Melatonin, a potent regulator during plant development and stress responses, affects diverse plastid-related processes. However, its role in the regulation of plastid gene expression is largely unknown. In this study, exogenous melatonin was shown to reduce the negative influence of excess light by increasing the efficiency of the photosystems and rearranging the expression of chloroplast- and nuclear-encoded genes in detached *Arabidopsis* leaves. The positive effects of melatonin predominantly occurred at lower concentrations, while high doses had an inhibitory effect. The impact of melatonin was not straightforward. It mainly influenced the expression of the genes encoding the chloroplast transcription machinery and housekeeping genes involved in maintaining transcriptional activity and the functional state of chloroplasts. Despite the fact that melatonin treatment improved photosynthetic parameters, the levels of photosynthesis gene transcripts and photosynthetic proteins remained practically unaltered suggesting that melatonin impact on photosynthetic apparatus which would allow the balancing of chloroplast functions with stress responses is highly complicated.

## 1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine), a derivative of tryptophan, is a multifunctional molecule that combines the signal properties of a regulator of integral physiological processes with the role of an anti-stress agent (Reiter et al., 2015; Tan et al., 2014). According to transcriptome data, exogenous melatonin is able to alter the expression of a significant number of genes involved in stress responses, signal transduction and hormonal regulation (Wan et al., 2018; Weeda et al., 2014). Melatonin was transiently induced in response to high light stress in *Arabidopsis thaliana,* with the simultaneous increase in the expression of melatonin biosynthetic genes conferring an efficient ROS-scavenging effect (Lee and Back, 2018). Furthermore, knockout mutants for the melatonin synthesis gene showed less resistance to light exposure, and mutants with overexpression revealed greater resistance, than the wild type.

In plants, chloroplasts are the major subcellular organelles that produce ROS under light conditions (Lee and Back, 2018). Along with that, a penultimate step for melatonin biosynthesis in plants was shown to occur in chloroplasts where serotonin is converted into N-acetylserotonin by serotonin *N*-acetultransferase1 (SNAT1) (Back et al., 2016; Byeon et al., 2014). This suggests that chloroplasts could be the targets of melatonin effects in plants. As such, melatonin might act both as a scavenger and as a modulator of gene expression, contributing to the regulation of chloroplast biogenesis and functioning (Wan et al., 2018; García et al., 2014). However, the mode of action of melatonin in chloroplasts and its potential effects on chloroplast gene expression remain unexplored despite the fact that melatonin and its synthetic enzymes were also identified in cyanobacteria (Byeon et al., 2013), from which plastids are believed to have evolved.

As the descendants of ancient cyanobacteria, chloroplasts possess their own genomes and transcription systems that require the coordinate expression of nuclear and plastid-encoded genes (Kusnetsov, 2018). Thus, plastids use two types of RNA polymerases: the plastid-encoded eubacterial-type enzyme (PEP) composed of four subunits (α, β, β′, β”) and the nuclear-encoded single subunit phage-type RNA polymerase (NEP). In dicots, NEP is represented by two proteins that are either targeted into plastids (RPOTp) or dually targeted into mitochondria and plastids (RPOTmp). In addition, PEP requires one of the several nuclear-encoded sigma factors for promoter recognition and 12 nuclear-encoded polymerase associated proteins (PAPs) (Pfannschmidt et al., 2015).

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In the course of the drastic reduction in coding capacity during evolution, chloroplasts retained genes for fewer than 100 proteins involved in photosynthesis and the regulation of plastid gene expression. It is generally assumed that PEP is mainly responsible for transcription of photosynthesis genes, while NEP is engaged in transcription of housekeeping genes. However, the ratio of PEP- and NEP-dependent transcription may change under adverse conditions since NEPs have the ability to transcribe even photosynthesis genes from remote promoters (Pfannschmidt et al., 2015; Zhelyazkova et al., 2012).

Maintenance of chloroplast gene expression under excess light requires an efficient scavenging system as well as other protective mechanisms, such as the dissipation of excess light as thermal energy, cyclic electron transport, light avoidant movements of chloroplasts and the induction of various antioxidant enzymes (Cortleven et al., 2014). Melatonin can directly influence at least some of these processes. Therefore, it is likely that melatonin may have an important role in the regulation of chloroplast gene expression under stress conditions.

In this study, using detached *Arabidopsis* leaves, a number of tests to clarify the molecular basis of the melatonin effects on plastid gene expression under photooxidative stress were performed. The results obtained revealed that the upregulation of some of the selected chloroplast genes was restricted to lower concentrations of exogenous melatonin, a response that should be interpreted as the participation of the substance in the signaling mechanisms regulating the expression of the chloroplast genome.

## 2. Materials and methods

### 2.1. Growth conditions and experimental design

*Arabidopsis thaliana* plants, ecotype Columbia 0, were used in the experiments. The seeds were stratified for 48 h at 4 °C in the dark and then sown in a mixture of soil and vermiculite. Plants were grown in a growth chamber under a 16 h light/8 h dark cycle at 23 °C and 75 $\mu E\, m^{-2}\, s^{-1}$ light intensity up to the age of four weeks. For the experiment, the 5th and 6th rosette leaves were selected. Detached leaves were incubated in Petri dishes on filter papers moistened with water (control) or melatonin solutions (10 μM, 100 μM or 1 mM). For mild light stress treatment, the dishes were transferred for 72 h under constant illumination generated by fluorescent lamps (OSRAM L58W/640, Russia) with a light energy flux of 250 $\mu E\, m^{-2}\, s^{-1}$. Control leaf blades were kept under 75 $\mu E\, m^{-2}\, s^{-1}$ light intensity. The air temperature was 23 °C. At the end of the exposure time, measurements were directly taken or samples were frozen in liquid nitrogen and stored at −70 °C.

### 2.2. Determination of pigments, proline, TBARS and SOD activity

Relative anthocyanin levels were determined according to the Giraud method (Giraud et al., 2008). Chlorophyll and carotenoid extraction and calculation were performed as described by (Lichtenthaler, 1987). The free proline content was estimated as proposed by (Bates et al., 1973). The level of the TBARs (secondary products of the lipid peroxidation of membranes that react with thiobarbituric acid) was measured according to the method of (Heath and Packer, 1968). SOD (EC 1.15.1.1) activity was assessed by the protocol of (Giannopolitis and Ries, 1977). All measurements were taken with a Pharmacia Biotech Ultrospec-2000 (UK) spectrophotometer.

