CO₂ supply modulates lipid remodelling, photosynthetic and respiratory activities in *Chlorella* species

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Abstract

Microalgae represent a potential solution to reduce CO₂ emission exploiting their photosynthetic activity. Here, the physiologic and metabolic responses at the base of CO₂ assimilation were investigated in conditions of high or low CO₂ availability in two of the most promising algae species for industrial cultivation, *Chlorella sorokiniana* and *Chlorella vulgaris*. In both species, high CO₂ availability increased biomass accumulation with specific increase of triacylglycerols in *C. vulgaris* and polar lipids and proteins in *C. sorokiniana*. Moreover, high CO₂ availability caused only in *C. vulgaris* a reduced NAD(P)H/NADP⁺ ratio and reduced mitochondrial respiration, suggesting a CO₂ dependent increase of reducing power consumption in the chloroplast, which in turn influences the redox state of the mitochondria. Several rearrangements of the photosynthetic machinery were observed in both species, differing from those described for the model organism *Chlamydomonas reinhardtii*, where adaptation to carbon availability is mainly controlled by the translational repressor NAB1. NAB1 homologous protein could be identified only in *C. vulgaris* but lacked the regulation mechanisms previously described in *C. reinhardtii*. Acclimation strategies to cope with a fluctuating inorganic carbon supply are thus diverse among green microalgae, and these results suggest new biotechnological strategies to boost CO₂ fixation.

KEYWORDS

carbon assimilation, *chlorella*, *Chlorophyta*, lipids, microalgae, photosynthesis, respiration, triacylglycerols

1 | INTRODUCTION

Microalgae emit half of the oxygen available in the atmosphere and contribute to half of the total organic carbon produced worldwide (Li-Beisson, Thelen, Fedosejevs, & Harwood, 2019). Thanks to the photosynthetic process, algae convert light energy into chemical energy to fix CO₂ in organic compounds. CO₂ is one of the main greenhouse gases responsible for global warming. CO₂ concentration in the atmosphere is constantly increasing reaching 407.4 ± 0.1 ppm for 2018, an

increase of 2.4 ± 0.1 ppm from 2017 (Dlugokencky et al., 2019). There is an urgent need for an efficient way to reduce the global carbon footprint, which is fundamental to reduce the effects of human activity in the worldwide poise.

Microalgae are emerging as a possible solution due to their ability to grow at high levels of CO₂ and to produce biomass that can be exploited for several applications: as food or feed supplement, biofuels or to produce high value products. Moreover, these photosynthetic organisms do not require arable land, have a fast growth

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rate and waste products as well as wastewater-derived effluent can be used as fertilizers for their cultivation (Lum, Kim, & Lei, 2013).

Light is harvested in the microalgal chloroplast by pigment binding protein complexes called Photosystem I (PSI) and II (PSII). These complexes are composed of a core complex, where photochemical reactions occur, and an external antenna system, which increases light harvesting efficiency and where several photoprotective reactions occur (Gao, Wang, Yuan, & Feng, 2018; Pan, Cao, Su, Liu, & Li, 2020). In oxygenic photosynthetic organisms, as eukaryotic microalgae, PSI and PSII work in series to strip electrons from water and transfer them to NADP⁺ producing NADPH. During this linear electron transport, protons are pumped from stroma to the lumen generating an electrochemical gradient used by ATPase to synthesize ATP. ATP and NADPH are then used by the Calvin Benson cycle to fix CO₂ into sugars. In parallel, another electron transport chain takes place in mitochondria, consuming oxygen and NADH and releasing NAD⁺ and ATP. A constant balance between chloroplast and mitochondrial activity is fundamental for cell survival and for adaptation to fluctuating environmental conditions.

It is important to point out that CO₂ diffusion in the water environments, where microalgae live, is strongly reduced compared to CO₂ diffusion in air. CO₂-limitation is known to reduce the consumption of ATP and NADPH by the Calvin Benson cycle leading to an over-reduced photosynthetic electron transport chain, which could potentially lead to oxidative stress (Y. Wang, Stessman, & Spalding, 2015). For this reason several microalgae species evolved an efficient system to enrich the CO₂ level inside the cell, that is, Carbon Concentrating Mechanism (CCM), a complex mechanism by which inorganic carbon is actively transported close to the enzyme responsible for its fixation, that is, the Ribulose-1,5-bisphosphate carboxylase-oxygenase (RUBISCO) enzyme (Y. Wang et al., 2015). The CCM mechanism is induced by low CO₂ concentrations (air level or lower) (Y. Wang et al., 2015). CO₂ availability plays a critical role in modulating photosynthetic efficiency and biomass accumulation in microalgal cultures. For example, in the model green alga *Chlamydomonas reinhardtii*, CO₂ has been reported to act as a molecular switch inducing a complex network of cell adaptation mechanisms including a translational up-regulation in the formation of PSII antenna complexes (Berger et al., 2014, 2016; Blifernez-Klassen et al., 2011; Mussnug et al., 2005; Wobbe et al., 2009). In conditions of low CO₂ availability, accumulation of the cytosolic RNA-binding protein NAB1 is triggered by the transcription factor LCRF (Low Carbon dioxide Response Factor) (Blifernez-Klassen et al., 2011). NAB1 then represses the translation of transcripts encoding light-harvesting antenna proteins (Berger et al., 2014; Mussnug et al., 2005). The translation repressor activity of NAB1 is controlled by two independent mechanisms related to the methylation of Arg90 and Arg92 residues (Blifernez, Wobbe, Niehaus, & Kruse, 2011) and to the redox state of Cys181 and Cys226 residues (Wobbe et al., 2009). NAB1 is highly active in the methylated state, while reduced Cys181 and reduced Cys226 are required for NAB1 RNA-binding activity. Nitrosylation of Cys181 and Cys226 has also been reported to inhibit the RNA binding activity of NAB1 (Berger et al., 2016). The truncation of the PSII antenna

reduces the excitation pressure on the photosynthetic apparatus as a response to diminished CO₂ availability (Berger et al., 2014). Accumulation of NAB1 and its post-translational regulation have been demonstrated to be finely tuned as an acclimation mechanism to different environmental conditions, including varying CO₂ availability (Berger et al., 2014; Berger et al., 2016; Wobbe et al., 2009).

