NKFIH OTKA K-128575

Comprehensive analysis of changes in global gene expression, full metabolome, lipid and hormone content to elucidate the mechanisms of light induced frost tolerance in cereals and in lower model plants

Final Report

The main aim of this project was to get a comprehensive picture about the molecular/metabolic changes during modulated light induced pre-hardening process in barley as specially by focusing on hormone homeostasis and lipid metabolism. It was well established that the alteration of membrane and chloroplast lipid composition is imperative in the cold acclimation process of higher plants. Our working hypothesis was that the lipid content of a higher plant, barley should also be affected by light quality resulting improved membrane freezing stability. Secondly, plant hormones also do have pivotal role in regulating the cold hardening process, consequently we hypothesized that most likely they are also being involved in the regulation of light induced pre-hardening process. There was very limited information how the spectral composition of light influences the lipid composition of lower plants in the time when the grant proposal was created (2018). The second important goal of the grant proposal was to elucidate whether light spectrum and temperature-dependent changes of the lipid content in the lower plant Chlamydomonas and photosynthetic prokaryotes, cyanobacteria are similar or different to those detected in higher plants. We could not completely fulfil this research goal until in this project was closed, but we have achieved significant progress in this regard as well, which is confirmed by this final report. The most important reason for the discrepancy was that Dr. Zoltán Gombos, who was one of the designers of the research program described in the application and the head of the lower plant program, died in 2019 at the beginning of the implementation of the application.

RESULTS

1. Temperature changes and decreased red: far-red (R:FR) ratio in incident white light affect the composition of barley leaf lipidome

The differences in the lipid composition of barley leaves illuminated with white light or white light supplemented with far-red light at 5 or 15 °C was studied. For the lipid analysis electrospray ionization triple quadrupole mass spectrometry was used in collaboration with the Kansas Lipidomics Research Center (https://www.k - state.edu/lipid/analytical_laboratory/lipid_profiling/index.html). According to LC-MS analysis, far-red light supplementation increased the amount of monogalactosyldiacylglycerol species (MGDG) 36:6, 36:5, and 36:4 after 1 day at 5 °C, and 10 days at 15 °C. Changes were observed in the levels of phosphatidylethanolamine (PE), and phosphatidylserine (PS) in leaves illuminated by modified white light (decreased R:FR) at 15 °C. The additional FR light also increased the amount of several PS species at 5 °C. At 15 °C, the amount of some

phosphatidylglycerol species increased transiently in leave samples after 1 day as a result of supplemental FR light illumination. The ceramide (42:2)-3 content increased regardless of the temperature. The double-bond index of phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylcholine (PC) ceramide together with total double-bond index changed when the plant was grown at 15 °C and illuminated with light by increased FR ratio. The FR supplementation also increased the monogalactosyldiacylglycerol/diacylglycerol ratio, as well. The gene expression changes are well correlated with the alterations in the lipidome (see the details: Kovacs et al. Int. J. Mol. Sci. 2020, 21, 7557; doi:10.3390/ijms21207557. Impact: 11).

As final conclusion of this subject:

The total lipid content of PS had increased both in 15˚C and 5˚C under increased FR ratio. (PS is involved both in cold and ABA signal transduction pathways.)

PG content increased, what should be important for maintaining the chloroplast function during cold stress.

The number of double bonds of the total lipid content increased by the supplemented FR light. This changes can be the indicator of higher membrane fluidity.

In the studied HexCer molecular class, only one type containing two double bonds (42:2)-3 increased significantly as a result of additional FR light.

The supplemented FR light decreased the PC/PE ratio. Consequently, by applying increased FR ratio in the incident white light we can mimic the effect of cold treatment to increase the membrane fluidity.

The MGDG and DGDG are the most important lipid components of chloroplast membranes. Although both of their amounts increased as a resulted of FR light treatment the increment in MGDG content was considerably higher resulting increased membrane fluidity.

2. The influence of incident light intensity on the light-quality-dependent freezing tolerance of barley

To elucidate the interaction between the ambient temperature and the light intensity on the cold hardening process, barley plantlets were grown under different light conditions with low (125 PAR), normal (250 PAR), and high (350 PAR) light intensities at 5 and 15 °C. The expression of the *HvCBF14* gene and two well-characterized members of the C-repeat binding factor (CBF)-regulon *HvCOR14b* and *HvDHN5* were studied. In general, the expression level of the studied genes was several folds higher at 5 °C than that at 15 °C independently of the applied light intensity or the spectra. The complementary far-red (FR) illumination induced the expression of *HvCBF14* and also its target gene *HvCOR14b* at both temperatures in the leaves of barley plants most effectively when the lowest light intensity (125 PAR) was applied. This phenomenon well illustrates that both the intensity and the quality of the incident light influence the gene expression. However, this supplementation did not affect significantly the expression of *HvDHN5*. To test the physiological effects of these changes in environmental conditions, freezing tests were also performed. In all the cases, we found that the reduced R:FR ratio increased the frost tolerance

of barley at each incident light intensity. These results show that the combined effects of cold, light intensity, and the modification of the R:FR light ratio can greatly influence the gene expression pattern of the plants, which can result in increased plant frost tolerance (See details: Ahres et al: Plants 2020, 9, 83; doi:10.3390/plants9010083. Impact: 21).