### 2.3. Fluorometry

The data for calculation of the parameters of photosynthetic activity of PSII (the maximum potential quantum yield in the dark Fv/Fm, quantum yield of PSII in the light Y(II), and the coefficients of photochemical (qP) and non-photochemical quenching (qN, NPQ)) were obtained using a DUAL-PAM-100 (Walz, Germany) according to (Kozuleva et al. (2017)). To evaluate the performance of photosystem I, the following parameters were assessed: quantum yield of PSI in the light Y(I) and the non-photochemical quantum yield caused by energy dissipation due to limitations on the donor side Y(ND) and on the acceptor side Y(NA). For measurements, we used the following parameters: measuring light – 460 nm, 9 μmol photons/$m^2$ per 1 s; saturating pulses – 500 ms, 635 nm, 4000 μmol photons/m2 per 1 s; actinic light – 635 nm, 37 μmol photons/$m^2$ per 1 s.

### 2.4. RNA isolation and quantitative real time (RT)-PCR

The expression levels of nuclear and chloroplast genes were evaluated by RT-PCR according to (Danilova et al., 2014) in a LightCycler 96 (Roche, Switzerland). The nucleotide sequences of the primers for quantitative RT-PCR analyses are presented in the Supporting Information section (Table S1).

### 2.5. Protein extraction and immunoblotting

Protein isolation and Western blot analysis were performed according to the protocol described in (Danilova et al., 2018). In brief, total proteins were extracted from the homogenized leaf material in a buffer containing 10 mM Tris-HCl, pH 8.0, 0.5% SDS, 4% glycerol, and 0.1 mM EDTA, at pH 8.0. Protein concentrations were determined by the bicinchoninic acid (BCA) method using the Pierce BCA protein assay kit (Thermo Scientific). Fifteen micrograms of total protein extract were separated by 10% and 12.5% SDS-PAGE (Laemmli, 1970). The samples were blotted to nitrocellulose membrane and stained with 0.5% Ponceau S (Sigma, USA) to check the correct loading. The blots were blocked in 2–5% of low-fat milk (Fluka, USA) and incubated with anti-PsbD (PSII; AS06 146), anti-RbcL (Rubisco; AS03 037), anti-PsaB (PSI; AS10 696), and anti-Lhcb2 (AS13 2705) primary antibodies (Agrisera, Sweden) overnight at 4 °C followed by the secondary antibody (anti-rabbit IgG horseradish peroxidases conjugated from Agrisera, ASO9 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 Licor Western blot imager (Licor, USA). Representative results are shown.

### 2.6. Statistical data processing

All experiments were performed using at least three biological replicates. Statistical analyses of data for physiological parameters were performed using SigmaPlot 12.3 (Systat Software Inc., USA) with one-way analysis of variance (ANOVA) followed by Duncan's method. Statistical analyses of gene expression data were performed with ANOVA with the post hoc Holm multiple-comparison test using the online calculator (astatsa.com/OneWay_Anova_with_TukeyHSD/). All data are presented as the mean values ± their standard errors (SE).

## 3. Results

### 3.1. Melatonin reduces stress in Arabidopsis leaves exposed to high light

First, we assessed the effectiveness of the model system in generating photooxidative damage. Under high-light conditions, the content of anthocyanins, proline, and TBARs was markedly elevated, as well as SOD activity and expression of the stress-induced genes *AOX1a* (alternative oxidase of plant mitochondrial electron transport chain) and *ELIP2* (early light inducible 2) compared to those in the control detached leaves exposed to 75 $\mu E\, m^{-2}\, s^{-1}$ lighting. In contrast, chlorophyll levels and *CAB2* expression (chlorophyll *a*/*b*-binding protein) were strongly reduced (Table 1). These results confirmed that the proposed experimental design worked as expected.

To evaluate the effect of melatonin, we selected indicator genes that, according to (Weeda et al., 2014), responded to melatonin

**Table 1**

Comparative characteristics of physiological parameters and transcript levels of indicator genes in detached leaves of *A. thaliana* under normal light and mild photooxidative stress (250 μE m$^{-2}$ s$^{-1}$). The 5th and 6th leaves were excised from 4-week-old plants and incubated on water for 72 h under 75 μE m$^{-2}$ s$^{-1}$ or 250 μE m$^{-2}$ s$^{-1}$. RNAs were analyzed by quantitative RT-PCR using *UBQ10* as an internal standard. The data presented in the table are the mean values ± standard errors (n ≥ 3). Different letters denote statistically significant differences at $p < 0.05$ (ANOVA followed by Duncan's test for physiological parameters or ANOVA with post hoc Holm's multiple-comparison test for gene expression data).

| Parameters | Light intensity | |
|---|---|---|
| | 75 μE m$^{-2}$ s$^{-1}$ | 250 μE m$^{-2}$ s$^{-1}$ |
| Anthocyanin content, relative units | 0.045 ± 0.002$^{a}$ | 1.172 ± 0.075$^{b}$ |
| TBARs content, μM/gfw | 1.643 ± 0.060$^{a}$ | 17.8 ± 0.130$^{b}$ |
| Proline content, mM/gfw | 0.134 ± 0.005$^{a}$ | 0.468 ± 0.015$^{b}$ |
| SOD activity, relative units | 1.000 ± 0.022$^{a}$ | 1.297 ± 0.043$^{b}$ |
| *AOX1a,*relative normalized expression | 1.000 ± 0.130$^{a}$ | 8.644 ± 1.484$^{b}$ |
| *ELIP2,*relative normalized expression | 1.000 ± 0.051$^{a}$ | 1.713 ± 0.031$^{b}$ |
| Chlorophyll (a + b) content, mg/gfw | 0.746 ± 0.013$^{a}$ | 0.476 ± 0.008$^{b}$ |
| *CAB2,*relative normalized expression | 1.000 ± 0.208$^{a}$ | 0.231 ± 0.059$^{b}$ |

treatment in 3-week-old *Arabidopsis* seedlings. Under mild photooxidative stress, these marker genes behaved similarly: melatonin upregulated the *AT5G39580* gene (peroxidase superfamily protein) and suppressed the activity of the *LTL1* gene (Li-tolerant lipase 1) (Fig. 1A and B), with the highest concentration of melatonin (1 mM) yielding the greatest effect. Thus, the transcriptional response of these genes proves the efficiency of melatonin treatment of detached leaves under excessive light. At the same time, melatonin treatment under normal light conditions had no significant effect on the transcript levels of the studied genes (Table 2Sa).

Exogenous melatonin did not change the level of COMT transcripts engaged in melatonin synthesis (Fig. 1D) but slightly increased the accumulation of SNAT1 transcripts (Fig. 1C). Elevation of SNAT1 transcript levels could be at least in part attributed to its additional physiological function as an active lysine acetyltransferase (Koskela et al., 2018).

