Short title: Light acclimation of *Chlamydomonas reinhardtii*

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Carbon supply and photoacclimation crosstalk in the green alga *Chlamydomonas reinhardtii*

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Summary: In this work we investigate high light acclimation strategies of *Chlamydomonas reinhardtii* cells grown in different carbon supply regimes.

Author contributions:

I.P and R.C planned and designed the research; I.P, R.F and E.D performed the experiments; I.P., R.F., P.C. and R.C analysed and interpreted the data; I.P and R.C. wrote the manuscript. All authors edited and commented the manuscript.

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Abstract:

Photosynthetic organisms are exposed to drastic changes in light conditions, which can affect their photosynthetic efficiency and can induce photodamage. To face these changes they have developed a series of acclimation mechanisms. In this work we have studied the acclimation strategies of *Chlamydomonas reinhardtii,* a model green alga that can grow using various carbon sources and it is thus an excellent system to study photosynthesis. Like other photosynthetic algae, it has evolved inducible mechanisms to adapt to conditions where carbon supply is limiting. We have analyzed how the carbon availability influences the composition and organization of the photosynthetic apparatus and the capacity of the cells to acclimate to different light conditions. Using electron microscopy, biochemical and fluorescence measurements, we show that differences in CO_2 availability do not only have a strong effect on the induction of the carbon concentrating mechanisms, but also change the acclimation strategy of the cells to light. For example, while cells in limiting CO_2 maintain a large antenna even in high light and switch on energy dissipative mechanisms, cells in high CO_2 reduce the amount of pigments per cells and the antenna size. Our results show the high plasticity of the photosynthetic apparatus of *Chlamydomonas reinhardtii*. This alga is able to use various photoacclimation strategies and the choice of which to activate strongly depend on the carbon availability.

Introduction:

Light sustains virtually all life on Earth through the process of photosynthesis. However, light can be very harmful for oxygenic photosynthetic organisms, as excess absorption can lead to the production of reactive oxygen species (ROS). In order to survive and grow, these organisms have developed various photoacclimation mechanisms operating on different time scales that protect the cell from photodamage. In the green alga *Chlamydomonas reinhardtii* (*C. reinhardtii*), these mechanisms vary from negative phototaxis and multicomponent non-photochemical quenching (NPQ), to a number of physiological and biochemical changes (Erickson et al., 2015). *C. reinhardtii* cells are around 10 μm in diameter, and more than half of their total volume is occupied by a single horseshoe-shaped chloroplast (Sager & Palade, 1957). The photosynthetic machinery responsible for the light reactions is located in thylakoid membranes and contains four major components: Photosystem II (PSII), cytochrome b_6f, Photosystem I (PSI) and ATP-synthase. Both PSs bind chlorophylls (Chls) and carotenoids (Car) and are composed of a core and several outer antennae pigment-protein complexes, the main function of which is light harvesting and its conversion into chemical energy. PSII core is composed of D1, D2, CP43 and CP47 pigment-protein complexes and several smaller subunits, the number of which varies between organisms (Shi et al., 2012). The outer antenna contains the light-harvesting complex II (LHCII), which in *C. reinhardtii* are encoded by nine LHCBM genes, and the minor antennae CP26 and CP29 (Nield and Kruse, 2000; Teramoto et al., 2001; Natali and Croce, 2015). These complexes are assembled together to form PSII-LHCII supercomplexes (Tokutsu et al., 2012; Drop et al., 2014). PSI core is composed of PsaA–PsaB heterodimer, and a number of smaller subunits (Jensen et al., 2007), and in *C. reinhardtii* the LHCI antenna consists of 9 Lhca proteins (Mozzo et al., 2010), which are associated with the core to form the PSI-LHCI complex (Stauber et al., 2009; Drop et al., 2011).

The composition and organization of the thylakoid membrane is light-dependent. Gene expression of different LHCs have been reported to be affected by light acclimation (Teramoto et al., 2002; Durnford et al., 2003; Yamano et al., 2008), and to be NAB1-regulated (Mussgnug et al., 2005). It has been observed that long term high light exposure of *C. reinhardtii* cells leads to a 50% decrease of Chl content (Neale and Melis, 1986; Bonente et al., 2012) and to changes in Chl to Car ratio (Niyogi et al., 1997a; Baroli et al., 2003; Bonente et al., 2012), suggesting

reduction of the antenna size (Neale & Melis, 1986), although, in a more recent report (Bonente et al., 2012), it was concluded that the antenna size is not modulated by light in this alga. Recently a dependence of the antenna components to the carbon availability was also reported. It was shown that when cells grown in acetate are shifted from high to low CO_2 concentration, the functional antenna size of PSII decreases and a downregulation of LHCBM6/8 occurs (Berger et al. 2014).

In short term, the main response to high light is the dissipation of energy absorbed in excess as heat in a process called qE, which is the fastest component of NPQ. In land plants the main player in this process is the protein PsbS (Li et al. 2002, 2004), while in *C. reinhardtii* the process is centered around LHCSR1 and LHCSR3 (Peers et al., 2009, Dinc et al., 2016). LHCSR3, the most studied of the two, is a pigment-protein complex that is expressed within one hour of high light exposure (Allorent et al., 2013) in combination with CO_2 limitation (Yamano et al., 2008, Muruyama et al., 2014). The qE onset is triggered by lumen acidification sensed by LHCSR3/1 (Bonente et al., 2011; Tokutsu & Minagawa, 2013; Liguori et al., 2013; Dinc et al., 2016).

Carotenoids are well known to be involved in photoprotection. They quench triplet chlorophyll (^{3}Chl*) and scavenge singlet oxygen ($^1O_2$) (Frank et al., 1996). In *C. reinhardtii* the antioxidant role of xanthophylls is well illustrated by the mutant (*npq1 lor1*) lacking lutein and zeaxanthin (Niyogi et al., 1997b). This mutant is deficient in qE, but compared with other qE-deficient mutants like npq4 (Peers et al., 2009) and npq5 (Elrad et al., 2002), which are LHCSR3 and LHCBM1 knock-outs, respectively, it is extremely light sensitive, due to the absence of quenching of ^{3}Chl* and $^1O_2$ by zeaxanthin and lutein.

Aquatic oxygenic photosynthetic organisms meet several challenges in CO_2 fixation (Moroney et al., 2007). First of all, the diffusion of CO_2 in water is 10,000 times slower than in air. Secondly, the CO_2 fixating enzyme ribulose bisphosphate carboxylase-oxygenase (Rubisco) is not selective for CO_2 and also binds O_2, resulting in the process of photorespiration. Thirdly, the form of inorganic carbon (C_i) depends on the pH, *i.e.* in alkaline pH it is HCO_3^-, while in acidic pH it is CO_2 (Beardall et al., 1981; Gehl et al., 1987). This diminishes even further the availability of CO_2 in the cell. In order to overcome these CO_2 fixation barriers, algae have developed Carbon Concentrating Mechanisms (CCMs) (Moroney et al., 2007). The essence of these processes lies

in the active pumping of C_i in the cell via a number of transporters that concentrate it in the pyrenoid, a ball-like structure containing Rubisco, Rubisco activase and intrapyrenoid thylakoids, and surrounded by a starch sheath. In the pyrenoid, HCO_3^- is converted to CO_2 by the carbonic anhydrase 3 (CAH3) (Sinetova et al., 2012; Blanco-Rivero et al., 2012), and then fixed by Rubisco in the Calvin-Benson-Bassham cycle. CAH3 is also suggested to provide HCO_3^- in the proximity of the oxygen evolving complex (OEC), where it may function as proton carrier, removing H^+ from water splitting to avoid photoinhibition (Villarejo et al., 2002; Shutova et al., 2008).