3. The impact of far-red light supplementation on hormonal responses to cold acclimation in barley

Cold acclimation, the necessary prerequisite for promotion of freezing tolerance, is affected by both low temperature and enhanced far-red/red light (FR/R) ratio. The impact of FR supplementation to white light, created by artificial LED light sources, on the hormone levels, metabolism, and expression of the key hormone metabolism-related genes was determined in winter barley at moderate (15 °C) and low (5 °C) temperature. FR-enhanced freezing tolerance at 15 °C was associated with promotion of abscisic acid (ABA) levels, and accompanied by a moderate increase in indole-3-acetic acid (IAA) and cis-zeatin levels. The most prominent impact on the plants' freezing tolerance was found after FR pre-treatment at 15 °C (for 10 days) followed by cold treatment at FR supplementation (7 days). The response of ABA was diminished in comparison with white light treatment, probably due to the elevation of stress tolerance during FR pre-treatment. Jasmonic acid (JA) and salicylic acid (SA) were transiently reduced. When the plants were exposed directly to a combination of cold (5 °C) and FR supplementation, ABA increase was higher than in white light, and was associated with enhanced elevation of JA and, in the longer term (after 7 days), with IAA and cis-zeatin increase, which indicates a stronger stress response and better acclimation. Cold hardening was more efficient when FR light was applied in the early developmental stage of the barley plants (three-leaf stage, 18 days), rather than in later stages (28-days). The dynamics of the phytohormone changes are well supported by the expression profiles of the key hormone metabolism-related genes. This series of treatments serves as evidence for the close relationship between plant hormones, light quality, and low temperature at the beginning of cold acclimation. Besides the timing of the FR treatments, plant age also represents a key factor during light spectrum-dependent cold acclimation (Ahres et al.: Biomolecules 2021, 11, 450.https://doi.org/10.3390/biom11030450. Impact: 9).

4. White light spectrum modifications by excess of blue light affecting the frost tolerance, lipid- and hormone composition of barley in the early pre-hardening phase

It is well established that cold acclimation processes are highly influenced, apart from cold ambient temperatures, by light-dependent environmental factors. In this study we investigated whether an extra blue (B) light supplementation would be able to further improve the well-documented freezing tolerance enhancing effect of far-red (FR) enriched white (W) light. The impact of B and FR light supplementation to white light (WFRB) on hormone levels and lipid contents were determined in winter barley at moderate (15 °C) and low (5 °C) temperatures.

Low R:FR ratio effectively induced frost tolerance in barley plantlets, but additional B light further enhanced frost hardiness at both temperatures. Supplementation of WFR (white light enriched with FR light) with B had a strong positive effect on abscisic acid accumulation while the suppression of salicylic acid and jasmonic acid levels were observed at low temperature which resembles the shade avoidance syndrome. We also observed clear lipidomic differences between the individual light and temperature treatments. WFRB light changed the total lipid content negatively, but monogalactosyldiacylglycerol (MGDG) content was increased, nonetheless. Our results prove that WFRB light can greatly influence phytohormone dynamics and lipid contents, which eventually leads to more efficient pre-hardening to avoid frost damage (Ahres et al. Plants 2023, 12, 40. https://doi.org/10.3390/plants12010040).

5. Light and temperature-dependent changes of the lipid content and metabolic changes in cyanobacteria

Based on the promising results of the two pilot experiments that were run previously, we design an experiment to reveal the effects of light supplementation on growth rate, pigment- and lipid content and gene expression of *Synechococcus sp*. (WH7803).

Following a 12 day pre-growth phase at 20°C under white (W) light illumination, the cultures were separated into two groups. The first group remained under W illumination at 20°C for further 7 days, the other group was moved into white + far-red (WFR) light and stayed there for further 7 days. Following that, the temperature was reduced to 15°C and the cultures remained there for further 7 days at both W and WFR illumination.

Three independent biological replicates were used at all light and temperature combination, cultures were grown in 500 mL Erlenmeyer flasks. During the experiment the growth of the cultures was followed by measuring the OD on every third or fourth day at 750 nm. Samples were taken for lipid-, pigment- and gene expression analysis on the 7th day of each temperature and light treatments. Then samples were sent to Dr. Blazenka Gasparovic (Laboratory for Marine and Atmospheric Biogeochemistry, Center for Marine and Environmental Research, Rudjer Boskovic Institute, Zagreb) for lipid analysis and to Dr. László Kovács (SZBK, Szeged) for pigment analysis.

Growth rate

Different growth rates of the Synechococcus cultures were observed under different spectral compositions (Figure 1), also the effect of temperature was observed. During the pre-growth phase, different between the growths of the cultures were not observed. Following the start of the light treatment cultures grown under WFR illumination had higher growth than the ones were under W. This slight difference was observed at 20°C, however this difference was increased significantly when the temperature was decreased to 15°C.