The accumulation of anthocyanins is among the most significant traits by which it is possible to evaluate the degree of stress in plants (Zenga et al., 2010). In our experiments, melatonin significantly reduced the accumulation of anthocyanins in detached leaves (Fig. 2A). A similar pattern was shown in the changes in TBAR content, the accumulation of which is currently used to assess the degree of damage to plasma membranes caused by reactive oxygen species (ROS) (Fig. 2B). In addition, there was a significant decline in the content of proline (Fig. 2C), a low molecular weight antioxidant generated by stresses of different origins (Kholodova et al., 2018), and a reduction in the activity of SOD, which is directly involved in scavenging of superoxide radicals, at the concentration of 10 μM (Fig. 2D) (Myouga et al., 2008). Thus, the similar impact of various melatonin concentrations on various stress indicators suggests that melatonin might alleviate photodamage *via* diverse regulatory mechanisms. At the same time, melatonin treatment under normal light conditions had no significant effect on the transcript levels of the studied genes (Table 2Sb).

### 3.2. Melatonin slows chlorophyll degradation and reduces photodamage of PSI and PSII

The rate of leaf senescence was assessed by chlorophyll degradation and the expression of *SAG12*, encoding cysteine protease (Brusslan et al., 2012), which is directly involved in protein degradation (Fig. 3A and C). With the experimental scheme used, 1 mM melatonin had less effect on both indicators than lower concentrations, probably due to some inhibitory effect (Fig. 3C). The mechanism of chlorophyll retention may be associated with a retardation of degradation, as indicated

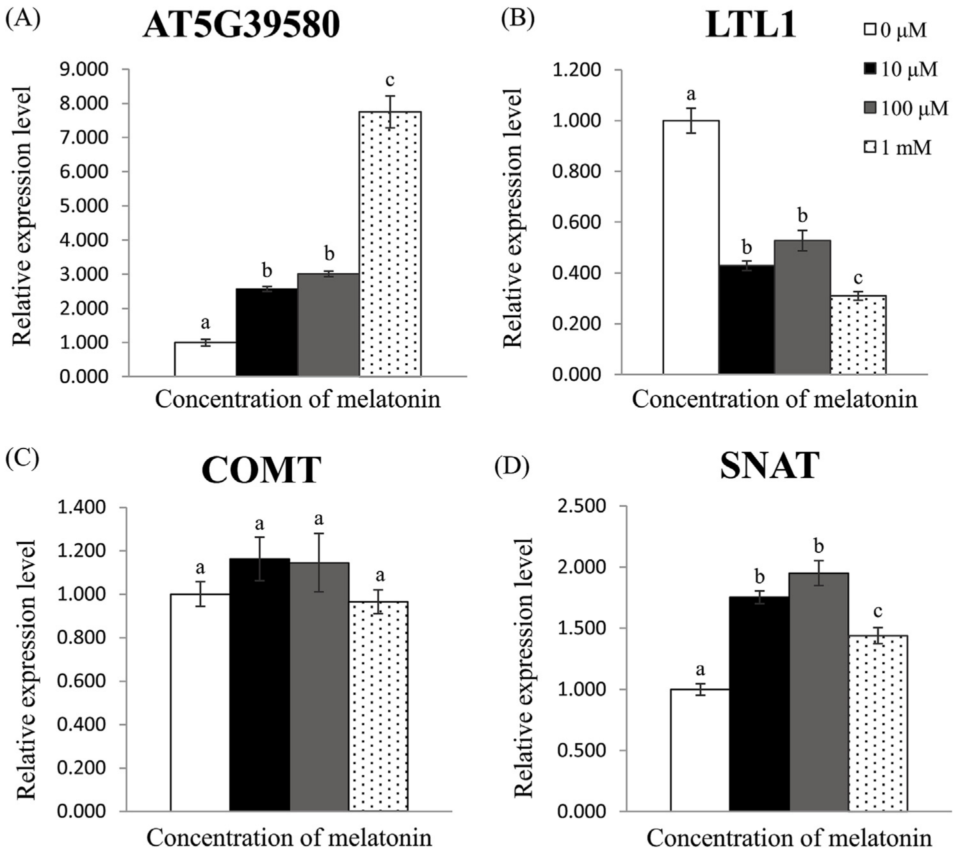

**Fig. 1.** The effect of mild photooxidative stress and exogenous melatonin treatment on the expression of melatonin indicator genes (A, B) and melatonin synthesis genes (C, D) in detached leaves of *A. thaliana*. The 5th and 6th leaves were excised from 4-week-old plants and incubated on water or melatonin solutions (10 μM, 100 μM or 1 mM) for 72 h under 250 μE m$^{-2}$ s$^{-1}$. RNAs were analyzed by relative quantitative RT-PCR using *UBQ10* as an internal standard. The data presented in the figure are the mean values (n ≥ 3). Error bars represent SEs. Different letters denote statistically significant differences at $p < 0.05$ (ANOVA with post hoc Holm's multiple-comparison test).

**Table 2**

The effect of mild photooxidative stress and exogenous melatonin treatment on the photosynthetic activity of photosystems I and II in detached leaves of *A. thaliana*. The 5th and 6th leaves were excised from 4-week-old plants and incubated on water or melatonin solution (10 μM, 100 μM or 1 mM) for 72 h under 250 μE m$^{-2}$ s$^{-1}$. The data presented in the table are the mean values ± standard errors (n ≥ 5). Different letters denote statistically significant differences at $p < 0.05$ (ANOVA followed by Duncan's test).

| Parameters | Melatonin concentration | | | |
|---|---|---|---|---|
| | 0 μM | 10 μM | 100 μM | 1 mM |
| Fs | $0.579 \pm 0.021^{a}$ | $0.664 \pm 0.030^{b}$ | $0.721 \pm 0.034^{b}$ | $0.610 \pm 0.034^{ab}$ |
| Fv/Fm | $0.586 \pm 0.021^{a}$ | $0.714 \pm 0.012^{b}$ | $0.711 \pm 0.015^{b}$ | $0.626 \pm 0.026^{a}$ |
| Y(II) | $0.494 \pm 0.028^{a}$ | $0.571 \pm 0.019^{b}$ | $0.563 \pm 0.021^{b}$ | $0.487 \pm 0.021^{a}$ |
| qP | $0.809 \pm 0.018^{a}$ | $0.862 \pm 0.019^{b}$ | $0.854 \pm 0.014^{b}$ | $0.784 \pm 0.020^{a}$ |
| qN | $0.174 \pm 0.010^{a}$ | $0.179 \pm 0.007^{a}$ | $0.182 \pm 0.006^{a}$ | $0.207 \pm 0.005^{b}$ |
| NPQ | $0.100 \pm 0.005^{a}$ | $0.116 \pm 0.009^{ab}$ | $0.126 \pm 0.012^{ab}$ | $0.139 \pm 0.006^{b}$ |
| Y(I) | $0.722 \pm 0.036^{a}$ | $0.821 \pm 0.025^{b}$ | $0.809 \pm 0.031^{ab}$ | $0.777 \pm 0.020^{ab}$ |
| Y(NA) | $0.139 \pm 0.028^{a}$ | $0.064 \pm 0.013^{b}$ | $0.045 \pm 0.015^{b}$ | $0.076 \pm 0.017^{ab}$ |
| Y(ND) | $0.139 \pm 0.021^{a}$ | $0.115 \pm 0.015^{a}$ | $0.146 \pm 0.021^{a}$ | $0.147 \pm 0.030^{a}$ |