C.reinhardtii can also grow mixotrophically using alternative organic carbon sources present in its environment. For example it can uptake acetate, which is then incorporated into citric cycle producing reducing equivalents and CO_2 (Johnson & Alric, 2012) and into glyoxylate cycle producing malate (Plancke et al., 2012; Lauersen et al., 2016). In the presence of acetate, it has been reported that the CO_2 uptake and O_2 evolution were decreased by half, under saturating CO_2 and light intensities without affecting PSII efficiency, respiration and cell growth (Heifetz et al., 2000). In addition, reactions of the oxidative pentose phosphate and glycolysis pathways, inactive under phototrophic conditions, show substantial flux under mixotrophic conditions (Chapman et al., 2015). Furthermore, acetate can replace PSII–associated HCO_3^-, reducing the $^1O_2$ formation and, therefore, acting as a photoprotector during high light acclimation (Roach et al., 2013).

In short, high light acclimation is a complex, multicomponent process that happens on different timescales. Furthermore, it is embedded in the overall metabolic network and is potentially influenced by different nutrients and metabolic states. A thorough understanding of this process and its regulation is crucial for fundamental research and applications. To determine if different carbon supply conditions trigger different light acclimation strategies and photoprotective responses, we systematically studied *C. reinhardtii* cells grown in mixotrophic, photoautotrophic and high CO_2 photoautotrophic conditions in different light intensities.

We show that *C. reinhardtii* cells use different strategies to acclimate to high light depending on the carbon availability and trophic status. These results underline the strong connection between metabolism and light acclimation responses and reconcile the data from various reports.

Furthermore, our study demonstrates how in a dynamic system such as *C. reinhardtii* a single change in growth conditions has large effects at multiple levels.

Results:

Induction of Carbon Concentrating Mechanisms:

C. reinhardtii wild-type strain CC-124 were grown mixotrophically (M) in the presence of acetate (TAP medium), photoautotrophically (P) in ambient air (~400ppm CO_2), and photoautotrophically in ambient air supplemented with 5% CO_2 (CO2). To study high light acclimation, the cells were grown in 500 μE m^{-2} s^{-1} of white continuous light (hl) for more than 6 generations, while the control cells were kept in 50 μE m^{-2} s^{-1} (nl). To study possible morphological differences between the cells in the various conditions we analyzed them by transmission electron microscopy (TEM). The pictures are presented in Fig.1.

The pyrenoid was not formed in the presence of high CO_2 (Fig.1), while a clearly formed pyrenoid (P+, Fig.1) was observed in Mhl, Pnl and Phl. Interestingly, a well formed pyrenoid was absent in Mnl as can be inferred by the lack of the starch sheath that usually surrounds it. Starch sheaths around the pyrenoid were shown to become thicker under low CO_2 condition (Kuchitsu et al., 1988 ; Ramazanov et al., 1994), and together with a low carbon induced (LCI) LCIB–LCIC complex layer are hypothesized to prevent CO_2 leakage from the pyrenoid (Yamano et al., 2010). Another interesting observation is that most mitochondria were in a central position in CO2nl, CO2hl and Mnl, but in Mhl, Pnl, Phl they were located at the periphery of the cell in close contact with the chloroplast. It has been observed previously that in low CO_2 the mitochondria migrate from the central part of the cell to its periphery, and it was suggested that this migration happens to provide ATP for the active transport of HCO_3^- inside the cell (Geraghty and Spalding, 1996). These results thus suggest that CCM is induced in Mhl, Pnl and Phl, but not in Mnl, a condition in which the cells are apparently not strongly CO_2-limited. This conclusion is supported by the increase of carbonic anhydrase 3 (CAH3) expression level in Mhl, Pnl and Phl by 50%, 70% and more than 100% respectively, compared to Mnl (Fig. 2). In CO_2-enriched condition the CAH3 level was even lower than in Mnl.

Architecture of *Chlamydomonas* thylakoid membranes

In land plants the changes in membrane organization upon long-term acclimation to different light conditions are well documented (Anderson et al., 2012; Kirchhoff et al., 2013; Pribil et al., 2014), while little is known about about how algae adapt to light conditions with respect to membrane organization. We used transmission electron microscopy (TEM) to visualize the structural changes that *C. reinhardtii* cells undergo in various carbon supply and light conditions (Fig. 3). In all cells the thylakoids form stacks that run throughout the chloroplast, while very few interconnecting regions (stroma lamellae) are observed (Fig. 3A), unlike in land plants, where stacks of thylakoid discs (grana) and stroma lamellae regions are visible (Shimoni et al., 2005). The number of thylakoids (with enclosed lumen) per stack ranges from 2 to 12 (Fig. 3B). This is in agreement with previous reports from *C. reinhardtii* (Engel et al., 2015). Interestingly, we observed that the amount of thylakoids per stack decreases in high light, unlike in land plants (Anderson et al., 2012), and is influenced by carbon supply regime. In normal light, there are on average 5 thylakoid double membranes in mixotrophic grown cells and photoautotrophic cells, independently of the CO2 concentration. In high light, the amount of membranes per stack decreased differently: 43% for Mhl, 23% for Phl and 55% for CO2hl.

Doubling time, pigment content and maximal PSII quantum efficiency

The cell division time of *C. reinhardtii* depends on carbon source and light intensity (Table 1). For Mnl and CO2nl cells, we observed a 9 hours cell doubling time in agreement with the literature (Harris et al., 2008). Pnl and Phl cells divided around once per day, which is similar to a previous report as well (Bonente et al., 2012), while CO2hl cells divided every 5 hours. In summary, cell doubling time is faster in high light, especially in CO2hl, where growth is sustained by higher carbon fixation rate in the presence of elevated CO2 levels. In Table 1 the PSII maximal quantum efficiency (Fv/Fm) is reported. The highest values level in this condition were observed for CO2 cells both in nl and hl, while a lower value for Phl cells suggests an overall higher stress.

On the one hand, Chl content per cell decreased to half in high light in M and P, and in CO2 to one third compared to cells grown in normal light in the same carbon condition (Table 1). On the other hand, even in normal light, the Chl content differed depending on carbon supply, with the

highest in Mnl, 3.9 pg/cell, then Pnl, 2.6 pg/cell, and the lowest in CO2nl, 2.1 pg/cell. Therefore, our data show that the amount of Chl per cell varies not only depending on light intensity as shown before (Bonente et al., 2011; Neale & Melis, 1986), but also depending on trophic state and CO_2 availability.

The Chl *a* to *b* ratio is indicative of changes in the composition of the photosynthetic apparatus as Chl *b* is only present in the outer antennae. In normal light the Chl *a/b* ratio was 2.55-2.57 in all growth conditions (Table 1). High light acclimation affected the Chl *a/b* ratio in a different way depending on the carbon supply. While the Chl *a/b* was the same (2.55 - 2.57) in Pnl and Phl in agreement with previous data (Bonente et al., 2012), it increased in Mhl and CO2hl (2.9-3) compared to nl, suggesting that in these conditions the antenna size decreases as part of the strategy of high light acclimation. However, changes in Chl *a/b* ratio can reflect both changes in the antenna size of the PSs and in their ratio. To disentangle these effects, PSI/PSII was estimated based on the electrochromic shift (ECS) of the carotenoid's absorption (Bailleul et al., 2010) (Table 1). In Mnl cells the ratio between PSI and PSII RCs was close to 1 as reported before (Allorent et al., 2013). In Mhl PSI/PSII ratio was very similar (1.1), while it increased in P cells (nl, 1.7; hl, 1.5). In CO2nl cells, PSI/PSII ratio was similar to M cells (1.0) while it decreased in hl (0.6). It is interesting to observe that the changes in PSI/PSII ratio are far larger than what reported for plants (Anderson et al., 1995; Ballotari et al., 2007; Wientjes et al., 2013), indicating that in *C. reinhardtii* the photosynthetic apparatus is very flexible and can change drastically in response to the metabolic state of the cell.