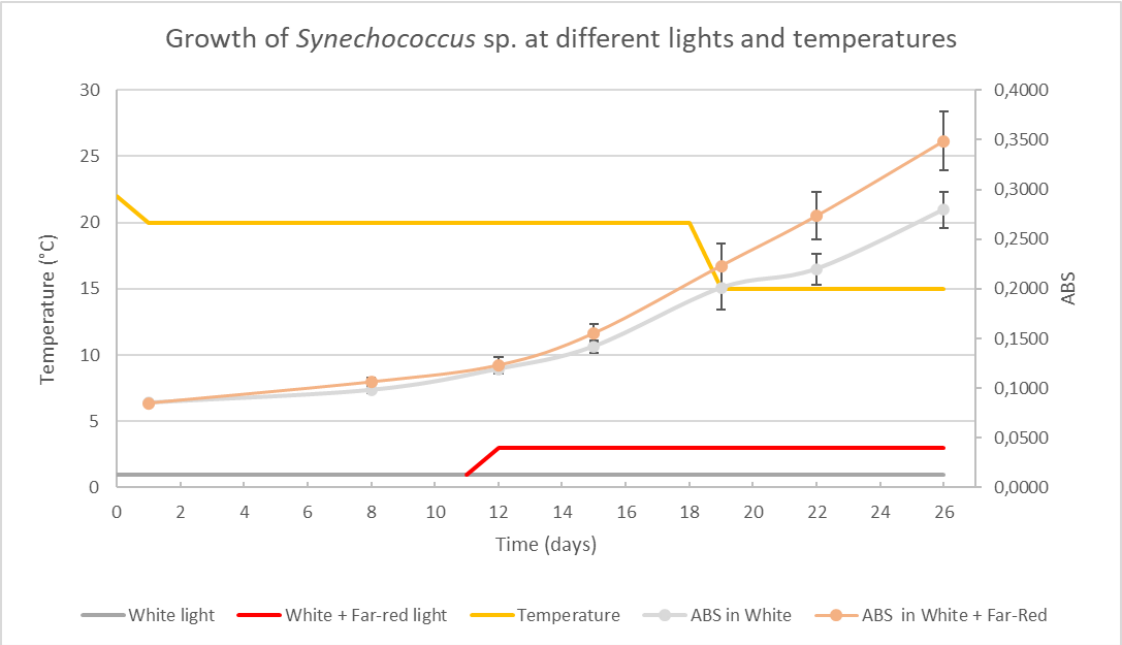

Figure 1 Growth of Synechococcus sp. at different light spectra and temperatures

Pigment and lipid composition

In our experimental setup, statistically significant changes in the concentration of Chl a was not observed. Slight differentiations could be found: at 20°C in WFR the level of Chl a was non significantly higher, in contrast, at 15°C we found higher Chl a level under W illumination. Similarly, in the levels of β-carotene statistically significant differences were not found. Highest level of Zeaxanthin was observed at 20°C in W. Significantly lower values were measured under WFR light at 20°C and in both lights at 15°C. In case of 9-cis-β-carotene at 15°C we found a statistically non-significant, but 15% differences according to the light treatments. The level of 9-cis-β-carotene was 15% higher under WFR illumination than under W light.

The most abundant lipids in our experiment were Monogalactosyl diglyceride (MGDG), Phosphatidylglycerol (PG), Sulfoquinovosyl diacylglycerol (SQDG) and Phosphatidylethanolamine (PE), these four are responsible for ~80% of the total lipids in average (Figure 2).

Two out of three examined glycolipids were dominant in our samples, only Digalactosyl diacylglycerols (DGDG) were presented in low amount The MGDG level was statistically significantly lower at 20°C in WFR compared to the W light, when the temperature was decreased to 15°C this difference caused by the light treatment has disappeared. At 15°C MGDG was responsible for the ~30% of the total measured lipids at both lights. The light treatment has not affected significantly the SQDG levels in our samples, however non-significant differences between the light treatments was found at 20°C, the SQDG content of WFR samples were ~2% higher compared to W. At 15°C the SQDG levels decreased significantly under both illuminations. Very small amount of DGDG was found, in average it was 1.6% in our samples.