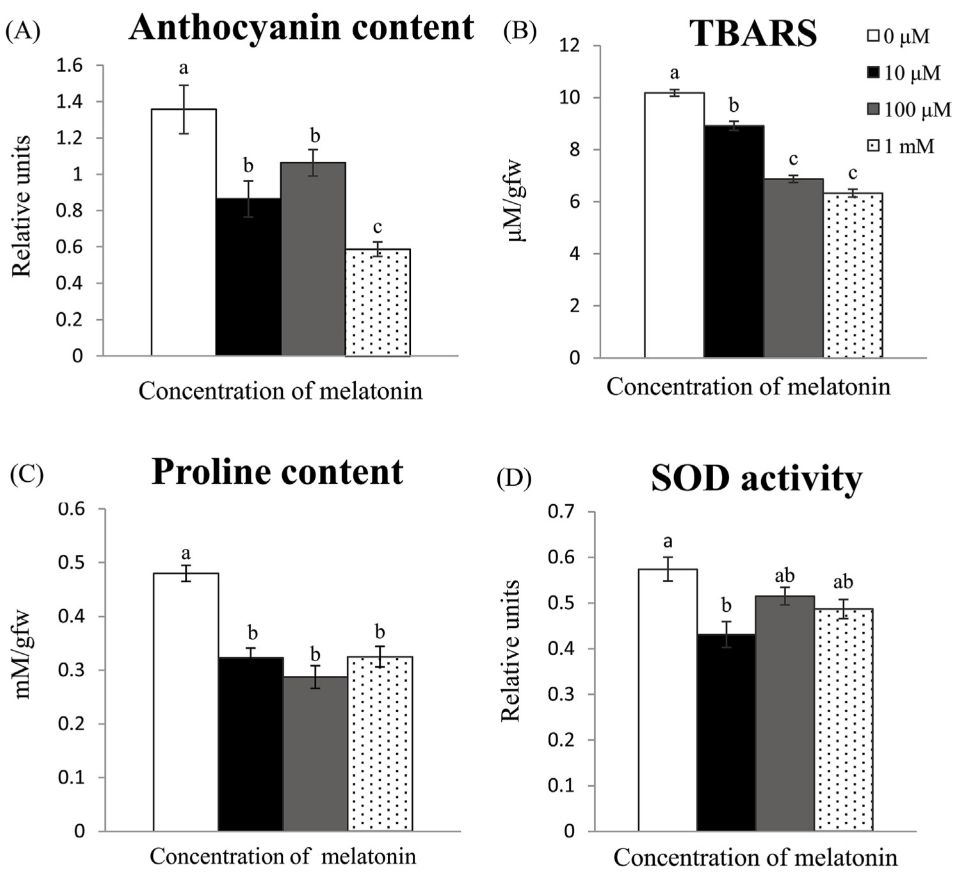

**Fig. 2.** The effect of mild photooxidative stress and exogenous melatonin treatment on stress indicators and elements of the antioxidant system in detached leaves of *A. thaliana*. A. Anthocyanin content. B. TBAR levels. C. Proline content. D. SOD activity. The 5th and 6th leaves were excised from 4-week-old plants and incubated on water or melatonin solution (10 μM, 100 μM or 1 mM) for 72 h under 250 μE m$^{-2}$ s$^{-1}$. The data presented in the figure are the mean values (n ≥ 3). Error bars represent SEs. Different letters denote statistically significant differences at $p < 0.05$ (ANOVA followed by Duncan's test).

by the suppression of the activity of the key gene for the chlorophyll degradation enzyme pheophorbide an oxygenase (PAO) (Fig. 3B). Melatonin also alleviated the decline in carotenoids, thus contributing to photoprotection (Fig. 3D).

There was a direct correlation between the total amount of chlorophyll and the activity of photosystem II (Table 2). The level of the stationary fluorescence signal ($F_s$) was higher in leaves treated with 10 and 100 μM melatonin than in the control. Lower concentrations of melatonin also increased the maximal fluorescence yield, the quantum yield in the light, and the coefficient of photochemical quenching PSII compared to those in the control. In addition, non-photochemical quenching was significantly higher under 1 mM melatonin treatment, as indicated by the qN and NPQ values, than under the other treatments. The increase in energy dissipation in the form of heat under high light prevents photoinhibition. However, in this case, elevated values of qN and NPQ did not have a positive effect on other indices, such as Fv/Fm and Y(II). This means that qN and NPQ levels do not reflect the optimization of the photosynthetic apparatus but only demonstrate the inhibitory effect of high melatonin concentrations.

Unlike the more dynamic and flexible PSII, photosystem I is quite stable (Schwarz et al., 2018). Melatonin showed an unambiguous positive effect on the PSI photochemical quantum yield only at the smallest concentration used (10 μM) (Table 2). Y(NA) and Y(ND) are quantum yields of non-photochemical energy dissipation due to acceptor-side and donor-side limitations in PSI, respectively. The non-photochemical quantum yield Y(NA) reliably decreased at all melatonin concentrations, but the dissipation associated with donor-side limitations in PSI did not change. This suggests the possible involvement of melatonin in the regulation of thermal dissipation of PSI.