To evaluate if the observed functional changes in PSI/PSII ratio are due to a change in the relative content of the photosystems or only to a change in their functionality, we performed immunoblotting on the total protein extracts using antibodies against PsaA (a subunit of PSI) and CP43 (PSII). The protein data show the same trend as the functional data, indicating that the changes in the PSI/PSII ratio are largely due to changes in the protein content (Fig. 4).

Combining Chl *a/b* and PSI/PSII, the number of LHCII trimers per PSII core monomer can be estimated (Table 1). According to our calculation (see M&M), in Mnl and CO2nl the LHCII/PSII ratio was around 5. A decrease in this ratio was observed upon high light acclimation although to a different extent, *i.e.* by less than 1 LHCII trimer in M (LHCII/PSII in Mhl = 4.3), and by 3 in CO2 (LHCII/PSII in CO2hl= 2.2). On the contrary, in Pnl and Phl the LHCII antenna was even

larger and did not change much under high light acclimation, 8 and 7.5 LHCII/PSII, respectively. Drop et al. (Drop et al., 2014), showed that the PSII monomer of *C. reinhardtii* can directly coordinate a maximum of 3 LHCIIs, leaving then many "extra" LHCII. This is especially true in Pnl and Phl conditions.

To support our calculations we have performed immunoblot analyzes using antibodies against LHC subunits, namely LHCBM1, LHCBM5, CP26 and CP29 (Fig. 5A). We present quantitative analyzes of these proteins in Fig. 5B. First of all, we observed that the amount of all four antenna proteins depends on both light and carbon availability. In Mhl the decrease of the antennae compared to normal light was very small for all the proteins except CP26. On the contrary, in high CO_2 the amount of all antenna complexes strongly decreased in hl. The situation was again different in P, which showed far higher levels of LHCBM1 in nl compared to all other conditions. In hl, no change in this complex was observed, while the amount of all other antennas decreased.

Carotenoid composition dependency on light and carbon availability

To determine the carotenoid involvement in high light acclimation in different carbon supply conditions, we analyzed the pigment extracts (Fig. 6). In normal light the carotenoid to chlorophyll ratio was independent of the carbon source and availability, with the exception of a slightly higher lutein and β-carotene content in Pnl. In high light, the carotenoid to chlorophyll ratio increased in all conditions due to a relative increase in lutein and violaxanthin+antheraxanthin+zeaxanthin. This increase was particularly remarkable in CO2hl cells (~4-fold increase) despite a strong reduction of the antenna size, which might indicate the presence of “free” xanthophylls in the membrane (also visible in the relative increase in β-carotene). At last, the deepoxidation state, calculated as the ratio between antheraxanthin+zeaxanthin and violaxanthin+antheraxanthin+zeaxanthin was higher in hl, the highest deepoxation state (~0.4) being observed in Phl (Fig.6B). Because deepoxidized forms of violaxanthin directly participate to photoprotection, this suggests a higher photoprotective capacity in Phl.

Non-photochemical quenching capacity

The above results show differences in long term high light acclimation strategies in different carbon supply conditions, suggesting possible differences in short term high light responses, namely NPQ. In order to systematically study the dependence of the heat dissipation capacity of the *C. reinhardtii* cells on the growth conditions, we measured NPQ induction and relaxation (Fig.7A). We observed that the NPQ induction also depends on both light and carbon supply conditions. The highest quenching is observed for cells grown photoautotrophically in high light (Phl), which is indeed the condition in which NPQ is normally monitored (Peers et al., 2009), and where the highest deepoxidation state of xanthophylls was observed. However, we also observed a buildup of NPQ in Pnl. In all other conditions NPQ was below 1 and did not fully relax in the dark suggesting the presence of long term quenching or photoinhibition. It has been shown that the fast rise of NPQ (qE) depends on LHCSR3, and this protein is expressed in high light and low CO_2 (Peers et al., 2009; Allorent et al., 2012; Maruyama et al., 2014). The LHCSR3 protein expression was then checked by immunoblotting (Fig. 7B). LHCSR3 was highly expressed in Phl, but it was also present in Pnl and Mhl cells, while it was not present in high CO_2, not even in high light.

LHCSR1 is another protein that was suggested to participate to NPQ (Peers et al., 2009, Berteotti et al., 2016) and was shown to induce a pH-dependent quenching in LHCII (Dinc et al., 2016). It has been shown at the RNA level LHCSR1 increases in medium light in high CO_2 and especially in high light and high CO_2 (Yamano et al., 2008). At a protein level though, our data showed that LHCSR1 is expressed in Mnl, CO2nl and in high light in all conditions and can be then probably responsible for the quenching observed in the absence of LHCSR3 (CO2hl) (Fig. 7).

Discussion:

C. reinhardtii is a model organism that can grow on different carbon sources. This metabolic flexibility is certainly an advantage for the study of photosynthesis because it permits the study of mutants that cannot perform photosynthesis (Harris et al., 2008). However, the carbon metabolism might affect the composition and functionality of the photosynthetic apparatus, making difficult the comparison of results obtained with cells grown in the presence of different carbon sources. To understand how the carbon supply influences the light reactions, we have

then studied long term (photosynthetic machinery remodeling) acclimation responses to light and the capacity of NPQ induction of *C. reinhardtii* cells grown mixotrophically in acetate and photoautotrophically in ambient CO_2 or CO_2-enriched conditions. In the following, we first discuss the activation of the CCM as a response to low CO_2 and how it is affected in the presence of acetate. Then, we compare the composition of the photosynthetic apparatus in the three different conditions under normal light and finally discuss the differences in the short and long term responses to high light.

CCM is not induced in cells growing in acetate in normal light.

We observed that the activation of CCM is lower in cells grown in the presence of acetate in low light (Mnl) than in cells grown in ambient CO_2 in low light (Pnl), high light (Phl), or high light in the presence of acetate (Mhl). Indeed CAH3 expression and pyrenoid formation in Mnl, although somewhat increased compared to high CO_2 conditions, were developed to a significantly lower extent than in Mhl, Pnl and Phl. Similarly, mitochondria migration towards the cell wall was observed in Pnl, Phl, Mhl, but not in Mnl. Altogether, these observations suggests that the cells in Mnl are not CO_2-limited. As the light intensity as well as the air ambient CO_2 concentration is the same in Pnl and Mnl, this absence of CO_2 limitation in Mnl cells is probably due to the increased CO_2 production by Krebs cycle activity in the mitochondria in the presence of acetate (Johnson and Alric, 2012), which in turn can be directly used by Rubisco in the chloroplast. On the other hand, in high light the amount of CO_2 produced in the mitochondria in M is insufficient to sustain photosynthesis as the cells switch on CCM.

The composition of the photosynthetic apparatus depends on the carbon availability

The composition of the photosynthetic apparatus in nl for cells that are not CO_2-limited (meaning M and CO2) is virtually identical in terms of antenna size, pigment composition, PSI/PSII ratio and NPQ capacity. The main difference is the amount of Chl per cell, which is much higher in M than in P and CO2. The higher number of thylakoids per stack in Mnl might partly accounts for this difference. It has been also reported that Chlamydomonas cells grown at 120 $\mu E * m^{-2} * s^{-1}$ are smaller in HSM medium (P) than in TAP medium (M) or HSM+CO_2 (CO2) (Fischer et al., 2006). In this respect, we cannot exclude the possibility that the cell volume is slightly different in our conditions, a 20% increase in cell diameter being enough to explain the difference in

chlorophyll content. In addition, it has been reported that in mixotrophic conditions PSII is more photoprotected than in photoautotrophic conditions showing less $^1O_2$ production (Roach et al., 2013). The decrease of $^1O_2$ under mixotrophic growth might be responsible to some extent for the overall higher Chl/cell content in mixotrophic compared to photoautotrophic conditions, as an accumulation of $^1O_2$ is one of the main triggers for the down regulation of the photosynthetic genes (Erickson et al., 2015).