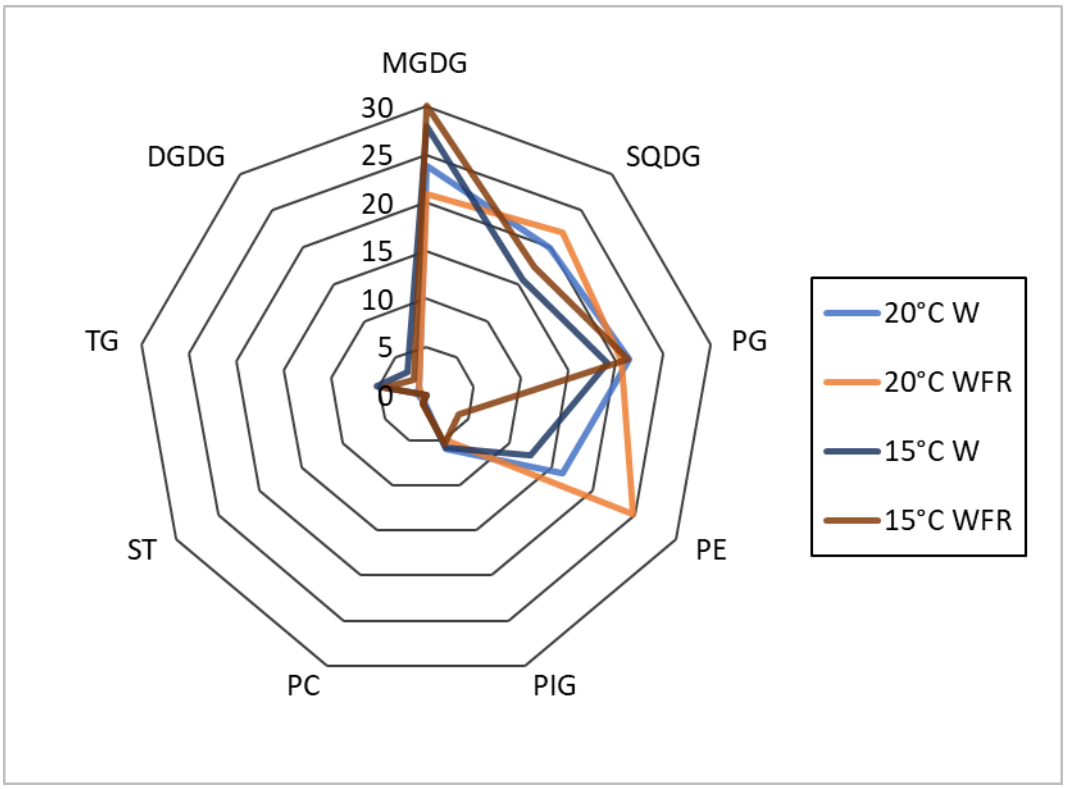

Figure 2 The relative amount (the values are given in % of the total lipid content) of the examined lipids at different light spectra and temperatures.

The MGDG level was statistically significantly lower at 20°C in WFR compared to the W light, when the temperature was decreased to 15°C this difference caused by the light treatment has disappeared. At 15°C MGDG was responsible for the ~30% of the total measured lipids at both lights. The light treatment has not affected significantly the SQDG levels in our samples, however non-significant differences between the light treatments was found at 20°C, the SQDG content of WFR samples were ~2% higher compared to W. At 15°C the SQDG levels decreased significantly under both illuminations. Very small amount of DGDG was found, in average it was 1.6% in our samples.

Out of the three examined Phospholipids also two were dominant and just Phosphatidylcholines (PC) were found in less amount. PG was responsible for ~20% of the total lipids in every sample, but did not show any light or temperature dependence. The level of PE was strongly affected by both light and temperature. At 20°C WFR light had statistically significant positive effect on the PE level, the light treatment increased the PE content with ~8% at 20°and WFR PE was the dominant lipid in our samples. In contrast, at 15°C the PE levels, both in W and WFR, has decreased compared to 20°C. At 15°C significant negative effect of WFR was observed. PC were found in our samples in low amount, however light treatment has significant effect: at both temperature WFR increased the level affected the amount of PC significantly.

The relative amount of the pigments was not affected significantly neither by the temperature nor by the light treatments, their average relative amount was 5.35% of the total lipids. Lower than 1% of the total lipids was provided by Triglycerides (TG) at 20°C. A remarkable increase to 5-6% in TG levels were found at 15°C, also a statistically non-significant, 1% difference was obtained between the two applied lights: at 15°C in W light the relative amount of TG was 6%

while in WFR light it was 5%. Small amount sterols (ST) were found only at 20°C, the WFR light reduced further amount of ST compared to W.

Table 1 Gene ID, description and pathway of the examined genes.

Gene ID	Description	Pathway
WH7803_0790	Photosystem II protein D1	Reference gene
WH7803_1539	possible processive Glycosyltransferase	Glycerolipid metabolism
WH7803_1831	Short-chain dehydrogenase/reductase	Glycerolipid metabolism
WH7803_0053	sulfolipid biosynthesis protein (UDP-sulfoquinovose synthase)	Glycerolipid metabolism
WH7803_0061	possible processive Glycosyltransferase	Glycerolipid metabolism
WH7803_2328	Diacylglycerol kinase	Glycerolipid metabolism
WH7803_0033	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	Fatty acid biosynthesis
WH7803_0196	3-oxoacyl-[acyl-carrier-protein] synthase II	Fatty acid biosynthesis
WH7803_0580	Long-chain acyl-CoA synthetase	Fatty acid biosynthesis
WH7803_1148	Acetyl-CoA carboxylase, beta subunit	Fatty acid biosynthesis
WH7803_1957	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	Fatty acid biosynthesis
WH7803_1861	3-oxoacyl-[acyl-carrier-protein] reductase	Fatty acid biosynthesis
WH7803_2128	Biotin carboxylase (A subunit of acetyl-CoA carboxylase)	Fatty acid biosynthesis
WH7803_2250	3-oxoacyl-[acyl-carrier-protein] synthase III	Fatty acid biosynthesis
WH7803_2251	Malonyl CoA-acyl carrier protein transacylase	Fatty acid biosynthesis

Low amount of lipid degradation indices was also found in our samples. Free fatty acids (FFA) were detected at 20°C (<1%), their amount was increased at 15°C to ~5%, their present was determined more by the temperature, whereas the light treatments not had significant effect. Some Fatty alcohols (ALC) also were found, but only in samples from 20°C, and WFR significantly decrease the amount of ALC compared to W light. Monoglycerides (MG) were also found, both temperature and light treatment had significant effect on the amount of MG. While at 20°C WFR significantly decreased the amount of MG, at 15°C WFR treatment resulted in a statistically non-significant increase of MG. The decrease of temperature increased the amount of MG regardless the light.