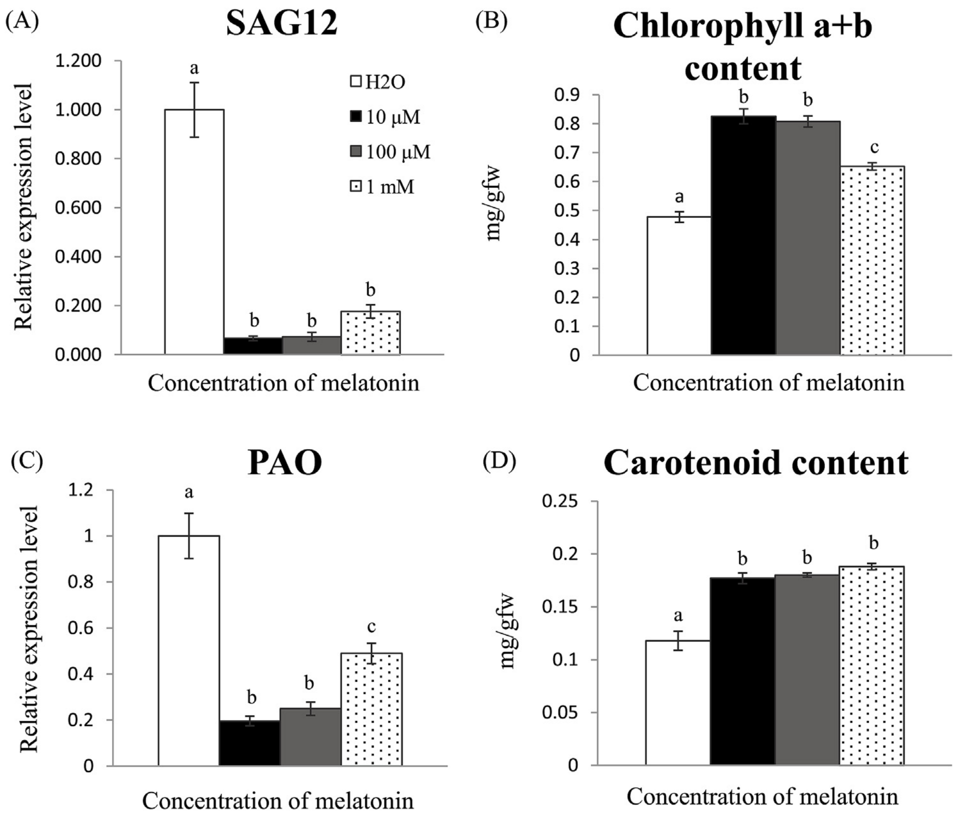

**Fig. 3.** The effect of mild photooxidative stress and exogenous melatonin treatment on the transcript levels of *SAG12* (an indicator of aging, A), and *PAO* (the chlorophyll degradation enzyme, B) and the content of photosynthetic pigments (C, D) in detached leaves of *A. thaliana*. The 5th and 6th leaves were excised from 4-week-old plants and incubated on water or melatonin solution (10 µM, 100 µM or 1 mM) for 72 h under 250 µE $m^{-2}$ $s^{-1}$. RNAs were analyzed by relative quantitative RT-PCR using *UBQ10* as the internal standard. Pigment measurements were performed according to Lichtenthaler (1987). The data presented in the figure are the mean values (n ≥ 3). Error bars represent SEs. Different letters denote statistically significant differences at $p < 0.05$ (ANOVA followed by Duncan's test for the content of photosynthetic pigments or ANOVA with post hoc Holm's multiple-comparison test for the transcript levels).

### 3.3. *Melatonin affects the expression patterns of the chloroplast-encoded genes*

To examine whether the beneficial effects of melatonin were associated with changes in the expression of the chloroplast genome, we studied the transcript levels of plastid-encoded genes belonging to different operons and functional complexes and transcribed by various RNA polymerases (Table 3). Quantitative RT-PCR revealed an essential impact of melatonin on the transcript levels of *rpoA* and *rpoB* genes (α and β subunits of PEP) transcribed by NEP as parts of two separate operons (Liere et al., 2011). The transcript levels showed a significant (2-fold) increase in the leaves treated with 10 µM and, to a lesser extent, those treated with 100 µM melatonin, compared to the water controls (Table 3). For the analysis of the expression data, 1.5- to 2-fold changes are often used as minimum cutoffs when identifying regulated genes (Bhargava et al., 2013). Taking into account this setting, we should admit that melatonin did not have a reliably significant stimulatory impact on the transcript abundance of housekeeping genes *accD* (acetyl-CoA carboxylase subunit) and *rps14* (ribosomal protein S14), which are involved in fatty acid biosynthesis and translation, respectively. The transcript levels of two other housekeeping genes, *rpl16* (ribosomal protein L16) and *clpP* (ATP-dependent protease), were slightly increased at concentrations of 10 µM and 100 µM and either were inhibited or did not differ from the control values at a concentration of

**Table 3**
The effect of mild photooxidative stress and exogenous melatonin treatment on the transcript levels of chloroplast- and nuclear-encoded genes in detached leaves of *A. thaliana*. The 5th and the 6th leaves were excised from of 4-week-old plants and incubated on the water or melatonin solutions (10 µM, 100 µM or 1 mM) for 72 h under 250 µE $m^{-2}$ $s^{-1}$. RNAs were analyzed by quantitative real-time (RT)-PCR using *UBQ10* as internal standard. The mRNA levels of the water control were set as 1.0 and the other values expressed relative to them. The data presented in the table are the mean values ± standard errors (n ≥ 3). Different letters denote statistically significant differences at $p < 0.05$ (ANOVA with post hoc Holm multiple-comparison calculation).

| Genes | Melatonin concentration | | | |
|---|---|---|---|---|
| | 0 µM | 10 µM | 100 µM | 1 mM |
| *rpoA* | 1.000 ± $0.064^{a}$ | 2.099 ± $0.108^{b}$ | 1.699 ± $0.097^{b}$ | 0.865 ± $0.049^{a}$ |
| *rpoB* | 1.000 ± $0.014^{a}$ | 2.042 ± $0.028^{b}$ | 1.306 ± $0.019^{c}$ | 1.102 ± $0.088^{ac}$ |
| *accD* | 1.000 ± $0.308^{a}$ | 1.266 ± $0.358^{a}$ | 1.206 ± $0.305^{a}$ | 1.083 ± $0.298^{a}$ |
| *rpS14* | 1.000 ± $0.204^{a}$ | 1.087 ± $0.274^{a}$ | 1.206 ± $0.302^{a}$ | 0.742 ± $0.193^{a}$ |
| *rpL16* | 1.000 ± $0.045^{a}$ | 1.521 ± $0.036^{b}$ | 1.310 ± $0.065^{b}$ | 0.332 ± $0.032^{c}$ |
| *clpP* | 1.000 ± $0.021^{a}$ | 1.591 ± $0.055^{b}$ | 1.464 ± $0.061^{b}$ | 0.946 ± $0.070^{a}$ |
| *psbA* | 1.000 ± $0.097^{a}$ | 0.596 ± $0.046^{b}$ | 0.904 ± $0.085^{a}$ | 0.785 ± $0.080^{ab}$ |
| *psbD* | 1.000 ± $0.047^{a}$ | 0.811 ± $0.027^{b}$ | 1.227 ± $0.079^{a}$ | 0.730 ± $0.039^{b}$ |
| *rbcL* | 1.000 ± $0.125^{a}$ | 0.748 ± $0.103^{a}$ | 1.039 ± $0.169^{a}$ | 0.237 ± $0.038^{b}$ |
| *psaA* | 1.000 ± $0.107^{a}$ | 0.693 ± $0.167^{a}$ | 1.057 ± $0.148^{a}$ | 1.301 ± $0.225^{a}$ |
| *psaB* | 1.000 ± $0.202^{a}$ | 0.835 ± $0.132^{a}$ | 1.137 ± $0.167^{a}$ | 1.479 ± $0.230^{a}$ |
| *petD* | 1.000 ± $0.048^{a}$ | 0.940 ± $0.028^{a}$ | 0.859 ± $0.057^{ab}$ | 0.676 ± $0.052^{b}$ |
| *ndhA* | 1.000 ± $0.107^{a}$ | 1.017 ± $0.114^{a}$ | 1.039 ± $0.138^{a}$ | 1.068 ± $0.145^{a}$ |
| *LHSB2* | 1.000 ± $0.056^{a}$ | 0.777 ± $0.065^{a}$ | 1.279 ± $0.142^{a}$ | 0.807 ± $0.067^{a}$ |