Among microalgae species discovered, *Trebouxiophyceae* represent an evolutionary defined class of green algae (*Chlorophyta*) comprising the green freshwater algae of the *Chlorella* genus, one of the first microalgae to be cultured on a large scale due to their easy cultivation and high resistance to stresses (Borowitzka, 2018; B. Yang, Liu, Jiang, & Chen, 2016). Species belonging to the *Trebouxiophyceae* class are evolutionarily separated from the model species of green alga, *C. reinhardtii*, belonging to the *Chlorophyceae* class. *Chlorella* species are interesting for industrial cultivation, being reported to rapidly accumulate biomass containing high lipid, protein, carotenoid and vitamin amounts (Camacho, Macedo, & Malcata, 2019; Cecchin et al., 2019; Li, Zheng, Yu, & Chen, 2013; Lum et al., 2013; Sarayloo et al., 2017; Treves et al., 2013). However, the lack of genetic resources and the low efficiency of transformation methods have limited the development of genetic engineering in these species (Cecchin et al., 2019; Lin, Tan, Hsiang, Sung, & Ng, 2019).

In this work two *Chlorella* species, *Chlorella sorokiniana* and *Chlorella vulgaris*, were investigated for their physiologic and metabolic responses to a fluctuating CO₂ availability. *Chlorella sorokiniana* and *C. vulgaris* were chosen among other *Chlorella* species being already selected as those with the highest biomass accumulation potential (Kobayashi et al., 2015). Our results highlighted contrasting metabolic responses to CO₂ availability among green algae.

2 | RESULTS

2.1 | Species of the genus *chlorella* adjust their carbon flow differentially in response to high CO₂ availability

Chlorella sorokiniana and *C. vulgaris* cells were grown in 80 ml batch airlift photobioreactors bubbled with air (CO₂ concentration of ~0.04%, defined as AIR condition throughout the manuscript) or air enriched with 3% of CO₂ (defined as CO₂ condition throughout the manuscript). *Chlorella* strains were cultivated at 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ until the saturation phase was reached. Growth kinetics were followed by measuring the optical density (OD) at 720 nm and fitted with sigmoidal function as showed in Figure 1a,b. In both species, the increased CO₂ concentration induced a faster growth rate, as highlighted by the first derivative of the growth curves (Figure 1c,d). Total biomass production was increased in CO₂ compared to AIR condition by 253% $\pm$ 52% in *C. sorokiniana* and 269% $\pm$ 13% in *C. vulgaris* (Figure 1e,f). Moreover, ~4-fold increase in maximal daily productivities was observed for both species in CO₂ condition. These data confirmed that in the cultivation conditions applied carbon fixation processes were stimulated in *Chlorella* species by high CO₂ suggesting

FIGURE 1 Growth curve and biomass productivity in AIR versus CO₂. Growth curve and biomass productivity are reported for *C. sorokiniana* in panel a, c, e and for *C. vulgaris* in panel b, d, f in AIR condition (□ 0.04% CO₂) compared to CO₂ condition (3% CO₂). (a, b): growth curve obtained measuring OD at 720 nm fitted with sigmoidal function. (c, d): first derivative of growth curves reported in panels a and b. (e, f) Dry weight (g/L), average and maximum daily productivity (g/L day⁻¹) obtained harvesting the biomass at the end of the growth curve. Data are means of four replicates and error bars represent standard deviation [Colour figure can be viewed at wileyonlinelibrary.com]

a possible carbon-limitation in AIR condition. Interestingly a reduction of dry biomass per cell was evident in both *C. sorokiniana* and *C. vulgaris* in CO₂ condition, with a stronger decrease compared to the AIR condition in the latter species (Figure S1).

Biomass composition at the end of the growth curves was evaluated, and this revealed a significant increase in total lipids in both species, while different effects were observed on protein and starch content per dry weight (Figure 2). Indeed, CO₂ condition triggered an accumulation of proteins per dry weight in *C. sorokiniana* with the amount of starch not being altered. In *C. vulgaris*, protein amounts remained stable, but a decrease in starch content per dry weight was observed. On a cell basis, despite the reduction of dry biomass per cell in CO₂ conditions compared to AIR in both *C. sorokiniana* and *C. vulgaris* (Figure S1), a slight increase of protein and lipid accumulation were measured in *C. sorokiniana* cells grown at high CO₂ availability, while only lipids remained constant in *C. vulgaris* in both AIR or CO₂ conditions (Figure S2). This suggests a different behaviour of the two species in macromolecule accumulation upon increased carbon availability underlying potential differences in metabolic rearrangement.

Fatty acid compositional and lipid class analyses revealed genus-specific response to varying CO₂ availability (Figure 3a–f). An increase in galactolipids under CO₂-replete conditions was observed in *C. sorokiniana* while triacylglycerol (TAG) was only increased in *C. vulgaris*. Differently, a decrease in phospholipids in CO₂ could be

FIGURE 2 Starch, lipids and protein content in AIR versus CO₂. Relative starch, protein and lipid content per dry weight in *C. sorokiniana* (Panel a) and *C. vulgaris* (Panel b) in AIR versus CO₂ condition. Data are means of three biological replicates with standard deviation shown. Significantly different values, respectively, for starch, protein and lipids content per dry weight in CO₂ versus AIR or in *C. vulgaris* versus *C. sorokiniana* are indicated with different letters ($p < .05$, $n = 3$) [Colour figure can be viewed at wileyonlinelibrary.com]

noted in both genera (Figure 3, Figure S3). Specifically, a strong increase in monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol

FIGURE 3 Lipid composition and fatty acids profile in AIR versus CO₂ conditions. Lipids composition of *C. sorokiniana* (panel a–c) and *C. vulgaris* (panel d–f) cells grown in AIR or CO₂ conditions. Panel a and d: lipid composition in AIR versus CO₂ condition in terms of phospholipids, galactolipids, DGTS and triacylglycerol (TAG). Panel b and e: Fatty acids profile obtained by gas chromatography. Panel c and f: Polar lipid profile obtained by thin layer chromatography. Data are means of three biological replicates with standard deviation shown. Significantly different values in CO₂ versus AIR are indicated by * ($p < .05$). MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DGTS, diacylglycerol *N,N,N*-trimethylhomoserine [Colour figure can be viewed at wileyonlinelibrary.com]

