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EDITED BY
Cristian Ilioaia,
UMR9198 Institut de Biologie Intégrative de la Cellule (I2BC), France

REVIEWED BY
Luke Colin Martin Mackinder,
University of York, United Kingdom

*CORRESPONDENCE
Ginga Shimakawa,
gshimakawa@panda.kobe-u.ac.jp
Yusuke Matsuda,
yusuke@kwansei.ac.jp

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Multiphase CO_2-dependent photosynthesis in marine diatoms

Ginga Shimakawa [1]* and Yusuke Matsuda [2]*

[1]Graduate School of Agricultural Science, Kobe University, Kobe, Japan, [2]Department of Bioscience, School of Biological and Environmental Sciences, Kwansei-Gakuin University, Sanda, Japan

Diatoms, one of the most ubiquitous phytoplankton in the oceans, have evolved a pyrenoid-based CO_2-concentrating mechanism (CCM) to utilize limited CO_2 in seawater for photosynthesis. Recent proteomics analyses and molecular biological tools have deepened our understanding of the molecular mechanisms involved in diatom chloroplast architecture and the CCM. Here, we provide an update to our knowledge of the processes involved in high affinity photosynthesis for dissolved inorganic carbon (DIC) in diatoms. Based on the phenotype of genome-edited mutants, we propose a model of the diatom CCM composed of four phases of CO_2-dependent photosynthesis at (I) less than 0.1 mM, (II) 0.1–2 mM, (III) 2–10 mM, and (IV) more than 10 mM of DIC concentrations, in which the rate-determining steps are the capture of unfixed CO_2 in the chloroplast stroma at Phases I and II, the evolution of CO_2 in the pyrenoid-penetrating thylakoid lumen at Phase III, and DIC transport to the stroma at Phase IV. Under natural seawater containing 2 mM DIC mainly in the form of HCO_3^-, the photosynthesis of marine diatoms is likely primarily in Phase III, shifting to Phase II when available CO_2 is limited.

KEYWORDS

photosynthesis, pyrenoid, pyrenoid-penetrating thylakoid membranes, marine diatoms, CO_2-concentrating mechanism

Introduction

Marine diatoms are widely distributed and highly diversified phytoplankton group in the global oceans. They are among the most productive phytoplankton groups, with their photosynthesis contributing nearly half of oceanic primary production (Falkowski et al., 1998). Since dissolved inorganic carbon (DIC) mainly exists in the ionic form in natural seawater (*ca.* 2 mM of HCO_3^- >> *ca.* 16 μM of CO_2 at 20°C), marine diatoms have evolved a system to utilize this large HCO_3^- pool for photosynthesis, the so-called biophysical CO_2-concentrating mechanism (CCM) (Kaplan et al., 1980; Matsuda et al., 2001). The pennate diatom *Phaeodactylum tricornutum* and the centric *Thalassiosira pseudonana* are model species for which a variety of genetic engineering techniques have been developed. To date, molecular studies of these species have revealed the functions of many CCM components (Figure 1). In both species, extracellular CO_2 is directly taken up by diatom cells. In *P. tricornutum*, external HCO_3^- is also directly taken up to the cytoplasm by active transporters such as solute carrier four family proteins (SLC4-1, SLC4-2, and SLC4-4) (Nakajima et al., 2013; Nawaly et al., 2023a), while *T. pseudonana* in contrast indirectly uptakes external HCO_3^- *via* dehydration by extracellular carbonic anhydrases (CA) (Tsuji et al., 2017a). Cytosolic pH was estimated to be 7.4 in *Thalassiosira weissflogii* (Hervé et al., 2012) and 7.9 in *P. tricornutum* (Shimakawa et al., 2023b), and at this pH DIC should be kept mainly as HCO_3^- in the cytosol. Diatom chloroplasts are comprised of four-layered membranes (Keeling, 2010; Flori et al., 2016; Cavalier-Smith, 2018). Chloroplast