1 mM. Hence, we can conclude that the transcript turnover of housekeeping genes followed no single pattern, suggesting that melatonin-induced changes under high light were gene-specific.

The expression patterns of selected photosynthesis genes transcribed by PEP were largely unaltered, although there was a downward trend under 10 μM and 1 mM melatonin treatment for the PSII genes *psbA* and *psbD* (D1 and D2 proteins of PSII) and for *rbcL* (the large subunit of Rubisco). No statistically significant changes were found in the transcription activities of *ndhA* (chloroplast NADH dehydrogenase) at any concentration or those of *petD* (subunit IV of cytochrome $b_6f$ complex) at concentrations of 10 μM and 100 μM. These last two genes are transcribed from both PEP and NEP promoters.

These data suggest that melatonin has little or no influence on the expression of photosynthesis genes. Thus, under stress conditions, melatonin primarily alters the expression of housekeeping genes involved in maintaining transcriptional activity and the functional state of chloroplasts.

### *3.4. Effects of melatonin on the expression of nucleus-encoded genes for the plastid transcription machinery under high light*

The changes in the expression patterns of plastid-encoded genes under high light might be associated at least in part with melatonin-induced alterations in the activity of the chloroplast transcription machinery. To test this assumption, the transcript abundance of the key players in the plastid transcription apparatus was studied.

In Arabidopsis, the NEP transcription system includes two types of nuclear-encoded polymerases: RPOTp and RPOTmp (Liebers and Pfannschmidt, 2017). The genes encoding both enzymes were strongly upregulated under high-light conditions when treated with 10 μM and to a lesser extent with 100 μM melatonin (Table 4), while the higher concentrations caused inhibition of the expression. However, no differences in the expression profiles of RNA polymerase genes under normal light were observed (data not shown). Thus, melatonin regulates both PEP and NEP transcript levels in excessive light.

The activity of PEP in *Arabidopsis* chloroplasts is mediated by six nucleus-encoded sigma factors and Ser/Thr protein kinase cpCK2, which, depending on the redox state of the components of the chloroplast electron transport chain, phosphorylates RNA polymerase subunits and sigma factors. Melatonin positively regulated the levels of *Sig1-Sig4* transcripts at a concentration of 10 μM and negatively regulated the levels at a concentration of 1 mM. *Sig6* was not reliably activated by lower concentrations, probably because it functions mainly at the early stage of chloroplast development (Ishizaki et al., 2005). The transcript abundance of stress-inducible *Sig5* remained elevated at all concentrations applied. The same regulation pattern was shown for *cpCK2*.

Beyond the aforementioned genes, the PEP transcription system contains 12 nuclear-encoded proteins (PAPs) crucial for the proper formation of the plastid-encoded RNA polymerase complex (Liebers and Pfannschmidt, 2017). The melatonin treatment significantly affected the expression profiles of all *PAP* genes (except *PAP5*), with the highest activation rates at a concentration of 10 μM. Notably, the expression levels of the genes implicated in ROS protection (*PAP4, 9*) and redox regulation genes (*PAP6, 10*) were usually higher than those of the genes involved in DNA/RNA metabolism and gene expression regulation. However, 1 mM melatonin in some cases caused severe inhibition of *PAP* transcript levels up to values inferior to those of the water controls.

Overall, the results from this study show that under light stress, exogenous melatonin modifies the expression profiles of the genes implicated in chloroplast transcription. Depending on the concentration used, melatonin can either activate or suppress their expression, thereby contributing to the transition from the antioxidant to the pro-oxidant type of regulation.

### *3.5. The effect of melatonin on the levels of photosynthetic proteins under light stress*

The fact that melatonin has no or only limited influence on the expression of photosynthesis genes contradicts its effect on photosynthetic activity under intensive light. Therefore, we wanted to determine whether photosynthetic proteins (e.g., PSI and PSII proteins,

**Table 4**

The effect of mild photooxidative stress and exogenous melatonin treatment on the transcript levels of nuclear genes encoding chloroplast transcription machinery in detached leaves of *A. thaliana*. The 5th and 6th leaves were excised from 4-week-old plants and incubated on water or melatonin solution (10 μM, 100 μM or 1 mM) for 72 h under 250 μE $m^{-2}$ $s^{-1}$. RNAs were analyzed by quantitative RT-PCR using *UBQ10* as an internal standard. The mRNA levels of the water control were set as 1.0, and the other values were expressed relative to that value. The data presented in the table are the mean values ± standard errors ($n \geq 3$). Different letters denote statistically significant differences at $p < 0.05$ (ANOVA with post hoc Holm's multiple-comparison test).