Cells grown in P at ambient CO_2 showed instead a different composition of the photosynthetic apparatus. In particular, the relative amount of PSII to PSI was strongly reduced compared to the other conditions, while the antenna increased. It is plausible that this difference is related to the presence of CCM that results in a higher need for ATP, which is supported by a change in the ratio between linear and cyclic electron transport explaining the higher relative amount of PSI. Indeed, a 33% increased CEF was observed for cells grown in low CO_2, compared to the high CO_2 (Lucker et al., 2013) and it was also shown that the intrapyrenoid thylakoids are enriched in PSI (Gunning et al., 1999, Blanco-Rivera et al., 2012).

The higher amount of LHCII compared to the PSII core in P at ambient CO_2, indicates a larger antenna size for PSII in these conditions, although we cannot exclude that part of LHCII acts as an antenna of PSI as observed in plants (Wientjes et al., 2013). Interestingly, the higher amount of LHCII is mainly due to an increased amount of LHCBM1. The increase of LHCBM1 in those conditions is particularly interesting because this gene product was reported to be important for the process of NPQ (Elrad et al., 2002; Ferrante et al., 2012). Indeed, Pnl cells also express LHCSR3, which is another protein essential for NPQ (Peers et al., 2009). These cells are able to develop NPQ although at a lower level than high-light grown cells. Notably, the same light intensity does not lead to expression of LHCSR3 in M and CO2 conditions. This suggests that despite the presence of CCM in Mhl, the cells are already light-saturated.

The acclimation responses to high light

For *C. reinhardtii* cells grown photoautotrophically a decrease in chlorophyll content upon exposure to high light was reported before (Bonente et al., 2011; Neale & Melis, 1986; Durnford et al. 2003). Here we show that this decrease occurs in all conditions upon exposure to high light, independent of the carbon availability. This effect is very large in M and P, where the amount of

342 Chl is reduced to half of what was in normal light, but even more extreme in CO2, where only
343 30% of the Chls remain upon exposure to high light.

344 While the reduction of the Chl content seems to be a common strategy, the way this reduction is
345 achieved differs in the different conditions. It was previously shown that no reduction of the
346 antenna proteins occurs upon high light acclimation (Bonente et al., 2012), while another report
347 (Neale & Melis, 1986) showed 40% reduction of the functional PSII antennae size in high light.
348 Here we show that there is no disagreement between these results, as the LHCII reduction
349 depends on the growth conditions. We indeed do not observe reduction of the antenna size in
350 cells grown photoautotrophically at ambient CO_2, which is the condition used by Bonente and
351 coauthors, while a large reduction occurred in photoautotrophic growth at high CO_2, which is the
352 condition used by Neale and Melis. Upon exposure to high light, a reduction of the antenna size,
353 although smaller, was also observed in the presence of acetate.

354 CO2 cells also show a strong reduction in PSI/PSII ratio under high light, while this value
355 decreases only slightly in P at ambient CO_2 and even increases in M cells. These results seem to
356 correlate with the presence of CCM and the CO_2 availability. CO2 cells, which are not limited by
357 CO_2 and do not need investments for CO_2 concentration, acclimate to high light by strongly
358 modulating the amount of proteins and the composition of the photosynthetic apparatus. This
359 allows them to optimize the light usage and the result is a very fast growth and a high PSII
360 efficiency (Fv/Fm).

361 Cells in M and P instead show a much limited capacity of re-designing the photosynthetic
362 apparatus in response to light. In particular, the CO_2 limitation in photoautotrophic cells seems
363 to lead to a different high light acclimation strategy: even in high light Phl cells maintain a very
364 large antenna, which is in principle harmful because it can easily lead to overexcitation, but at
365 the same time express LHCSR3, thus activating dissipative processes. It is possible that the large
366 antenna is needed to support the relative low amount of PSII and the high energetic needs of the
367 cells to sustain CCM. In these conditions, it would then be more effective for the cell to
368 maintain a large antenna and to activate NPQ when needed. This is in agreement with the
369 increase in P cells of LHCBM1, the LHCBM subunit that was shown to be involved in NPQ
370 (Elrad et al. 2002)

The high light acclimation strategy of the cells growing in the presence of acetate also differs. In M conditions, the relative amount of PSI increases, probably to support CCM, which was not active in normal light. The antenna size decreases only slightly and NPQ is activated via the expression of LHCSR3, although at a lower level than in P cells.

Interestingly, a strong increase in the amount of xanthophylls with respect to Chls is observed in all conditions upon exposure to high light, not only in photoautotrophic conditions at ambient CO_2 concentration as shown before (Niyogi et al. 1997a; Baroli et al. 2003; Bonente et al. 2012), but also in high CO_2 and in the presence of acetate. This also means that the increase in xanthophylls is independent of the changes in the antenna size and it is indeed even more pronounced in CO2 cells, where the antenna is strongly reduced. This suggests that there is a large pool of xanthophylls free in the membrane, probably acting as singlet oxygen scavengers (Frank et al., 1996). The need for it seems to be higher in CO2hl cells, which perform a very low NPQ.

NPQ and the expression of LHCSR1 and LHCSR3

NPQ in *C. reinhardtii* was shown to depend on the LHCSR genes (Peers *et al*., 2009). Two LHCSR gene products are present in *C. reinhardtii* cells, LHCSR1 and LHCSR3. These two proteins have an high sequence identity, but their RNA transcript levels were shown to be controlled differently: LHCSR3 mRNA was shown to be expressed in high light in low CO_2, while LHCSR1 mRNA was expressed in medium light in high CO_2 and especially in high light and high CO_2 (Yamano et al., 2008). Here we show on the one hand that LHCSR1 seems to be constitutively expressed (with the exception of Pnl) although its expression level increases in high light, as previously reported at RNA level (Maruyama et al. 2014). On the other hand LHCSR3 protein is present in photoautotrophic conditions in ambient CO_2 but also in the presence of acetate when the cells are in CO_2-limitation (Mhl), a condition where CCM is induced. The amount of NPQ directly correlates with the amount of LHCSR3 in the cells which follows the trend: Phl>Pnl>Mhl. Conversely, LHCSR3 is absent in Mnl, where there is no CO_2-limitation. Interestingly, unlike its RNA (Yamano et al., 2008), the LHCSR3 protein is absent in CO2hl. The data at the protein level than show that the expression of LHCSR3 is not triggered directly by light, but rather by limitation in the energy usage.

Conclusions

This work shows that *C. reinhardtii* cells can use a large set of strategies to acclimate to long and short term changes in light intensity. The choice of which set of responses is activated depends on the availability of the carbon and the resources that need to be used for carbon concentration. This capacity makes *C. reinhardtii* an highly adaptable organism capable to grow in many different conditions. A summary of the different strategies is shown in Fig. 8.

It is particularly interesting to observe that the largest changes upon long term acclimation occur in phototrophic conditions in the presence of high CO_2 concentration. In these conditions, the cells are able to optimize the light use efficiency by strongly reducing their absorption cross section while at the same time avoiding quenching mechanisms. This is the strategy that has been proposed to increase the photosynthetic efficiency for algae growing in photobioreactors, a strategy that apparently *C. reinhardtii* have also evolved and one that can be optimized for maximal productivity.