(DGDG) per dry weight was observed in *C. sorokiniana*, when the availability of CO₂ was high (CO₂ condition). TAGs can be derived from the recycling of pre-existing membrane glycerolipids as well as from de novo biosynthesis of fatty acids (Simionato, Basso, Giacometti, & Morosinotto, 2013). In *C. vulgaris*, all polar lipids were decreased in CO₂-replete conditions, likely due to a redirection of the metabolism to TAG biosynthesis. Thus, at high CO₂ concentration *C. vulgaris* redirects its carbon flow from the storage of starch to the storage of TAGs, a more energy-dense carbon sink, while *C. sorokiniana* increased the fraction of lipids involved in thylakoid assembly. Interestingly, high CO₂ availability led to an increase in the betaine lipid diacylglycerol *N,N,N*-trimethylhomoserine (DGTS) with a decrease in phosphatidylcholine (PC) in both species (Figure 3c–f). The fatty acid profile of *C. vulgaris* and *C. sorokiniana* grown in AIR and CO₂ is reported in Figure 3b–e: *C. vulgaris* cells grown in high CO₂ were characterized by a strong increase in palmitic acid (16:0), hexadecadienoic acid (16:2), stearic acid (18:0) and oleic acid (18:1) together with a decrease in 3-hexadecenoic

acid, that is, 16:1 (3 t). In *C. sorokiniana*, cells were grown in high CO₂, an increase in palmitoleic acid (16:1 [9]), hexadecadienoic acid (16:2), hexadecatrienoic acid (16:3), linoleic acid (18:2) and α -linolenic acid (18:3) and a decrease in oleic acid (18:1) were observed. The strong increase in palmitic and oleic acid observed in *C. vulgaris* either on dry weight (Figure 3) or on a cell basis (Figure S3), is consistent with the increased TAG accumulation observed in this species, being C16:0 and C18:1 fatty acids the main constituent of TAG in lipid droplets in microalgae (Siaut et al., 2011).

2.2 | Photosynthetic properties of *Chlorella vulgaris* and *Chlorella sorokiniana* are differently influenced by CO₂ availability

Photosynthetic properties of *Chlorella* species were investigated to determine the influence of different CO₂ concentration on chloroplast metabolism. The amount of RUBISCO, being the key enzyme

responsible for CO₂ fixation in organic molecules, was first quantified. RUBISCO amount was similar in both species in the two conditions analysed on a cell basis (Figure S4), suggesting that RUBISCO accumulation was not tuned by CO₂ concentration in *Chlorella*. This result is consistent with previous findings in soybean leaves exposed to different CO₂ concentrations (Campbell, Allen, & Bowes, 1988). We then analysed PSII maximum quantum yield by measuring the fluorescence parameter F_v/f_m , which is often used as a general indicator of the fitness of the culture. As reported in Figure 4c similar F_v/f_m values were found in both conditions, suggesting a minor, if any, impact of CO₂ concentration in maximum PSII quantum yield. Interestingly, a strong decrease in chlorophyll (Chl) content per cell was observed in *C. sorokiniana* grown in CO₂ condition (Figure 4a). Moreover, in *C. sorokiniana* an increased Chl *a/b* ratio was evident in CO₂ condition (Figure 4b): Chl *b* is bound only to the Light Harvesting Complex (LHC) subunits, the external antenna proteins of photosystems, while Chl *a* is bound to both antennae and core complex. A variation of the Chl *a/b* ratio suggests a change in the antenna/core complex stoichiometry, suggesting a rearrangement of the photosynthetic machinery. This adaptation in *C. sorokiniana* is consistent with results previously reported for the model alga *C. reinhardtii*, where a similar strong reduction of Chl content per cell was observed at high CO₂ concentration (Polukhina, Fristedt, Dinc, Cardol, & Croce, 2016). In contrast, this was not the case for *C. vulgaris*, where Chl/cell content was not significantly different in CO₂ compared to AIR condition (Figure 4a).

To further investigate remodelling of the components of photosynthetic complexes, PSI/PSII ratio and LHCII/PSII ratio was evaluated by immunoblot analysis (Figure 5a,b). In *C. sorokiniana*, both PSI/PSII and LHCII/PSII ratio were decreased in CO₂ compared to AIR condition, while no significant differences were observed in *C. vulgaris* (Figure 5c,d). PSI photochemical activity can be measured by transient absorption: oxidation of the reaction centre of PSI, called P700, causes an increased absorption at 830 nm, thus following transient

absorption kinetics at this wavelength it is possible to evaluate the PSI activity. Transient absorption measurements at 830 nm were performed in *Chlorella* species in the presence of DCMU, which inhibits linear electron transport from PSII to PSI, and ascorbate and methyl viologen as an electron donor and acceptor, respectively. In the case of *C. sorokiniana* a ~40% reduction of the maximum PSI activity was observed in CO₂ condition compared to the AIR case (Figure 5e), consistent with the reduced PSI/PSII ratio observed by immunoblot analysis (Figure 5d).

The reduced LHCII/PSII ratio observed in *C. sorokiniana* is consistent with the increased Chl *a/b* ratio (Figure 4b). To investigate whether the different LHCII/PSII ratio induced by CO₂ availability in *C. sorokiniana* influences a functional light harvesting capacity of PSII, as previously observed in *C. reinhardtii* (Berger et al., 2014), fast Chl *a* fluorescence emission spectrum in the presence of the inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) was measured for both *C. vulgaris* and *C. sorokiniana* grown in AIR and CO₂ condition. Light harvesting capacity, or functional antenna size, of PSII was indeed reported to be inversely proportional to the time required to reach 2/3 of the maximum Chl *a* fluorescence emission upon inhibition of PSII electron transport activity (Malkin, Armond, Mooney, & Fork, 1981). As shown in Figure 5f and Figure S5 no differences in PSII functional antenna size were observed in *C. sorokiniana* or in *C. vulgaris* depending on CO₂ availability. This result indicates that the reduction of LHCII/PSII ratio measured in *C. sorokiniana* did not affect the PSII light harvesting capacity, being thus likely related to LHCII subunits poorly connected to PSII.

LHC complexes are also involved in the process called state transitions, where a fraction of the antenna complexes bound to PSII moves to PSI to maintain the excitation balance between the two photosystems. This process is triggered in *C. reinhardtii* by LHC phosphorylation catalysed by a kinase enzyme called STT7 (Depege, Bellafiore, & Rochaix, 2003). State 1 (S1) or State (S2), being