| Genes | Melatonin concentration | | | |
|---|---|---|---|---|
| | 0 μM | 10 μM | 100 μM | 1 mM |
| *RPOTp* | $1.000 \pm 0.041^{a}$ | $3.950 \pm 0.504^{b}$ | $2.803 \pm 0.483^{b}$ | $0.609 \pm 0.032^{c}$ |
| *RPOTmp* | $1.000 \pm 0.039^{a}$ | $3.260 \pm 0.077^{b}$ | $2.065 \pm 0.197^{c}$ | $0.289 \pm 0.024^{d}$ |
| *SIG1* | $1.000 \pm 0.093^{a}$ | $2.594 \pm 0.219^{b}$ | $1.181 \pm 0.122^{a}$ | $0.662 \pm 0.018^{a}$ |
| *SIG2* | $1.000 \pm 0.078^{a}$ | $2.007 \pm 0.114^{b}$ | $1.682 \pm 0.093^{b}$ | $0.454 \pm 0.026^{c}$ |
| *SIG3* | $1.000 \pm 0.104^{a}$ | $2.185 \pm 0.136^{b}$ | $1.650 \pm 0.328^{ab}$ | $0.488 \pm 0.101^{c}$ |
| *SIG4* | $1.000 \pm 0.024^{a}$ | $1.548 \pm 0.054^{b}$ | $0.933 \pm 0.125^{ab}$ | $0.276 \pm 0.010^{c}$ |
| *SIG5* | $1.000 \pm 0.010^{a}$ | $2.387 \pm 0.074^{b}$ | $2.667 \pm 0.038^{b}$ | $1.790 \pm 0.030^{c}$ |
| *SIG6* | $1.000 \pm 0.112^{a}$ | $1.218 \pm 0.289^{a}$ | $1.079 \pm 0.218^{a}$ | $0.294 \pm 0.067^{b}$ |
| *PAP1* | $1.000 \pm 0.057^{a}$ | $1.608 \pm 0.102^{b}$ | $1.569 \pm 0.070^{b}$ | $0.693 \pm 0.017^{c}$ |
| *PAP2* | $1.000 \pm 0.031^{a}$ | $2.204 \pm 0.046^{b}$ | $1.434 \pm 0.039^{c}$ | $0.375 \pm 0.011^{d}$ |
| *PAP3* | $1.000 \pm 0.118^{a}$ | $4.993 \pm 0.423^{b}$ | $3.482 \pm 0.435^{b}$ | $1.145 \pm 0.108^{a}$ |
| *PAP4* | $1.000 \pm 0.029^{a}$ | $4.500 \pm 0.086^{b}$ | $2.848 \pm 0.043^{c}$ | $0.667 \pm 0.049^{d}$ |
| *PAP5* | $1.000 \pm 0.059^{ab}$ | $1.439 \pm 0.129^{a}$ | $1.259 \pm 0.195^{a}$ | $0.348 \pm 0.024^{ab}$ |
| *PAP6* | $1.000 \pm 0.025^{a}$ | $2.549 \pm 0.045^{b}$ | $1.847 \pm 0.237^{b}$ | $0.521 \pm 0.073^{a}$ |
| *PAP7* | $1.000 \pm 0.065^{a}$ | $2.899 \pm 0.090^{b}$ | $1.580 \pm 0.022^{c}$ | $0.702 \pm 0.043^{a}$ |
| *PAP8* | $1.000 \pm 0.014^{a}$ | $2.990 \pm 0.265^{b}$ | $2.799 \pm 0.026^{b}$ | $0.446 \pm 0.012^{c}$ |
| *PAP9* | $1.000 \pm 0.085^{a}$ | $3.182 \pm 0.191^{b}$ | $2.809 \pm 0.182^{b}$ | $1.087 \pm 0.076^{a}$ |
| *PAP10* | $1.000 \pm 0.139^{a}$ | $4.098 \pm 0.407^{b}$ | $1.028 \pm 0.161^{a}$ | $0.655 \pm 0.120^{a}$ |
| *PAP11* | $1.000 \pm 0.124^{a}$ | $2.107 \pm 0.286^{b}$ | $2.478 \pm 0.161^{b}$ | $0.543 \pm 0.073^{a}$ |
| *PAP12* | $1.000 \pm 0.118^{a}$ | $1.847 \pm 0.026^{b}$ | $2.035 \pm 0.035^{b}$ | $0.557 \pm 0.029^{c}$ |
| *cPCK2* | $1.000 \pm 0.016^{a}$ | $2.949 \pm 0.026^{b}$ | $2.549 \pm 0.011^{c}$ | $2.078 \pm 0.036^{d}$ |
| *Abi4* | $1.000 \pm 0.048^{a}$ | $3.658 \pm 0.124^{b}$ | $3.779 \pm 0.497^{b}$ | $2.567 \pm 0.133^{b}$ |

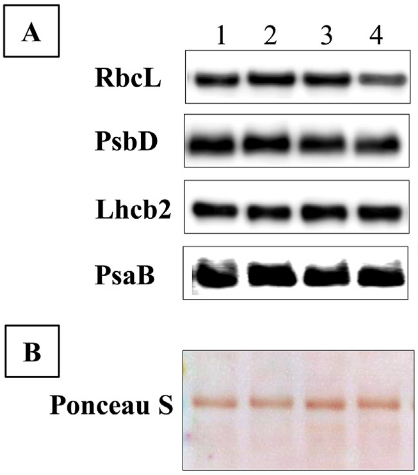

**Fig. 4.** Immunoblot analysis (A) of the photosynthetic proteins on the basis of equal total (B) Ponceau S dye stained blot with proteins (15 μg) from the 5th and 6th leaves of *A. thaliana* excised from 4-week-old plants and incubated on water (1) or melatonin solution 10 μM (2), 100 μM (3) or 1 mM (4) for 72 h under 250 μE $m^{-2}$ $s^{-1}$. Proteins were visualized by immunoblotting using antibodies specific for RbcL, PsaB, PsbD and Lhcb2 proteins.

RbcL and LHCBs) accumulate in the same manner as their corresponding mRNAs. The immunodetection analysis showed a noticeable increase in the protein levels of RbcL under 10 μM melatonin treatment (approximately 35%) and a mild decrease (78%) at 1 mM melatonin compared to the level in the water controls (Fig. 4; Table 3S). Levels of PSI and PSII proteins were insignificantly increased (PsaB) or remained practically unchanged (PsbD) when treated with 10 μM melatonin compared to the levels in the water controls. However, higher concentrations resulted in lower amounts of PS proteins compared to the amounts in the other treatments. In contrast, no changes were observed in the protein levels of LHCb2 at any of the three melatonin concentrations. We conclude from these experiments that under melatonin treatment, the levels of photosynthetic proteins correlate poorly with the accumulation of their transcripts and the parameters of photochemical processes. This suggests that the effect of melatonin on the photosynthetic apparatus which would allow the balancing of chloroplast functions with stress responses is highly complicated.

## 4. Discussion

Melatonin has remarkably diverse effects combining properties of both anti-stress agents and signaling molecules. It is generally assumed that at low concentrations, melatonin exhibits mainly its regulatory function compatible with its phytohormone role and at high concentrations, it acts as an antioxidant (Hernández et al., 2015), though analysis of the literature does not confirm this unambiguously. According to the assumption proposed by Hardeland (Hardeland 2016), low levels of melatonin should act through high-affinity binding sites. At the same time, at strongly elevated levels, antioxidant properties are believed to take over the signaling function, since high-affinity binding sites would be completely saturated or desensitized, as observed for many hormone receptors. This requires the existence of additional low-affinity binding sites that would contribute to efficient stress protection.