Material and methods:

***Cell growth*:** *Chlamydomonas reinhardtii* wild type cells (CC-124) were acclimated to high light (hl = 500 μE* m^{-2}* s^{-1}) in mixotrophic (M), photoautotrophic (P) and photoautotrophic plus 5% CO_2 bubbled (CO2) culture conditions, and in normal light (nl = 50 μE* m^{-2}* s^{-1}) as a control. For CO2 cells pH was kept constant. The cells were grown for more than 6 generations in all conditions, to reach an acclimated state. Prior to that, cells were grown mixotrophically in Tris-Acetate-Phosphate medium (TAP) in nl, and collected in their exponential growth phase at the concentration of $6*10^6$cells/ml. Cells were pelleted down and resuspended in high salt medium (HSM) in case of photoautotrophic growth. In all conditions, cells were kept in exponential growth phase.

***PAM measurements*:** Fv/Fm was measured in parallel with NPQ using a Dual PAM-100 (Walz). Cells were dark adapted for 40 min before the measurements. TAP grown cells were resuspended in HSM before dark adaptation in order to remove acetate, which keeps the PQ pool reduced. NPQ measurement script recorded actinic-light-induced quenching of fluorescence

during 9 min and 7 min of quenching recovery after the actinic light was switched off (act. light – 500 μE* m^{-2}* s^{-1} ; sat. pulse – 3000 μE* m^{-2}* s^{-1}, 250msec pulse duration).

PSI/PSII measurements: PSI/PSII was measured as in Bailleul et al., 2010, using a JTS-10 pump-probe spectrophotometer. The measurement is based on the electrochromic shift (ECS) of carotenoid absorption caused by single charge separation. The 520nm values were corrected by subtracting the signal at 546nm, to avoid absorption changes caused by energization of the membrane. ECS was first recorded with both PSII and PSI active. Then, PSII was inactivated by applying a saturating pulse in the presence of 200 μM DCMU and 1 mM hydroxylamine (inhibitors of PSII at acceptor (Q_B site) and donor (oxygen evolving complex) sides (Negent et al., 2003), respectively). After that, PSI response was recorded. To correct for different electron turnover rate of PSII and PSI induced by a single flash of the xenon lamp, a correction factor of 1.6, obtained upon calibration of the xenon lamp with a laser, was used (Wientjes et al., 2011). Before the measurements the cells were dark adapted, and TAP grown cells were resuspended in HSM.

Pigment analysis: Cells were pelleted down at 2k *g* for 2min, 4 °C and the growth medium was removed and the cells were resuspended in the same volume of 80% acetone to extract the pigments. The samples were vortexed and the cell debris was pelleted down at 20k *g*, 2min, 4 C°. The pigment composition was analyzed by fitting the spectrum of the 80% acetone extracted pigments with the spectra of the individual pigments, and HPLC was performed as described previously (Croce et al., 2002).

Total protein extracts preparation and immunoblot analyzes: Total protein extracts were prepared as in Ramundo et al., 2013. Western blots were performed as in Dinc *et al*., 2014. The primary antibodies (Agrisera) were prepared in PBS salt solution, containing 5% non-fat dry milk (Elk) and 0.1% Tween-20 in following dilutions: CP43 (1:2500), PSAA (1:1000), LHCSR3 (1:1000), LHCBM5(1:5000), LHCBM1 (1:2000), CP29 (1:5000), CP26 (1:5000), CAH3 (1:2000). 10 μg of total protein extracts (TPE) was loaded per well. To correct for a loading error, protein densities were normalized to the total protein loading density of Ponceau Red stained nitrocellulose membranes and analyzed using GelPro31 software.

Calculation of PSII antenna sizes: LHCII antennae size calculation is based on Chl *a/b* ratio values obtained from the fitting of absorption spectra of 80% acetone extracts from the cells, and on the PSI/PSII ratio obtained by ECS measurements. We considered 35 Chl *a* per PSII monomeric core, 42 Chls (24 Chls *a* and 18 Chls *b*) for LHCII trimer (Liu et al., 2004), 226 Chls per PSI (Le Quiniou et al., 2015), 13 Chls per CP29 (9 Chls *a* and 4 Chls *b*) (Pan et al., 2011), and 12 Chls per CP26 (8 Chls *a* and 4 Chls *b*) as for a monomeric LHCII. CP29 and CP26 are always in 1 to 1 ratio with the core and we have assumed that no changes in the PSI/LHCI ratio occur in the different conditions. Moreover, a decrease in Lhca content would not affect the LHCII/PSII ratio dramatically because of a much larger Chl content in LHCII than in Lhcas.

Electron Microscopy: *C. reinhardtii* cells were diluted in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS, pH 7.4, for 1 h at room temperature then incubated at 4°C overnight. Then, 0.5% of tannic acid was added to the cells and incubated for an hour at room temperature. The cells were then washed five times in 0.1 M PBS buffer and post-fixed in a solution of 1% OsO_4 in PBS, pH 7.2–7.4. The combination of tannic acid/glutaraldehyde/paraformaldehyde followed by osmification increased the staining of the membranes. The samples were washed four times in sodium acetate buffer, pH 5.5, block-stained in 0.5% uranyl acetate in 0.1 M sodium acetate buffer, pH 5.5, for 12 h at 4°C. The samples were dehydrated in graded ethanol (50%, 75%, 95%, 100%, 100%, 100%) 10 minutes each, passed through propylene oxide, and infiltrated in mixtures of Epon 812 and propylene oxide 1:1 and then 2:1 for two hours each. The cells were infiltrated in pure Epon 812 overnight. Embedding was then performed in pure Epon 812 and curing was done in an oven at 60°C for 48 h. Sections of 60 nm thickness (gray interference color) were cut on an ultramicrotome (RMC MTX) using a diamond knife. The sections were deposited on single-hole grids coated with Formvar and carbon and double-stained in aqueous solutions of 8% uranyl acetate for 25 min at 60°C and lead citrate for 3 min at room temperature. Thin sections subsequently were examined with a 100CX JEOL electron microscope at different resolutions.

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Table 1

Properties of *Chlamydomonas reinhardtii* acclimated to different light and carbon supply regimes

	Doubling time (h)	Chl/cell (pg)	Chl *a/b*	PSI/PSII ECS	PSI/PSII WB	LHCII/PSII	Fv/Fm
Mnl	**9.0** ± 0.0	**3.9** ± 0.21	**2.57** ± 0.00	**1.05** ± 0.34	**1.00** ± 0.00	4.9	**0.71** ± 0.01
Mhl	**7.3** ± 0.4	**2.0** ± 0.11	**2.87** ± 0.03	**1.13** ± 0.02	**1.35** ± 0.30	4.3	**0.70** ± 0.02
Pnl	**25.0** ± 1.4	**2.6** ± 0.02	**2.55** ± 0.03	**1.67** ± 0.11	**1.95** ± 0.31	8	**0.68** ± 0.01
Phl	**22.5** ± 2.1	**1.3** ± 0.02	**2.57** ± 0.02	**1.54** ± 0.58	**1.65** ± 0.28	7.5	**0.59** ± 0.00
CO2nl	**8.8** ± 0.4	**2.1** ± 0.12	**2.56** ± 0.05	**0.96** ± 0.04	**1.04** ± 0.17	4.9	**0.75** ± 0.01
CO2hl	**4.6** ± 0.5	**0.6** ± 0.02	**3.00** ± 0.05	**0.62** ± 0.04	**0.66** ± 0.13	2.2	**0.76** ± 0.01

Table 1. Changes in *Chlamydomonas reinhardtii* phenotype upon acclimation to different light and carbon supply regimes: M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO_2, nl – normal and hl – high light. 1) Cell number doubling time in hours. 2) Chlorophyll content (in picograms) per cell. 3) Chl a/b ratio calculated from the fitting of absorption spectra of 80% acetone extracted pigments form cells. 4) PSI/PSII ratio, measured based on the ECS signal. 5) PSI/PSII ratio, obtained by immunoblot quantification of PsaA and CP43. 6) LHCII/PSII monomer calculations based on Chl a/b and PSI/PSII data, as described in M&M. 7) Maximal quantum efficiency of PSII (Fv/Fm). The data is averaged and standard error (SE) is derived from a minimum of 2 biological replicas, each with 3 technical replicas.