FIGURE 4 Chlorophyll content and PSII maximum yield in AIR versus CO₂ conditions. (a) Chlorophyll content per cell, (b) chlorophyll *a/b* ratio and (c) PSII maximum quantum yield expressed as $F_v/f_m = (F_m - F_0)/F_m$ in *C. sorokiniana* (grey colour) and *C. vulgaris* (red colour) in AIR (full colour) or CO₂ (dash colour) condition. Data are means of three biological replicates with standard deviation shown. Significantly different values in CO₂ versus AIR are indicated by * ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 5 Analysis of PSI, PSII and LHCII content by immunoblots, P700 activity and functional PSII antenna size. (a, b) Immunoblot analysis of PSI (α-PsaA antibody), PSII (α-CP43 antibody) and LHCII (α-LHCII antibody). Loading was performed on a chlorophyll basis: total µg of chlorophylls loaded in each lane is reported on the top of Panel a and b. (c, d) PSI/PSII (c) and LHCII/PSII (d) ratios calculated by densitometry of immunoblot signals for *C. sorokiniana* (panel a, grey colour) and *C. vulgaris* (panel b, red colour) in AIR (full colour) or CO₂ (dash colour) condition. (e) Maximal P700 oxidation on a chlorophyll basis in *C. sorokiniana* (left, grey colour) and *C. vulgaris* (right, red colour) in AIR (full colour) or CO₂ (dash colour) normalized to AIR condition. (f) Functional antenna size of the photosystem II (1/τ_{2/3}) normalized to AIR condition in *C. sorokiniana* (grey colour) and *C. vulgaris* (red colour). Data are means of three biological replicates with standard deviation shown. Significant different values in CO₂ versus AIR are indicated by ** ($p < .01$) and by * ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

respectively, the conditions with minimum or maximum migration of LHCII to PSI, can be induced by consuming or increasing the reducing power in the chloroplast as described in (Fleischmann et al., 1999). In particular, S1 or S2 state can be measured on whole cells by measuring Chl fluorescence emission at 77 K, where both PSI and PSII

emission is detectable. As reported in Figure 6, both *C. vulgaris* and *C. sorokiniana* were able to undergo state transitions in both AIR and CO₂ with an increased PSI contribution in S2 compared to S1. However, *C. sorokiniana* cells grown in CO₂ exhibited an increased capacity for state transitions compared to cells grown in AIR. When 77 K fluorescence emission in S1 or S2 were compared to 77 K fluorescence of cells directly harvested in their growing conditions, it was possible to observe that both *C. vulgaris* and *C. sorokiniana* were essentially growing in S2 state in AIR condition. A different behaviour was instead observed in CO₂ condition between the two species herein analysed: while *C. vulgaris* cells were characterized by an intermediate state between S1 and S2, *C. sorokiniana* was essentially in S1. These results indicate that the reduction in *C. sorokiniana* grown in CO₂ of LHCII/PSII ratio was related to a decrease in LHCII preferentially connected in AIR to PSI, suggesting a compensatory regulation between antenna regulation and state transitions.

Photosynthetic electron transport is coupled to the formation of a proton gradient across the thylakoid membrane, exploited by ATPase as proton motif force to produce ATP (Tikhonov, 2013). ATPase content on Chl basis and proton-motive force (*pmf*) upon exposure to different light intensity was evaluated in CO₂ and AIR grown cells (Figure 7). The *pmf* can be estimated by measuring the light dependent-electrochromic shift of carotenoid absorption (Bailleul, Cardol, Breyton, & Finazzi, 2010). In this case, the behaviour of the two *Chlorella* species was similar with a reduced *pmf* in CO₂ compared to AIR condition. At the same time, an increase in ATPase content under CO₂ condition was detected for both *C. vulgaris* and *C. sorokiniana*. Likely, the higher level of ATPase in CO₂ condition improved proton movement back to the stroma resulting in reduced *pmf* and higher ATP production in CO₂ condition. Furthermore, we investigated the influence of cyclic electron flow (CEF) around PSI measuring electrochromic shift (ECS) in the presence of DCMU inhibiting PSII and thus linear electron flow. Only a 2–7% of residual *pmf* was detected in DCMU treated samples, indicating a low level of CEF operating in *C. vulgaris* and *C. sorokiniana*, not significantly influenced by CO₂ concentration.

PSI is a plastocyanin-ferredoxin oxidoreductase that reduces NADP⁺ to NADPH by a ferredoxin–NADP⁺ reductase (FNR) enzyme. In parallel, the mitochondrial respiratory electron transport chain oxidase NADH releasing NAD⁺. Chloroplasts and mitochondria communicate to balance the NAD(P)⁺/NAD(P)H pool (Dang et al., 2014; Johnson & Alric, 2013; Uhmeyer, Cecchin, Ballottari, & Wobbe, 2017). We evaluated the light dependent NADPH formation rate by following NAD(P)H fluorescence changes upon exposure to actinic light of 300 µmol photons m⁻² s⁻¹ for 120 s. It is important to note that NADH and NADPH cannot be distinguished by fluorescence, both contributing to the signals herein detected. In both species either in AIR or CO₂ conditions the rates of NAD(P)H fluorescence during actinic light exposure were negative, indicating that NAD(P)H consumption exceeds light dependent NADPH production (Figure 8). It is interesting to observe that in *C. sorokiniana* the same balance between NAD(P)H formation and consumption was maintained comparing AIR versus CO₂ condition, while in *C. vulgaris* a higher rate of NADPH consumption was observed in CO₂ condition.

FIGURE 6 State transitions in AIR versus CO₂. State transition analysis by 77 K fluorescence emission spectra in state 1 (S1, red lines) or state 2 (S2, blue lines) conditions in *C. sorokiniana* (a–c) and *C. vulgaris* (d–f) in AIR (A, D) or CO₂ (b, e). S1 was induced by shaking vigorously cells in a low light ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$) with $10 \mu\text{M}$ of DCMU for at least 15 min to oxidize the plastoquinone pool while S2 was induced by adding $250 \mu\text{M}$ sodium azide to inhibit mitochondrial respiration and to reduce the plastoquinone pool as described in Fleischmann et al. (1999). Black lines are related to cells harvested in the different growing conditions (AIR or CO₂) and dark adapted for 1 min before freezing at 77 K. maximum capacities for state transitions were then quantified from the maximum fluorescence emission at 720 nm as $(F_{S2} - F_{S1})/F_{S2}$. Data reported are means of three biological replicates with standard deviation shown. Significant difference in CO₂ versus AIR are indicated with different letters (a, b, or c, $p < .05$)

2.3 | The response of mitochondrial respiratory pathways to CO₂ availability

Mitochondrial respiration is a fundamental process that allows producing ATP while releasing NAD⁺ that can return to the chloroplast. The mitochondrial electron transport chain, also called cytochrome pathway, includes an ATP synthase complex, called also complex V, and four oxidoreductase complexes that oxidize the reducing power and produce ATP thanks to the electrochemical gradient that is formed across the membrane. In addition, an alternative oxidase (AOX) might operate directly coupling ubiquinol oxidation with the reduction of O₂ to H₂O serving as an alternative route bypassing the electron transport chain thus dramatically reducing the energy (ATP) yield. AOX was reported having a role in the protection mechanism for the respiratory chain (Boekema & Braun, 2007; Vanlerberghe, 2013).