The analysis of the data obtained in this study suggests that positive results associated with the regulatory function, as a rule, were acquired using a minimum melatonin concentration of 10 μM. However, the fact that both minimum and maximum concentrations had approximately the same effect on such indicators as the accumulation of proline, anthocyanins and carotenoids (Figs. 2 and 3) implies that signaling as well as antioxidant mechanisms are involved in improving these parameters, thereby eliminating the negative aftermath of light stress. This finding is partly supported by chlorophyll retention data and the activity of the photosystems.

The additional mechanism by which melatonin confers efficient protection against photooxidative stress is the regulation of chloroplast gene expression. Melatonin did not cause general activation or repression of chloroplast-encoded genes. Rather, the data indicate that melatonin provided gene-specific regulation of housekeeping genes involved in maintaining basic cellular functions and viability under stress conditions. The upregulation of *ClpP* transcripts by melatonin is of special interest. The ClpP protease does not play a role in the proteolytic breakdown of the components of the photosynthetic apparatus during senescence but rather has a function in non-senescing chloroplasts showing high photosynthetic activity (Humbeck and Krupinska, 1996). However, photosynthetic chloroplast genes were practically unregulated by melatonin in this experimental setting. Such a response is probably explained by acclimation to high-light conditions, which is accompanied by a reduction in the photosynthetic reaction centers. Different responses of chloroplast genes to melatonin treatment can also be explained by differences in the transcriptional intensity of genes from various operons and differences in transcript stability. Remarkably, transcript levels of photosynthetic *petD* (an encoding subunit of the cytochrome $b_6f$ complex) and housekeeping *rpoA* (for α subunit of PEP), which are co-transcribed as a single polycistronic pre-RNA, differed, clearly pointing to posttranscriptional regulation of their accumulation under melatonin treatment.

The fact that photosynthetic genes were practically unregulated by melatonin may be attributed in part to acclimation to high-light conditions, in accordance with the finding that after perceiving a stress signal, plants reallocate resources to defense at the expense of growth (Cortleven et al., 2019) and hence at the expense of photosynthesis events. At the same time, the ability of melatonin to improve chloroplast function without increasing plastid-encoding photosynthesis gene transcription and the levels photosynthesis proteins, which are directly involved in the photochemistry of PSII and PSI, suggests that the effects of melatonin are quite complex. In fact, such multidirectional regulation may be performed through a multiplicity of pathways and none of them individually ensures a positive effect.

Chloroplast RNA polymerases and their regulatory proteins were among the main targets of melatonin. Both *NEP* and *PEP* genes and the majority of *PAPs* and sigma factor genes were activated by low concentrations of the compound (10 μM) and inhibited by high doses (1 mM).

Elevated transcript levels of *Sig5* were expected, given its stress-related functions and participation in rapid reconstruction of the photosynthetic reaction center (Kanamaru and Sugita, 2013). However, meticulous examination indicated that melatonin alters the transcript accumulation of all other sigma family genes, except *SIG6*, suggesting the possible involvement of highly conserved C-terminal domains or some other additional actors in regulatory changes, such as SIB1 (a sigma factor-binding protein 1) or a pentatricopeptide repeat protein DGI 1 (DELAYED GREENING 1) (Kanamaru and Sugita, 2013). The finding that *SIG6* does not show significant regulation by melatonin can be explained by the fact that it functions mainly at the early stage of chloroplast development (Ishizaki et al., 2005). In addition, the activity of sigma factors is regulated by their phosphorylation status. The melatonin-induced increase in transcript levels of plastid-targeted casein kinase cPCK2 may contribute to concerted changes in the phosphorylation of sigma factors and cause a consequent conformational change that would alter the transcriptional activity or specificity of the plant sigma factors in response to developmental and/or environmental conditions (Kanamaru and Sugita, 2013).

Despite their highly diverse functions and structure, PAP genes are considered to be coordinately expressed, suggesting that they generate a regulon (Pfannschmidt et al., 2015). In this study, they also displayed a high degree of similarity in their transcript patterns since most of them were upregulated by 10 μM melatonin and partly downregulated by higher concentrations. Among the most upregulated PAP genes, we found redox-related genes and *PAP3*-encoding RNA binding proteins. PAP3 can be one of the substrates of Ser/Thr protein kinase cPCK2 (Reiland et al., 2009), transcript levels of which were coordinately upregulated in response to melatonin treatment (Table 4), as noted above. The only exception among the *PAP* genes that were not reliably regulated by melatonin treatment was *PAP5*. PAP5 protein was shown to be localized to both chloroplasts and the nuclear phytochrome bodies, where it acts in proteolysis (Chen et al., 2010). Hence, its activation could cause undesirable protein degradation.

Thus, low concentrations of melatonin may provide efficient protection against photo-oxidative stress by regulating chloroplast gene expression, thereby revealing the possible role of melatonin in signal transduction pathways. Under conditions of high irradiance, plastid retrograde signals can be transmitted to the nucleus and rearrange gene expression *via* a set of distinct transcription factors (ABI4, NACs, WRCYs, etc.) (Kmiecik et al., 2016). In our tests, transcript levels of *ABI4,* one such transcriptional factor, were strongly upregulated by melatonin treatment (Table 4). It should be mentioned, however, that a recent comprehensive analysis by Kacprzak et al. obviously contradicts a deep involvement of ABI4 in chloroplast-to-nucleus retrograde signaling, although the induction of ABI4 expression under stress conditions was confirmed in the study (Kacprzak et al., 2019). It is of interest that Weeda and colleagues revealed a total of 29 genes for transcription factors that were upregulated by melatonin treatment, including eight WRKY and five NAC domain-containing proteins (Weeda et al., 2014). All but one were related to stress responses. It is likely that chloroplast genes could belong to their downstream targets either directly or through regulation of the chloroplast transcriptional machinery.

## Author contributions

I. Bychkov designed and conducted the experiments, analyzed the data, wrote the manuscript and accomplished pictures for the manuscript. N. Kudryakova contributed to the design of the experiments, performed the experiments, analyzed the data and wrote the manuscript. E. Pojidaeva and A. Andreeva conducted protein analysis by Western blotting and analyzed the data. V. Kusnetsov conceived and designed the experiments, analyzed the data, and finalized the manuscript. E. All authors read and approved the final draft of the manuscript.

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.013.

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