Gene expression changes

The changes of the expression of some glycerolipid metabolism- and fatty acid biosynthesis related genes were examined (Table 1). The gene expression changes of the examined genes showed similar pattern: at 20°C at the beginning of the light treatment differences between W and WFR was not found, the two exceptions were WH7803_0196 and WH7803_1148, where WFR increased the gene expression even after 1 hour. After 7 days of light treatments the examined genes showed similar pattern: the WFR increased the gene expression of all examined gene. At 15°C we found reduced levels of gene expression of the examined gene compared to

20°C. At 15°C WFR decreased the expression of all glycerolipid metabolism and fatty acid biosynthesis related examined genes. (Our plan is to submit a paper based on the above presented results the latest in March 2024.).

6. Functional role of MAP kinase signalling in photosynthesis and carotenoid metabolism in the green algal model species, *Chlamydomonas reinhardtii*

A unicellular plant model offers an efficient experimental system and an evolutionary framework to study cellular processes in the plant kingdom. The photosynthetic microalga *Chlamydomonas reinhardtii* is a suitable laboratory model species, and has been utilised to study photosynthesis, lipid biosynthesis or the cell cycle. Nevertheless, despite their importance and their increasing utilisation as a model system, very little is known about cellular signalling mechanisms in algae. Therefore, our goal is to functionally study mitogen-activated protein kinase (MAPK) genes in *Chlamydomonas*. The MAPK pathways are central regulatory mechanisms in all eukaryotes, they play key roles in regulating stress responses in plants.

Our work focused on revealing how a MAPK component affects the function and pigment content of photosynthetic apparatus in normal environmental light and in excess light (stress condition) in *C. reinhardtii*. In our experiments a *mpk8* strain was involved – an insertion mutant for CrMPK8 (Cre01.g010000, XP_001700291), a MAPK cascade component, most related to the best studied group of higher plant MAPKs (e.g. AtMPK3/6). Presence of the insert was confirmed at the expected site, where we could detect two insertion events in an inverted repeat arrangement, which certainly inhibits proper gene function. To ensure that the examined functions and features of the mutant strain are indeed caused by the loss of *MPK8* gene functions, the mutant was complemented. It was successfully transformed with a wild-type MPK8-containing plasmid construct by electroporation. Three positive clones, containing the transformed MPK8 gene construct were used in further experiments.

We first studied the impact of *mpk8* mutation on the growth rate and the Chl content of the cultures. Optical density at 720 and 680 nm was monitored for four days in WT and mutant cultures, under normal and high light conditions (120 and 900 µmol photon/m2/s). At 720 nm, we obtained the same sigmoidal curves for each setting, indicating that the mutation did not influence the overall vitality of the cells. We could observe just slight differences between the growth of WT and *mpk8* mutant in both normal and high light, after three days. At 680 nm, the OD increased faster at normal light than at high light intensities in case of both strains, however, the WT strain saturated at higher level and showed faster growth rate compared to the *mpk8* strain at the corresponding light intensities. Since changes of OD680 nm mainly reflect changes of chlorophyll content, while OD720 represents cell density, we can suppose that the cellular chlorophyll content decreased in *mpk8* mutant line. Chl a/b ratio also decreased in the mutant strain in comparison with the WT and complemented lines.

Western blot analysis of PSII and PSI core subunit proteins (D1 (PsbA), CP43, PSBO (PSII) and PsaA (PSI)) did not detect any significant difference in these protein amounts.

In order to study the effect of light stress on the function of the photosynthetic apparatus of the *mpk8* mutant, we exposed the cultures, grown at 120 µmol photon/m^2/s, to 900 µmol

photon/m^2/s white light illumination for 24 hours, then the photosynthetic parameters were compared between WT and *mpk8*. We estimated and compared various photosynthetic parameters by measuring the changes of Chl a fluorescence yield and by following the absorbance change at 820 nm at different light intensities, using a DUAL-PAM 100 setup. We also measured the kinetics of the fast fluorescence transient by Handy PEA instrument. The overall shape of the OJIP transient was just moderately affected by the presence of the *mpk8* mutation in both normal and high light-exposed cultures. The F_v/F_m which is an indicator of PSII maximal efficiency did not show significant differences. The capacity of qE type quenching that is a prominent form of NPQ proved to be very similar in both strains in the applied light intensity range. In line with the Chl a fluorescence transient measurements, this suggests that the *mpk8* mutation does not affect PSII photochemistry. We also could not find differences in the PSI yield at any examined light intensities.

The qT component of NPQ plays an important role in the regulation of light-harvesting in *C. reinhardtii*, due to state transition. We compared the capacity of qT between WT and the *mpk8* mutant strain. ST1 to ST2 transition was induced by anaerobiosis, ensured by the addition of glucose and glucose oxidase in the dark. ST1 was restored from ST2 by illumination (22 µmol photon/m^2/s). The kinetics of the state transitions in both directions was followed by measuring the changes in Chl a fluorescence yield. The difference between the F_m values, measured in ST1 and ST2 was larger in *mpk8* than in the WT. This difference is reflected by the corresponding NPQ, attributed to qT, calculated from F_m' and F_m" (Fig. 3 A).