The contribution of cytochrome and alternative pathways (Figure 9) was investigated by measuring the dark respiration in the

presence of two specific inhibitor: SHAM (salicylhydroxamic acid) that inhibits AOX and so the alternative pathway, and myxothiazol that locks the complex III, therefore, blocking the cytochrome pathway (Dang et al., 2014). We observed that the total dark respiration on a cell basis is essentially unaffected in *C. sorokiniana*, while a strong reduction was reported for *C. vulgaris* in CO₂ condition. In both species a reduction of the fraction of dark respiration operating through AOX was evident, leading to an increased efficiency of ATP production by NADH oxidation through the cytochrome pathway.

2.4 | NAB1-like proteins in *C. vulgaris* and *C. sorokiniana*

Acclimation to different carbon availability has been reported in *C. reinhardtii* to involve the translational repressor NAB1. NAB1 acts as a molecular switch triggered by the redox state of the cell, which is

FIGURE 7 ATPase content and electrochromic shift in AIR versus CO₂. Immunoblot analysis of ATPase content (atpC subunit antibodies) and ECS measurements in *C. sorokiniana* and *C. vulgaris* in AIR (solid line,) or CO₂ (dashed line) condition. ECS results in presence of DCMU are also reported with open symbol. Data are means of three biological replicates with standard deviation shown. Significantly, different values in CO₂ versus AIR are indicated by * ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 8 NAD(P)H formation rate in AIR versus CO₂. Light dependent rate of NAD(P)H formation upon exposure to light (300 μmol photons m⁻² s⁻¹) for 120 s in *C. sorokiniana* (grey colour) and *C. vulgaris* (red colour) in AIR (full colour) or CO₂ (dash colour) condition. The data reported were calculated from the slope of the NAD(P)H fluorescence emission curve upon exposure to actinic light. Data are means of three biological replicates with standard deviation shown. Significantly, different values in CO₂ versus AIR are indicated by * ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 9 Dark respiration in *C. sorokiniana* and *C. vulgaris* in AIR versus CO₂ conditions. The relative contribution of cytochrome (filled bars) and alternative respiration (empty bars) was reported normalized to cell content. Data are means of three biological replicates with standard deviation shown. Significant difference in CO₂ versus AIR are indicated by * ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

in turn strongly influenced by the carbon availability, repressing the translation of specific transcripts, including those for LHC subunits. To evaluate the possible conservation of NAB1 in the *C. vulgaris* and *C. sorokiniana* species, BLAST search was performed using the *C. reinhardtii* protein sequence as query. It is important to note that

functional NAB1 in *C. reinhardtii* is composed of a Cold-shock domain (CSD) at the N-terminus and an RNA recognition motif (RRM) at the C-terminus (Mussnug et al., 2005). Among the putative protein sequences identified by BLAST, only in *C. vulgaris* the g211.t1 locus containing both CSD and RRM domains were conserved. Both CSD and RRM domain were found also in a predicted mega-protein in

C. sorokiniana (CS12_123000002385-RA) where two additional V-ATPase proteolipid subunit C-like domains were present, suggesting that this polypeptide has likely different functions compared to the *C. reinhardtii* NAB1 (Figure S6). Consistent with the bioinformatic analysis, immunoblot analysis using anti-NAB1 antibody revealed a band at the expected molecular weight (27 kDa) in both *C. reinhardtii* and *C. vulgaris* but not in *C. sorokiniana* (Figure S7). However, while the accumulation of NAB1 was increased in AIR compared to CO₂ in *C. reinhardtii* condition, as previously described, a similar level of NAB1-like protein was observed in *C. vulgaris* cells grown in AIR or CO₂ (Figure S7). Increased accumulation of NAB1 in *C. reinhardtii* at low CO₂ availability has been recently reported as being related to a transcriptional activation mediated by the transcription factor LCRF, which belongs to the Squamosa promoter binding protein (SBP) family of transcription factors (Blifernez-Klassen et al., 2011). Possible homologous of *C. reinhardtii* LCRF was searched in *C. vulgaris* and *C. sorokiniana* by BLAST search but no putative candidate gene could be found in these *Chlorella* species.

Sequence analysis of the identified NAB1-like protein in *C. vulgaris* demonstrated that among the key residues involved in NAB1 activity regulation in *C. reinhardtii* (H Berger et al., 2016; Blifernez et al., 2011; Wobbe et al., 2009), only Cys181 and Arg92 were conserved, while Cys226 or Arg90 are substituted by a valine and a serine residue, respectively, in the NAB1-like subunit in *C. vulgaris* (Figure S8). It is important to note that the substitution of Cys226 in *C. reinhardtii* NAB1 arrested the protein in its active state and abolished the redox control mechanism leading to a pale green phenotype in strains exclusively expressing NAB1_{Cys226Ser} (Wobbe et al., 2009). As reported in Figure S5, when comparing the functional antenna size of *C. reinhardtii*, *C. vulgaris* and *C. sorokiniana* grown in AIR or CO₂ conditions, a smaller light harvesting capacity was evident in the *Chlorella* species compared to *C. reinhardtii* in CO₂, while similar PSII antenna size was observed in AIR. This result suggests that NAB1 dependent adaptation of PSII antenna size to different CO₂ concentration is not conserved in *C. vulgaris* and *C. sorokiniana*, because either the protein is not present (*C. sorokiniana*) or the redox control is not functional (*C. vulgaris*). As another important difference, the NAB1 homolog in *C. vulgaris* does not accumulate upon CO₂ limitation.

3 | DISCUSSION

Atmospheric CO₂ concentration has significantly increased over the last 100 years and is continuing rising at an unprecedented speed. This greenhouse gas strongly contributes to climate change and global warming leading to a potential severe environmental crisis. Microalgae are promising platforms to capture CO₂, possibly integrating microalgae cultivation with CO₂ recovery from flue gases, thus reducing industry derived CO₂ emission and carbon footprint (Cheng et al., 2019; Collet et al., 2011; Dineshkumar, Chauhan, & Sen, 2020; Garcia-Cubero, Moreno-Fernandez, & Garcia-Gonzalez, 2018). For this reason, understanding the cell acclimation process involved in CO₂ metabolism is crucial to develop new strategies for improving the ability of