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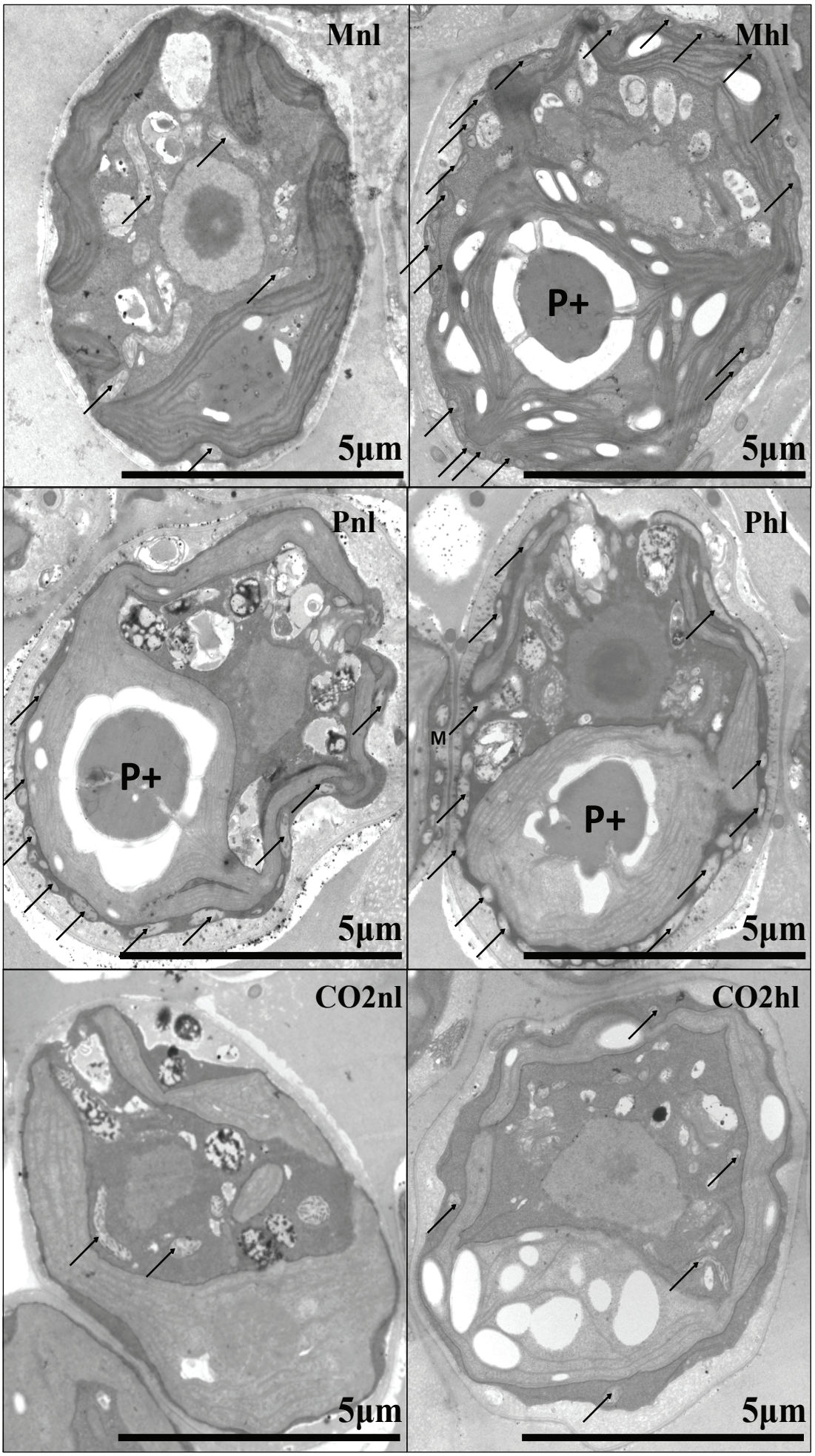
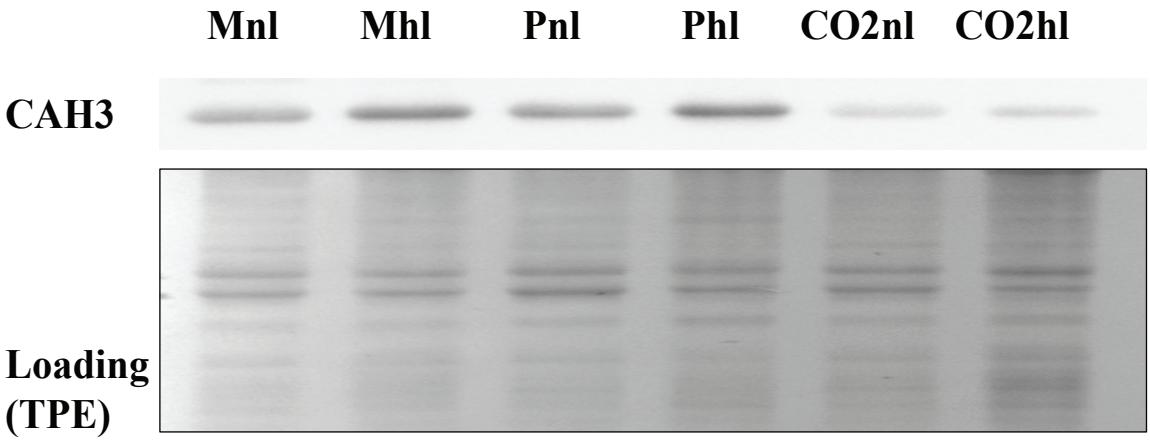