Fluorescence changes due to state transition are also manifested in the fluorescence emission spectra, measured at 77K. In the low-temperature spectra the fluorescence – originated from PSI-associated chlorophylls – appears at around 710 nm, while the peaks around 685-695 nm can be assigned to the PSII core complexes, CP43 and CP47. During the ST1-ST2 transition we could observe opposite change in the PSII and PSI peaks. In ST2 – induced by dark anaerobiosis – the PSI peak was higher and the PSII peak was lower, than in ST1 (illuminated samples). This difference can be described by the change in the ratio of the PSI and PSII emission peaks. The PSI/PSII ratio increased more in the *mpk8* mutant than in the WT in ST2 (Table 1), in good agreement with the observed changes in the Chl a fluorescence yield, measured at room temperature.

Table 2. The ratio of PSI and PSII peaks of 77K fluorescence emission spectra of WT and mpk8 C. reinhardtii *strains, in ST1 (in light) and ST2 (dark, glucose/glucose oxidase). The emission of PSI is at 710 nm, while that of PSII is at 685 nm. Data points are means of 2 technical and 3 biological replicates ±SE. Significance levels of differences in means were determined by Tukey multiple comparison test (different letters designate significantly different means, p <0.1).*

	F710/F685 ± SE
WT ST1	1,15 ± 0,028 [a]
WT ST2	1,30 ± 0,013 [b]
***mpk8* ST1**	1,04 ± 0,110 [c]
***mpk8* ST2**	1,45 ± 0,023 [b]

Low-temperature fluorescence emission was also measured after high light treatment. The alga cultures grown at normal light intensity for three days were exposed to 900 µmol photon/m^2/s white light. The fluorescence emission spectrum of the WT strain did not change when exposed to high light for 24 hours (Fig. 3 B). In the case of the *mpk8* mutant strain, the ratio of PSI/PSII fluorescence emission peak decreased (Fig. 3 C), with a concomitant shift of the PSI peak to shorter wavelengths (Fig. 3 E). From the emission spectra recorded from the samples grown under low light and high light, a difference spectrum was calculated. It revealed that the decrease of PSI peak is accompanied by the emergence of a fluorescence peak at around 680 nm (Fig. 3 D), which can be assigned to a detached population of LHC.

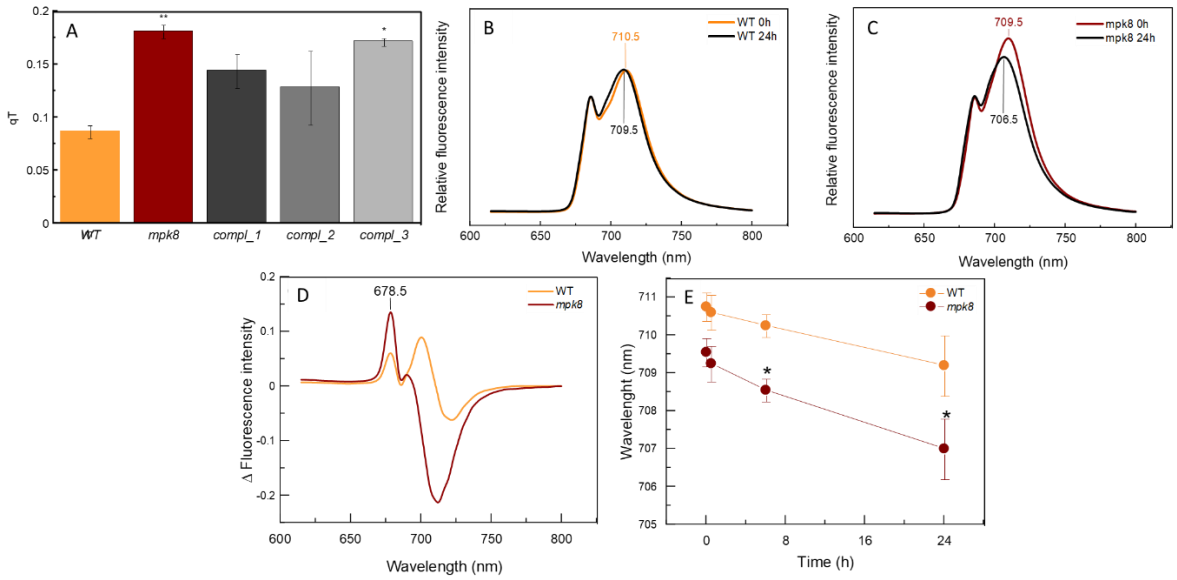

Figure 3 State transition of WT, mpk8*, and complemented strains; the NPQ of qT was calculated as (F_m'-F_m'')/F_m' (A). Significance levels of differences in means were determined by one-way ANOVA with Dunnet post hoc test using WT as control: *<0,1; **<0,05. The effect of high light on the 77 K fluorescence emission of WT and* mpk8 *strains (B-E). Cells were illuminated with white light (900 µmol photon/m^2/s) for 24 h and fluorescence emission spectra were measured in control conditions and 24 h after high light treatment. The WT (B) and* mpk8 *(A) samples had equivalent Chl content and their spectra were recorded at 436 nm excitation and were normalized to their maxima at 685 nm. Panel D shows the difference spectra of the 24 and 0 h measurements. The shift of PSI and PSII peaks of fluorescence emission spectra towards lower wavelengths in response to high light illumination is presented in panel E. Data points are representing the mean of 2 technical and 3 independent biological replicates ±SE. Level of significance of differences in means were determined by two-way mixed-designed ANOVA with Tukey post hoc multiple comparison test (* designate significantly different means between WT and* mpk8 *strains at the same time, p <0.1).*

Carotenoids are secondary metabolites that play crucial roles in protection against light and oxidative stress in green algae. To find out whether the absence of MPK8 and high-light stress cause changes in the carotenoid content and composition in *C. reinhardtii*, we performed HPLC

measurements on the WT and *mpk8* mutant strains after long-term (72 h) and short-term (30 m) high-light illumination (900 μmol photon/m^2/s).