microalgae to acquire and accumulate carbon. In this work we focused on two of the most promising species for microalgae cultivation at industrial level, *C. sorokiniana* and *C. vulgaris* (Bernaerts, Gheysen, Foubert, Hendrickx, & Van Loey, 2019; Camacho et al., 2019; Kobayashi et al., 2015; Li et al., 2013; Niccolai, Zittelli, Rodolfi, Biondi, & Tredici, 2019; Sun, Chen, & Du, 2016). Moreover, high quality annotated genome and transcriptomes are available for these species (Cecchin et al., 2019; Hovde et al., 2018), paving the way for possible future biotechnological manipulation on the base of the results obtained. *Chlorella sorokiniana* and *C. vulgaris* were grown in airlift photobioreactors under atmospheric level of CO₂ (~0.04% CO₂, AIR condition) or 3% CO₂ (CO₂ condition). We discuss the metabolic consequence of the different photosynthetic and respiratory responses to high or low CO₂ levels in two *Chlorella* genus. It worth to note that CO₂ concentration in flue gases can reach higher concentration than 3%, being usually in the 10–20% range (X. Wang & Song, 2020). Previous work demonstrated that increase CO₂ availability in the range of 1–8% saturated biomass productivity in *C. vulgaris*, while higher CO₂ concentration caused a strong reduction in biomass yield (Garcia-Cubero et al., 2018). Nevertheless, cultivation of different *Chlorella* species at higher CO₂ concentration (up to 20%) was described in some reports (Cheng et al., 2019; Freitas, Morais, & Costa, 2017; Garcia-Cubero et al., 2018; Zhang et al., 2019). The metabolic changes herein described upon cultivation of *C. vulgaris* and *C. sorokiniana* providing 3% CO₂ compared to the AIR condition could thus be further modulated upon cultivation of these species at industrial level for partially capturing CO₂ in flue gases. Nevertheless, the highlighted adaptation responses in CO₂ conditions compared to the AIR case, provide useful insights to understand the different cell strategies evolved in these *Chlorella* species to manage high carbon availability.

3.1 | CO₂ availability boosts biomass accumulation

Increasing CO₂ supply boosted (~260% increase) in biomass yield in both species. It is worth to note that the increased biomass productivity observed was accompanied in both species with a decrease in dry weight per cell at high CO₂ availability with a consequent strong increase in cell density (Figure S1). Likely, high carbon flux in CO₂ conditions caused increased cell divisions, reducing the cell size. Moreover, it is possible that the higher production of macromolecules per dry weight, as reported in Figure 2, allow the cells grown at high CO₂ to support cell replication for longer even when nutrient in the medium become limiting. Interestingly, differential response was observed between the two strains in terms of biomass composition (i.e., protein, starch and lipid amount). In *C. vulgaris* the fractions of dry biomass related to starch and lipids were, respectively, reduced and increased, with in particular a strong increase of TAG accumulation either on dry weight or on a cell basis. This suggests a redirection of the energy reserves from starch to TAG accumulation, a class of macromolecules with a higher energy content per gram, indicating an improved light energy conversion. In *C. sorokiniana* not only lipids, but also protein content increased, the latter being an additional carbon

sink in cells grown at high CO₂ concentration. Comparing the productivity of the two *Chlorella* species herein investigated, *C. vulgaris* was characterized by an increased biomass productivity at high CO₂ availability, making this species the best candidate for industrial application. The high protein content observed in *C. vulgaris* suggests a possible food application of this organism, which is already widely considered a novel food (Bernaerts et al., 2019; Camacho et al., 2019; Niccolai et al., 2019). The carbon storage as TAG observed in CO₂ condition in *C. vulgaris* is also a desirable trait for possible use of this species for biodiesel production, even if the feasibility of biofuels production by microalgae cultivation is highly debated (Koyande et al., 2019). Finally, the high starch content in *C. sorokiniana* suggests a possible biotechnological application of this species to convert CO₂ into sugars to be used for different purposes as for fuelling fermentative process by different microorganisms' communities to produce bioethanol, biomethane or other high value metabolic compounds.

3.2 | Lipid remodelling and CO₂ availability

Both *Chlorella* genus showed an increase in total lipid composition of dry biomass accumulated under high CO₂, and they further remodelled their fatty acid as well as lipid class composition albeit in different ways. Phospholipids were reduced whereas the betaine lipid DGTS were increased in both species by high CO₂ level. Betaine lipid, a non-phospholipid, has been observed often increased during phosphate limitation, presumably replacing the function of phospholipids in cell membranes (Hidayati et al., 2019; Murakami, Nobusawa, Hori, Shimojima, & Ohta, 2018; Riekhof, Naik, Bertrand, Benning, & Voelker, 2014). For still unknown reasons, a similar response between P limitation and high CO₂ was observed. We can speculate that increased CO₂ availability required phosphate reallocation to the different macromolecules produced, requiring partial phospholipids substitution with betaine lipid DGTS.

In *C. vulgaris*, the increase in lipid content per dry weight was mostly due to an increase in TAG, whereas, in *C. sorokiniana* the increase in total lipids was mainly due to an increase in the two galactolipids (MGDG and DGDG), the major lipids of photosynthetic membranes (Li-Beisson et al., 2019). The differential response in lipid classes in the two strains to high CO₂ level is further supported by fatty acid compositional alterations. Among other reasons, lipid compositional changes are mostly likely results of altered redox status brought about by differential chloroplast and mitochondrial energetic activities in response to varying CO₂ availability in the two *Chlorella* genus (Figures 6 and 8) (Burlacot, Peltier, & Li-Beisson, 2019).

3.3 | Thylakoid reorganization and photosynthetic activity under varying CO₂

The increase in DGDG and MGDG (Figure 3, Figure S3), surprisingly, was observed together with a reduction in Chl content per cell in *C. sorokiniana* under high CO₂. Nevertheless, the observed reduction