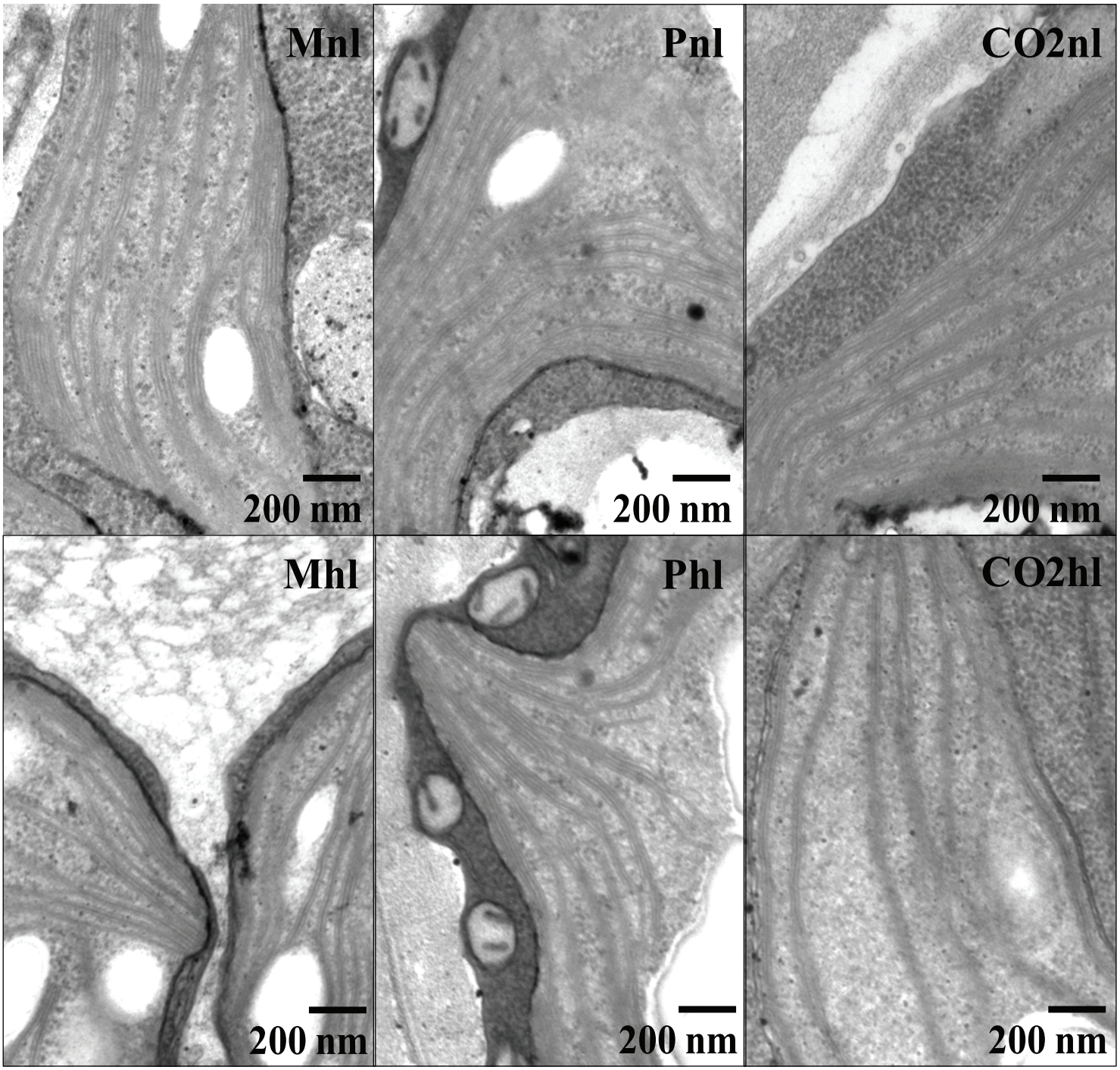
Figure 1. Transmission Electron Microscopy images of the Chlamydomonas reinhardtii cells grown in different carbon and light regimes. M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light. P+ shows a well formed pyrenoid, arrows indicate mitochondria.



	Mnl	Mhl	Pnl	Phl	CO2nl	CO2hl
CAH3/TPE	1	**1.54** ± 0.05	**1.70** ± 0.13	**2.15** ± 0.34	**0.82** ± 0.14	**0.53** ± 0.09

Figure 2. Expression of the carbonic anhydrase 3. M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light. CAH3 expression was determined on total protein extracts from cells. 10 µg of proteins– were loaded in each well. For quantitative densitometry analysis, CAH3 signal was normalized to the total protein loading to correct for possible loading errors. For digital analysis Gel-Pro software was used. The quantitative data was obtained from a minimum of 2 biological replicas and 3 technical repetitions.

A



B

	Mnl	Mhl	Pnl	Phl	CO2nl	CO2hl
Thyl/stack	**4.7** ± 0.22	**2.7** ± 0.11	**4.0** ± 0.21	**3.1** ± 0.12	**4.0** ± 0.20	**1.8** ± 0.05

Figure 3. Changes of thylakoid stacking in Chlamydomonas reinhardtii cell grown in different carbon supply and light regimes. M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light. A. Electron microscopy pictures, recorded under 60K magnification, with 200nm scale bar. B. Thylakoid stacks number (double layers with enclosed lumen), counted from a minimum of 50 different stacks from at least 5 different C. reinhardtii cells.

	Mnl	Mhl	Pnl	Phl	CO2nl	CO2hl
PsaA/CP43	1	**1.35** ± 0.30	**1.95** ± 0.31	**1.65** ± 0.28	**1.04** ± 0.17	**0.66** ± 0.13
PsaA						
CP43						

Figure 4. PSI/PSII ration changes in Chlamydomonas reinhardtii cell grown in different carbon supply and light regimes. M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light. PSI/PSII obtained by immunoblot quantification of PsaA and CP43. The data is averaged and standard error (SE) is derived from 2 biological replicas, each with 3 technical replicas.

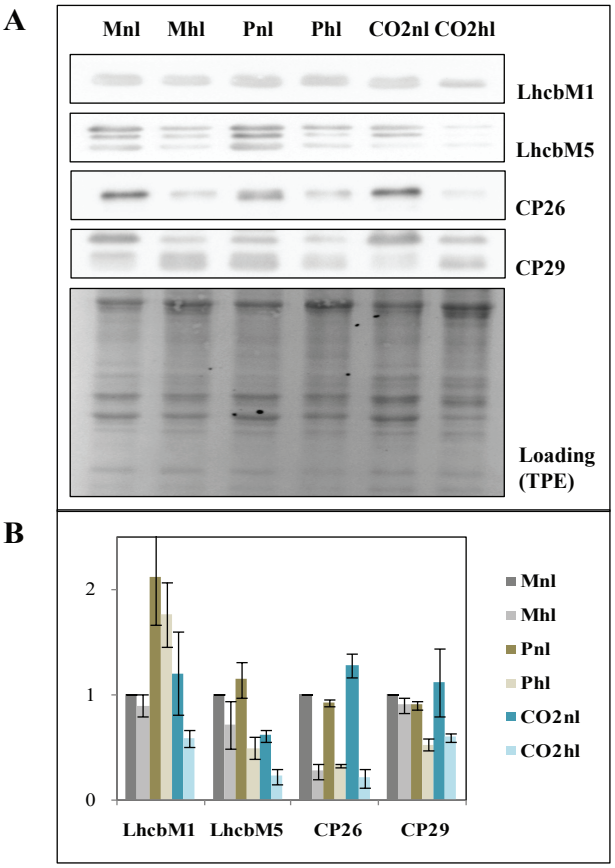
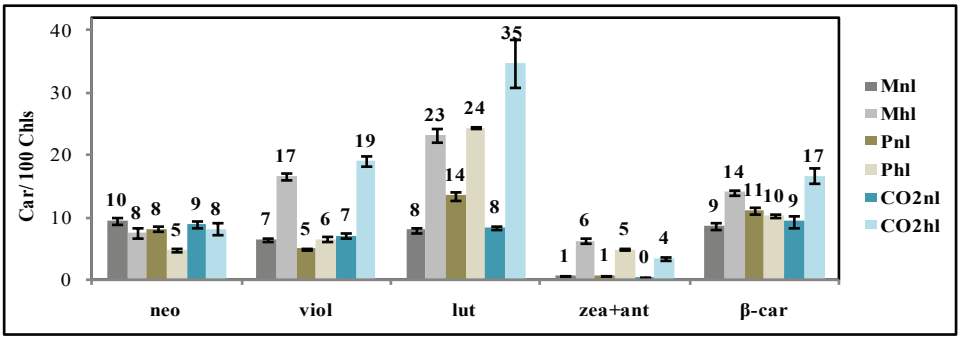


Figure 5. PSII antennae size alteration during Chlamydomonas reinhardtii light acclimation in different carbon supply regimes. M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light. A. Immunoblot data of PSII antennae proteins LHCBM1, LHCBM5, CP26 and CP29. 10µg of the total protein extract was loaded per well. B. Densitometry analysis of LHCBM1, LHCBM5, CP26, and CP29, normalized to loading, averaged and SE derived from 2 biological replicas, 3 repetitions each.