Under low and moderate light intensities the major carotenoid components are β-carotene (β-Car), 9-cis-neoxanthin (Nx), lutein (Lut) and violaxanthin (Vx). β-Car content was lower while the Nx was higher in the *mpk8* strain relative to WT, while no differences in lutein content were detected in normal light (Fig. 4 A). In high light, the β-Car, Nx, and Lut exhibited the same level as in their corresponding normal light controls, while the acclimation to the long-term high light treatment resulted in the de-epoxidation of Vx to antheraxanthin (Ax) and zeaxanthin (Zx), via the xanthophyll cycle. Vx and Zx conversion appeared at a suppressed extent in the *mpk8* mutant (Fig. 4 B). When the cultures grown under normal light conditions were exposed to a short-term 900 μmol photon/m^2/s white light illumination, for 30 minutes, it was sufficient to achieve Vx de-epoxidation in both strains (Fig. 4 C, D). However, in *mpk8*, de-epoxidation was markedly retarded indicating a hampered xanthophyll cycle (Fig. 4 E, F). Other carotenoid components did not show differences under long- and short-term high light stress.

The xanthophyll cycle is activated by the trans-thylakoidal pH gradient. To investigate the possible difference in the ΔpH between the WT and the mutant, we measured the proton motive force (pmf) in the thylakoid, following the absorbance change at 515 nm. From the kinetic changes of the 515 signal, we could estimate the pmf and partition it into two components, ΔpH (proton gradient component) and ΔΨ (electric component). In the *mpk8* mutant, the pmf and its partitioning into ΔpH and ΔΨ did not change compared to WT.

CD spectroscopy is a non-invasive technique that proves information on the organization of the Chl chromophores bound to pigment-protein complexes, embedded in stacked thylakoid membranes. The Ψ-type CD bands of thylakoids, measured in *C. reinhardtii* intact cells can be found at around (+)693 nm and (–)676 nm. The main positive Ψ-type bands ((+)693 peak) have been shown to depend largely on the LHCII content of the membranes, while the (–)676 peak mainly depends on the grana stacking. The negative peak is profoundly suppressed while the (+)693 band slightly increased in the *mpk8* mutant in comparison to WT. The effect of *mpk8* mutation on the grana structure in *C. reinhardtii*, revealed by CD spectroscopy, was confirmed by transmission electron microscopy (TEM). Fig. 5 shows the TEM images of the WT and *mpk8* thylakoid membranes, after 30 minutes, 120 μmol photon/m^2/s white light illumination. A significant difference was observed in the thylakoid membrane repeat distance: it was reduced in the case of the *mpk8* mutant compared to the WT.