of Chl content per cell in *C. sorokiniana* grown in CO₂ condition compared to AIR was in line with the results obtained previously in *C. reinhardtii* (Polukhina et al., 2016), while this was not the case for *C. vulgaris*, where Chl content per cell is independent from CO₂ availability. Again, similar to *C. reinhardtii*, *C. sorokiniana* grown in high CO₂ was characterized by reduction of LHCII/PSII content and a reduction in PSI/PSII ratio, while these adaptations were not observed in *C. vulgaris*. The reduced LHCII/PSII content observed in *C. sorokiniana* grown in CO₂ condition did not affect the functional antenna size of PSII: differently from previous observation in *C. reinhardtii*, functional antenna size of PSII was not influenced by CO₂ availability in both *Chlorella* species herein investigated (Figure 5f, Figure S5). In *C. reinhardtii* it was indeed reported that high CO₂ availability caused an increase in the functional antenna size of PSII, due to suppressed accumulation at high CO₂ concentration of NAB1, the translation repressor specific for LHCII encoding mRNAs (Berger et al., 2014). Here, we report the identification of a NAB1-like protein in *C. vulgaris* only, which however, is not differently accumulated in AIR versus CO₂ conditions. Accordingly, homologous protein of the transcription factor LCRF, the transcriptional regulator of NAB1 recently identified in *C. reinhardtii* (Blifernez-Klassen et al., 2011) could not be found in either *C. vulgaris* or *C. sorokiniana*. Moreover, in the *C. vulgaris* NAB1-like subunit only Cys181 and Arg92 residues are conserved among the two cysteine (Cys181, Cys226) and two arginine (Arg90, Arg92) residues reported in *C. reinhardtii* NAB1 to be involved in its activity as translational repressor. Taken together, the NAB1-dependent regulation of PSII antenna size at different CO₂ concentration in *C. reinhardtii* is absent in both *C. sorokiniana* and *C. vulgaris*, where NAB1 homolog was, respectively, not identified or is missing the crucial redox control of its translational inhibition activity (Figures S6 and S7). Indeed, the PSII antenna size of both *C. sorokiniana* and *C. vulgaris* was not affected by different CO₂ availability, being similar to the PSII antenna size measured in the case of *C. reinhardtii* grown in AIR (Figure S5). Regulation of PSII antenna size by NAB1 is thus an acclimation mechanism finely controlled by the redox state of the cell, which is not conserved among *Chlorophyta*.

It is important to note that the effect of CO₂ availability on *C. reinhardtii* LHCII/PSII ratio is still under debate, with Polukhina and coworker reporting a general reduction of LHCII/PSII content in *C. reinhardtii* grown in high CO₂, in parallel with a decrease in PSI/PSII ratio (Polukhina et al., 2016). Considering the possibility of LHCII proteins to function as PSI antenna, it was not excluded by Polukhina and coworkers that the decrease of LHCII content per PSII observed at high CO₂ might be mainly related to the amount of LHCII proteins acting as PSI antenna. Here we report a similar acclimation mechanism only in the case of *C. sorokiniana*, with the difference that PSII antenna size was not modulated by CO₂ availability.

3.4 | Cellular redox balance and CO₂ availability

Cellular reducing power is crucial for the overall carbon flow and cell metabolism: catabolic process generate reducing power, which can be

used by oxidative phosphorylation to generate ATP or by the anabolic pathways. In photosynthetic organisms, the NADP⁺/NADPH balance influence both the light phase of photosynthesis and the carbon fixation reactions. In general it is possible to hypothesize that the increased capacity of Calvin Benson cycle to regenerate NADP⁺ and ADP, thanks to the increased CO₂ availability, trigger the light phase of photosynthesis in order to keep the NADPH/NADP⁺ ratio similar to the AIR condition, as reported in Figure 8: this occurs by increasing the total amount of PSII compared to PSI and relatively redistributing the excitation pressure among PSI and PSII reducing the excitation pressure at the level of the former reducing the LHCII content bound to PSI. This acclimation process would explain the reduced LHCII/PSII content despite the similar PSII antenna size observed. The absence of such acclimation mechanisms in the case of *C. vulgaris* could be at the base of the strong reduction of NAD(P)H/NAD(P)⁺ ratio observed at high CO₂ concentration, as a consequence of increased NADPH consumption by the Calvin Benson cycle. Alteration of RUBISCO content was not detected (Figure S4), suggesting an enhanced RUBISCO activity due to the higher availability of substrate rather than an upregulation of enzyme to exploit the higher CO₂ availability. CO₂ fixation requires both ATP and NADPH: in both *Chlorella* species a decrease of *pmf* and an increased ATPase content was measured in CO₂ condition (Figure 6). Likely, the higher level of ATPase in CO₂ condition prevents the accumulation of the electrochemical gradient, suggesting a higher ATP production.

Interestingly, dark respiration is differentially regulated in the two *Chlorella* species: in *C. sorokiniana* total dark respiration was similar in AIR compared to CO₂ condition, with an increased NADH oxidation through the cytochrome pathway and reduced AOX activity. Accordingly, in *C. sorokiniana* we observed the same balance of the NAD(P)H redox state: the rearrangements of the photosynthetic machinery in CO₂ condition improved the pool of NADPH and ATP, likely matching the increased substrate (CO₂) availability for sugar production by the Calvin Benson cycle. In contrast, in *C. vulgaris* a strong reduction of dark respiration in CO₂ condition was evident, despite an increase of cytochrome/alternative pathway ratio. In addition, in *C. vulgaris* there was a higher NAD(P)H consumption in CO₂ suggesting that chloroplast acts as a sink of reducing power subtracting them from the mitochondrion. Moreover, the relative reduction of starch accumulation and the increase of TAG suggested a redirection of photosynthates to other metabolic pathways. Consumption of triose phosphates by the glycolytic pathway leading the acetyl-CoA production could be a possible link between reduced starch accumulation and increased TAG content. Indeed, an increase of acetyl-CoA and reducing power was reported at the base of the increased TAG accumulation observed in the diatom *Phaeodactylum tricornutum* (Li-Beisson et al., 2019; Valenzuela et al., 2012; Z. Yang et al., 2013).

In conclusion, high CO₂ availability caused an increased biomass accumulation in both *C. vulgaris* and *C. sorokiniana*, likely related to increased photosynthates production. The increased carbon assimilation in high CO₂ redirected the metabolism toward biosynthesis of lipids (TAG) in *C. vulgaris*, and proteins in *C. sorokiniana*, respectively. Increased carbon fixation at high CO₂ concentration requires an

increased NADPH and ATP availability: while increased ATPase content and reduced *pmf* suggesting indeed an increased ATP regeneration in both *C. vulgaris* and *C. sorokiniana*, the increased NADPH requirement was differently satisfied in *C. vulgaris* and *C. sorokiniana* in CO₂. In the case of *C. vulgaris*, chloroplast acted as a sink for reducing power, inducing consequently a reduced NADH availability for mitochondrial respiration, reducing in particular the relative contribution of alternative pathways, not related to ATP biosynthesis. In *C. sorokiniana* a reduced PSI/PSII ratio and reduced LHCII binding to PSI was observed in CO₂, allowing an increased electron flow toward NADP⁺ to NADPH regeneration and ensuring a similar NAD(P)H/NAD(P)⁺ ratio in both AIR or CO₂ conditions. A summary of the adaptation to CO₂ condition is shown in Figure S9.