A

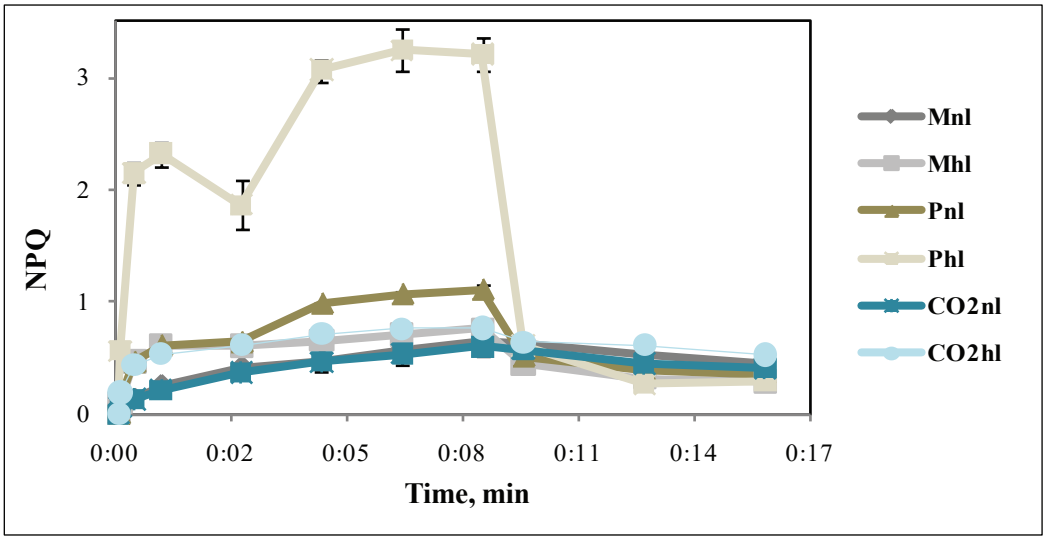


B

	Mnl	Mhl	Pnl	Phl	CO2nl	CO2hl
Deepoxidation state	0.087	0.275	0.122	0.429	0.064	0.156

Figure 6. Carotenoid content changes within Chlamydomonas reinhardtii cells acclimated to different carbon and light supply regimes. M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light. A. Amount of individual carotenoids normalized to 100 Chls, based on HPLC analysis. Standard error is indicated. B. Deepoxidation state

A



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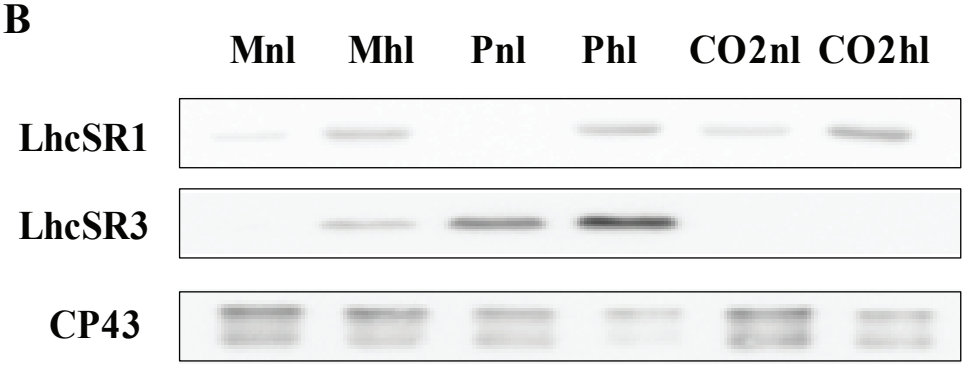

	Mnl	Mhl	Pnl	Phl	CO2nl	CO2hl
LhcSR1	**0.17** ± 0.02	**1**	0	**1.74** ±0.21	**0.35** ±0.05	**2.15** ±0.25
LhcSR3	0	**1**	**2.05** ±0.10	**10.5** ± 4.53	0	0

Figure 7. Changes in Chlamydomonas reinhardtii' non-photochemical quenching capacity upon different acclimations. M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light. A. NPQ measured on DualPAM-100. B. Immunoblotting detection of LHCSR1, LHCSR3 and CP43, 10µg per well of total protein extract from different cell samples is loaded.

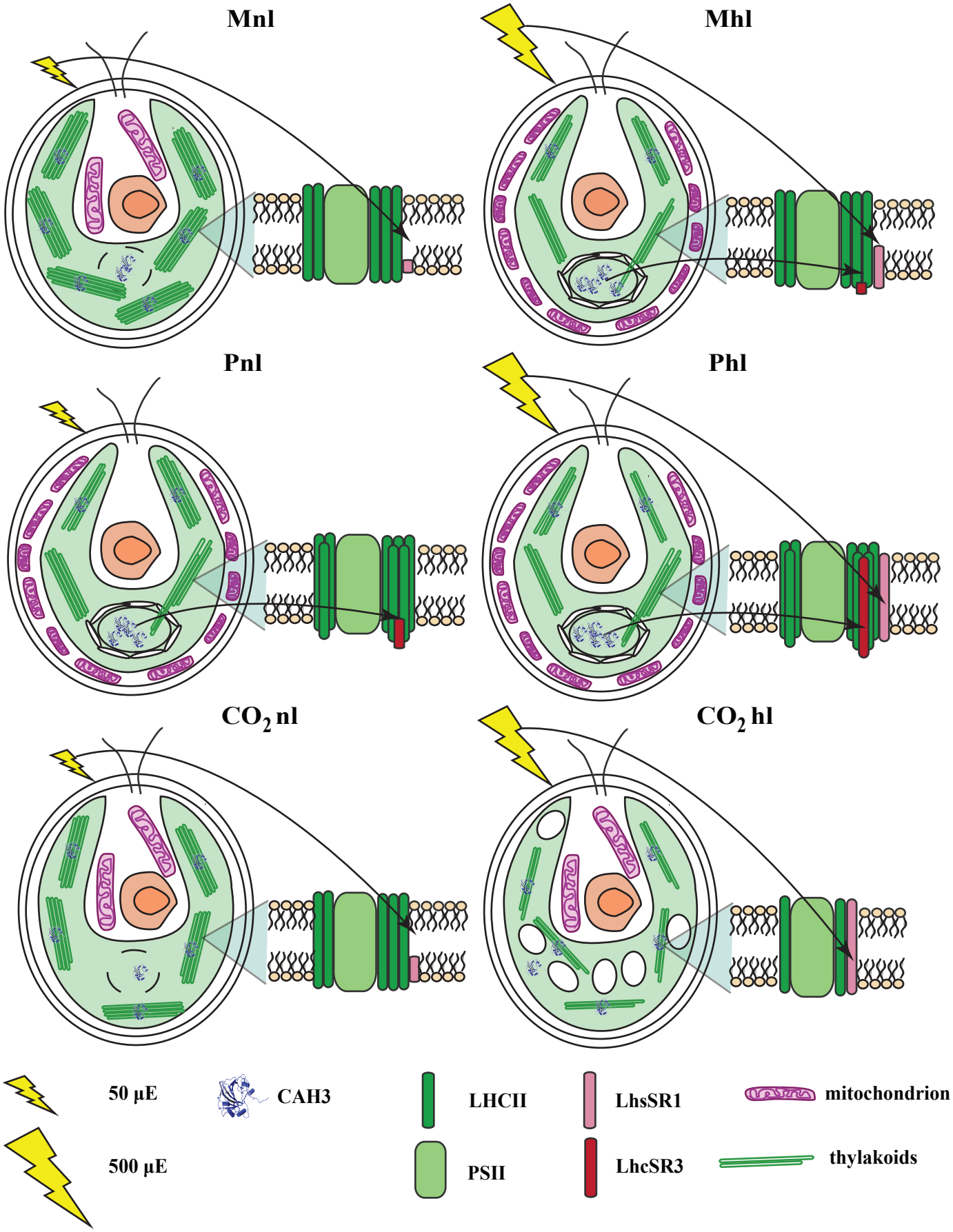

Figure 8. Carbon supply and PSII Photoacclimation crosstalk model in Chlamydomonas reinhardtii.
M – mixotrophic, P – photoautotrophic, CO2 – photoautotrophic with 5% CO2, nl – normal and hl – high light.

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