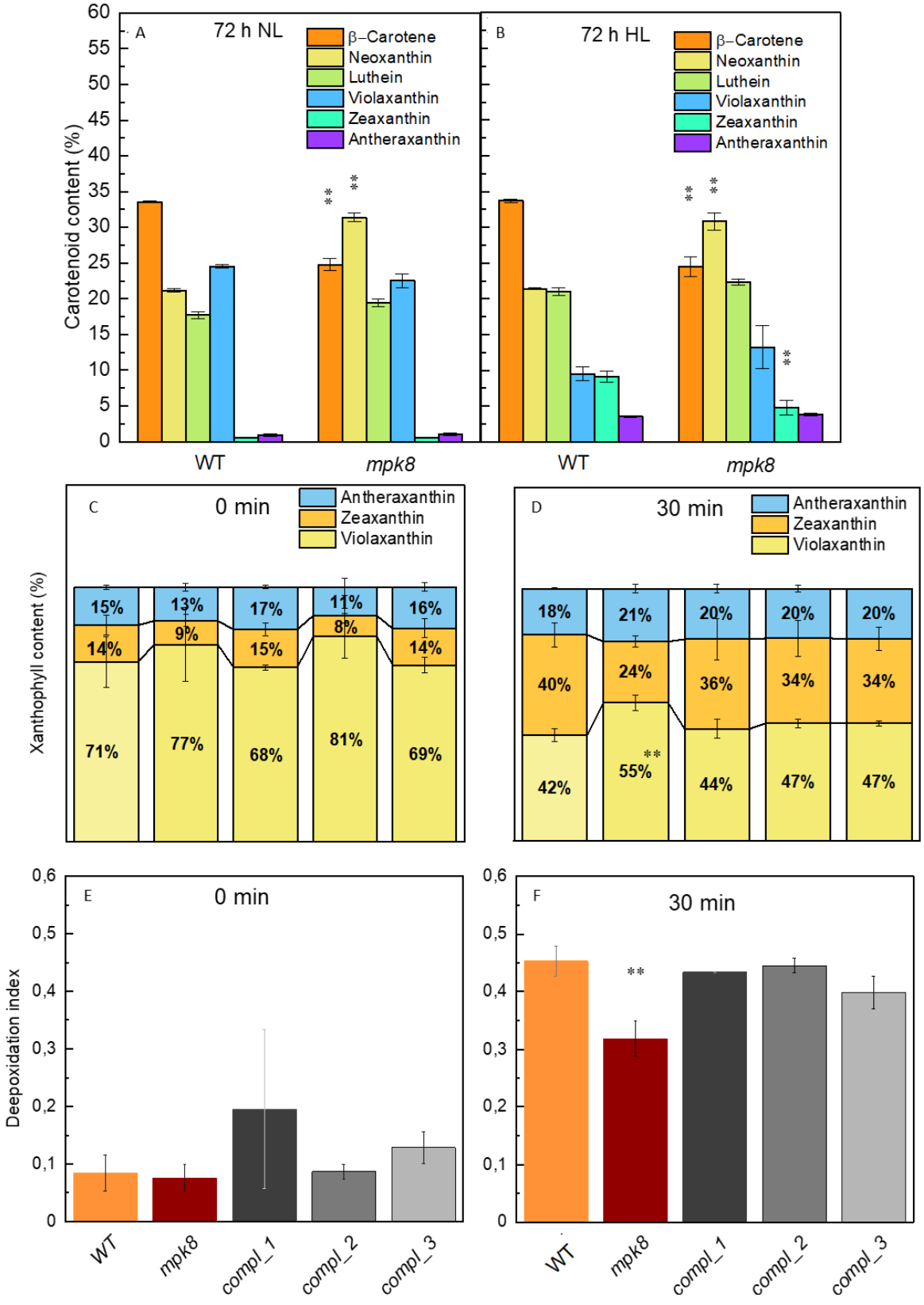

Figure 4. Carotenoid composition of WT and mpk8 C. reinhardtii *cultures grown at normal and high light. WT and mpk8 strains were grown under normal (NL) – 80 µmol photon/m²/s – (A), or at high white light (HL) – 900 µmol photon/m²/s – (B) illumination for 72 h. Data points are representing the mean of 3 independent biological replicates±SE. Significance levels of differences between WT and* mpk8*, measured at the same growth light intensity were determined by Student t-test (*<0.1, **<0.05,*

****<0.01). Amounts of xantophyll-cycle components and de-epoxidation index of WT,* mpk8 *and the MPK8 complemented strains of* C. reinhardtii*. Cultures grown at normal light intensity were exposed to 900 µmol photon/m2/s white light for 30 minutes. Antheraxanthin, zeaxanthin and violaxanthin content was determined by HPLC before (C) and after (D) light treatment. The corresponding de-epoxidation index was calculated as (Zea+0.5*Ant)/(Vio+Zea+Ant) (E, F). Data points represent the mean of 3-7 independent biological replicates ±SE. Significance levels of differences were determined by one-way ANOVA with Dunnett's post hoc test (**<0.05).*

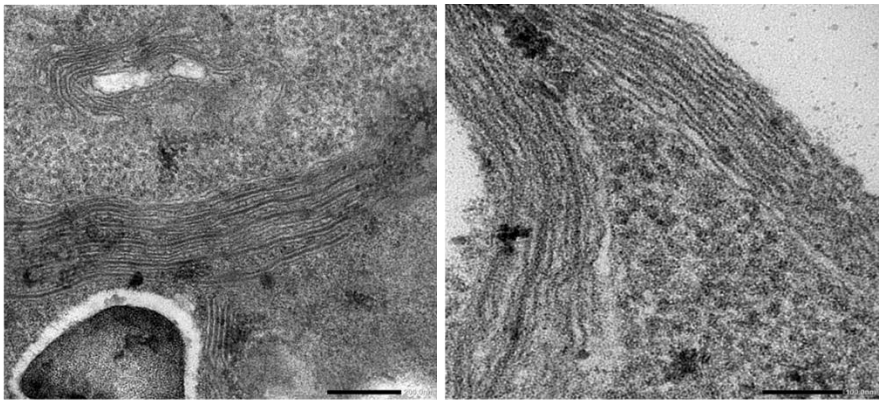

Figure 5. The ultra-structure of chloroplast granum of WT and mpk8 *strains. TEM images of thin-layer section of WT and* mpk8 *cells, grown at 120 µmol photon/m^2/s white light, were taken. The repeat distance (RD) of granum thylakoid membranes were determined by analysing digitalized pictures. RD values, indicated in the figure represent the mean of 100 technical and 2 independent biological replicates ±SE. Student's t-test showed significant difference between the mean of WT and mpk8 samples (p<0.1).*

To the best of our knowledge, this is the first functional characterization of a MAP kinase gene in the unicellular model green alga, *C. reinhardtii*. Our results reveal a role of this highly-conserved signalling mechanism in photosynthesis. Photosynthesis is the most distinctive characteristic of the plant kingdom, involvement of MAPK signalling in this process in algae represents a profound regulatory role in plant life. Moreover, carotenoids produced in algal species are highly relevant for biotechnology, thus our findings revealing the involvement of MPK8 in carotenoid metabolic processes warrant further studies. A manuscript presenting the above results is nearly complete and will be submitted to a high-impact journal shortly.