Detailed multi-omics analysis would be required to investigate the molecular mechanisms at the base of the different responses of *C. sorokiniana* and *C. vulgaris* to increased CO₂ availability. Increased TAG accumulation in *C. vulgaris* could be related to increased accumulation or activation of chloroplast fatty acid synthase or transporters and enzymes involved in the Kennedy pathway. Alternatively, a different metabolic contribution of glyoxylate cycle could be related to the different carbon storage strategies in the two *Chlorella* species herein investigated: the use of TAG as storage macromolecule require an efficient strategy for acetyl-CoA assimilation upon hydrolysis of these macromolecules, which in microalgae are usually assimilated through the glyoxylate cycle (Combres, Laliberte, Reyssac, & Delanoue, 1994; Plancke et al., 2014). While in *C. vulgaris* the key enzymes involved in glyoxylate cycle has been reported to be upregulated in growth conditions inducing high carbon flow (Cecchin et al., 2019) this was not the case for *C. sorokiniana* where the metabolic contribution of glyoxylate cycle appears to be limited (Cecchin et al., 2018; Xie et al., 2016). Further research efforts are required to support this hypothesis.

Elucidation of the molecular rearrangements in enriched CO₂ condition could be useful to develop strategies to improve in these species and in other microalgae of industrial interest their carbon assimilation efficiency to improve sustainability, biomass yield and productivity of specific compounds.

4 | MATERIAL AND METHODS

4.1 | Microalgae cultivation

Chlorella sorokiniana UTEX 1230 and *C. vulgaris* 211/11P strain (Culture Collection of Algae at Goettingen University CCAP 211/11P strain) cells were grown in the Multi-Cultivator MC 1000 tubes aerated with air or with 3% CO₂-enriched air obtained by a gas mixing system. Cells were grown in BG11 medium starting from 1*10⁶ cell/ml at 300 µmol photons m⁻² s⁻¹ (Allen & Stanier, 1968). Cell number was determined Countless® II FL automated cell counter (Thermo Fisher). The cell density was automatically monitored every 10 min by measuring the absorption at 720 nm. For physiological measurements, cultures were harvested during the exponential growth phase. At the end of the growth curve the dry weight

determination was performed: cell culture was harvested by centrifugation at 4,500g for 5 min at 20°C then drying in a lyophilizer for 48 h and then net dry weight was calculated.

4.2 | Biomass composition analysis

Lipid, starch and protein content of the biomass harvested at the end of the exponential phase were analysed as previously reported in Cecchin et al. (2019).

4.3 | Photosynthetic parameters and pigments extraction

Pigments were extracted with 100% DMSO at 60°C in dark conditions and measured with Jasco V-550 UV/VIS spectrophotometer. Proton motive force upon exposure to different light intensities was measured by ECS with MultispeQ v2.0 (PhotosynQ) according to (Kuhlgert et al., 2016) and normalized to the Chl content of the sample. PSII activity was analysed by fluorescence measurements on whole cells using a Dual-PAM 100 instrument (WALZ). 77 K fluorescence emission spectra were acquired with a charge-coupled device spectrophotometer (JBeamBio) as previously described (Allorent et al., 2013). State transitions were measured on whole cells induced to state 1 or state 2 as described in (Fleischmann et al., 1999). PSII functional antenna size was measured from fast Chl induction kinetics induced with a red light of 11 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ on dark-adapted cells incubated with 50 μM DCMU (Malkin et al., 1981). The reciprocal of time corresponding to two-thirds of the fluorescence rise ($\tau_{2/3}$) was taken as a measure of the PSII functional antenna size (Malkin et al., 1981). P700 activity was measured with the DUAL-PAM-100 (Heinz-Walz) following the transient absorption at 830 nm upon exposure to actinic light. Maximum P700 activity was measured after a pulse of saturating light in whole cells treated with DCMU (3-[3,4-dichlorophenyl]-1,1-dimethylurea), ascorbate and methylviologen, as described in Bonente et al. (2012). The formation rate of NADPH was determined with the NADPH/9-AA module of the Dual-PAM 101 (Schreiber & Klughammer, 2009). Cells were harvested and resuspended in BG11 medium with 10% of Ficoll to reduce cells precipitation. Measurement was performed as described in (Schreiber & Klughammer, 2009) at the same light intensity of growth (300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The slope during the light phase, between 60 and 120 s, was used to determine the rate of NADPH formation.

4.4 | SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed as described in (Bonente et al., 2011). The following antibodies obtained from Agrisera company (<https://www.agrisera.com/>) were used: α -RbcL AS03 037, α -PsaA AS06 172, α -PsbC (CP43) AS11 1787, α -AtpC

AS08 312. α -NAB1 antibody was kindly provided by Prof. Dr. Kruse from university of Bielefeld (Germany).

4.5 | Mitochondrial respiration

Samples in the exponential phase were subjected to respiratory rate measurements in the dark using a Clark-type O_2 electrode (Oxygraph Plus; Hansatech Instruments). Respiratory rates were normalized to cells number obtained by Countless[®] II FL automated cell counter (Thermo Fisher). To discriminate between the individual contributions of the alternative and the cytochrome pathway dark respiration measurements were conducted as follows: cell samples (5×10^7 cell/ml) were transferred to the measurement chamber of the Clark electrode, respiration rates were recorded for 3 min prior to the addition of the first inhibitor, then respiration rates were recorded for three additional min finally the second inhibitor was added and measurements were continued for another 3 min. Alternative respiration was inhibited by adding 2 mM SHAM (salicylhydroxamic acid), while the cytochrome pathway (complex III) was inhibited by adding 5 μM myxothiazol. To assess the relative contribution of the cytochrome pathway, respiration was first measured in the absence of inhibitors (total dark respiration) before alternative respiration was inhibited by adding SHAM. Cytochrome dependent respiration was then inhibited using myxothiazol and the residual respiration determined in relation to the uninhibited state. The contribution of alternative respiration was determined by reversing the order of inhibitor addition (myxothiazol followed by SHAM) (Bailleul et al., 2015).

4.6 | NAB1 sequence analysis

Chlamydomonas reinhardtii NAB1 protein (Cre06.g268600.t1.2) was blasted against *C. vulgaris* protein database (Cecchin et al., 2019) and *C. sorokiniana* protein database (<https://greenhouse.lanl.gov/greenhouse/>) (Hovde et al., 2018). Protein domains were obtained and aligned with Scanprosite tool of Prosite database (de Castro et al., 2006; Sigrist et al., 2013). Sequence alignment was visualized with Clustal Omega (Goujon et al., 2010; Sievers et al., 2011).

4.7 | Experimental replication and statistical treatment

All the experiments herein reported were performed at least three times. Errors are reported as standard deviations. Statistical significance was tested by Tukey's test.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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