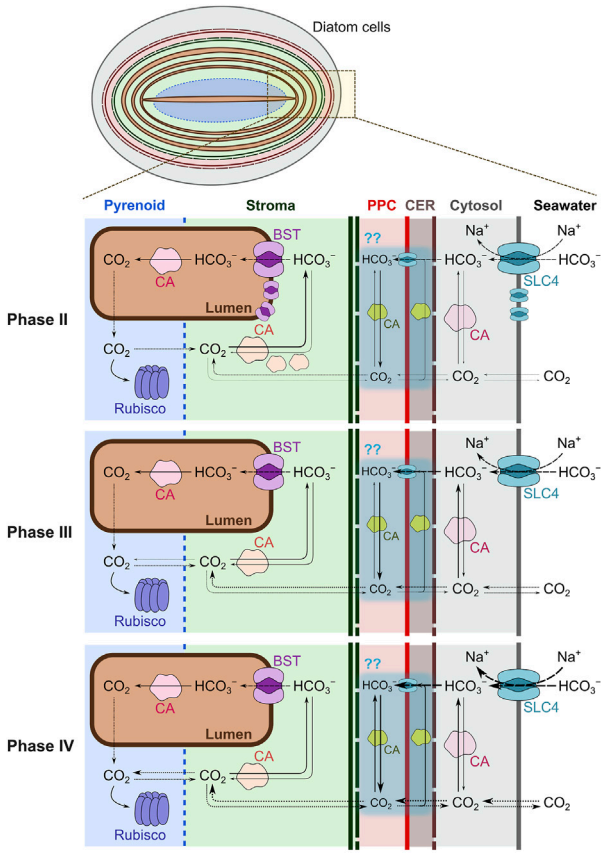

FIGURE 1
Multiphase CO_2-dependent photosynthesis in the marine diatom *Phaeodactylum tricornutum*. Cytoplasmic membranes (grey line), chloroplast endoplasmic reticulum (CER) envelopes (wine red dashed line), periplastidal membranes (red line), outer and inner plastid membranes (dashed and solid green lines), thylakoid membranes (brown line), and pyrenoid shells (blue dotted line) are drawn to separate each compartment. *P. tricornutum* possesses carbonic anhydrases (CAs) in the cytosol (θ-type), periplastidal compartment (PPC; α- and possibly ι-types), stroma (β-type), and the lumen of pyrenoid-penetrating thylakoid membranes (θ-type; Tachibana et al., 2011; Kikutani et al., 2016; Tsuji et al., 2017b). Solute carrier four family proteins (SLC4), stromal β-CAs, and the major bestrophin-like protein (BST1) are highly expressed in low dissolved inorganic carbon (DIC) concentrations. Thickness of each arrow roughly indicates the flux of diffusion, transport, and reaction. Phase I could be illustrated similarly to Phase II. Dynamics of DIC across four-layer chloroplast membranes (sky blue shadings) still remains to be demonstrated.

intermembrane CAs have been found in both *P. tricornutum* and *T. pseudonana* (Tachibana et al., 2011; Jensen et al., 2019). The periplastidal compartment (PPC) between the two outer and two inner membranes is maintained at an acidic pH, implying that cytosolic HCO_3^- is actively transported into PPC and then converted to CO_2 by intermembrane CAs to be passively diffused across the two inner chloroplast envelopes to the stroma (Shimakawa et al., 2023b). Meanwhile, it is also possible that intermembrane CAs rather convert CO_2 to HCO_3^- to suppress CO_2 leakage from the chloroplast stroma (Tsuji et al., 2017b; Jensen et al., 2019). Assuming the four-layer membrane system is beneficial to the diatom CCM, the CA-dependent CO_2 diffusion barrier would be located between the cytosol and PPC, i.e., chloroplast endoplasmic reticulum (CER) lumen, although this model of DIC dynamics across the chloroplast membranes still remains to be demonstrated (Figure 1). Stromal pH is 8.0 or higher (Anning et al., 1996; Shimakawa et al., 2023b), and CO_2 is trapped there in the form of HCO_3^-, presumably *via* hydration by stromal CAs (Tsuji et al., 2017b; Nawaly et al., 2023b). Overall, extracellular DIC in alkaline seawater is taken up across the plasma membrane with a strategy either extracellular-CA mediated CO_2 diffusion or direct HCO_3^- transport, and they are finally pumped into the chloroplast stroma mainly *via* active transport across chloroplast membranes (Hopkinson et al., 2011). The HCO_3^- pumping process across chloroplast membranes is likely to be fast enough to maintain the cytosolic CO_2 lower than external CO_2, thus enabling the influx of external CO_2 even without plasma membrane HCO_3^- transporter (Hopkinson et al., 2011). In either case, external DIC acquisition across the plasma membrane is, in principle, not the rate-determining step for diatom photosynthesis.

In diatom chloroplasts, there is a triple-layered thylakoid membrane called the girdle lamella with a layered stromal thylakoid in its interior, where the photosynthetic electron transport chain produces NADPH and ATP for CO_2 assimilation in the Calvin-Benson-Bassham cycle. In this process, H_2O is oxidized with O_2 evolution at the luminal side of photosystem II (PSII), and the electrons passed to the PSII reaction center are transported to photosystem I (PSI) through plastoquinone, cytochrome (Cyt) b_6f complex, and Cyt c_6. This linear electron transport process is accompanied by the generation of a proton concentration gradient (ΔpH) and an electric field gradient (ΔΨ) across the thylakoid membranes, which both function in the *proton motive force* driving ATP production by chloroplast ATP synthase (Witt, 1979). In *P. tricornutum*, the bestrophin-like protein (BST) is widely distributed throughout the stroma thylakoid, playing a role in transporting HCO_3^- into the lumen (Nigishi et al., 2024). In the middle of the stroma, there is a phase-separated proteinaceous body called the pyrenoid (Bedoshvili et al., 2009), which is a condensate body of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) interlinked by a intrisically disorder protein, PYCO1 (Oh et al., 2023). Two layered thylakoid membranes traverse along with the long axis of the oval shaped pyrenoid (Flori et al., 2016), which are termed the pyrenoid-penetrating thylakoid (PPT) membrane (Nigishi et al., 2024). The θ-type CA exists on the luminal side of PPT to convert HCO_3^- to CO_2 in the low pH of the PPT lumen under illumination (Kikutani et al., 2016; Shimakawa et al., 2023a). Due to the high membrane permeability of CO_2, evolved luminal CO_2 quickly effluxes to the pyrenoid and is fixed by Rubisco. This system was previously defined as the "CO_2-evolving machinery" (Shimakawa et al., 2023a). Further, unfixed CO_2 should be converted back to HCO_3^- by stromal CAs and transported again into the lumen of stroma thylakoid membranes by BST. It has been shown recently that the pyrenoid is surrounded by a sheet composed of a lattice-like protein sheath named Pyrenoid-Shell (PyShell) (Shimakawa et al., 2024a). It is possible that the PyShell sheet functions as a part of CO_2 diffusion barrier between the chloroplast stroma and the pyrenoid matrix. The strategy to utilize external DIC for photosynthesis should be diversified among diatom species dependent on their ecological niche. Nevertheless, central CCM components in the chloroplast, including stromal/luminal CA, BST, and PyShell are widely

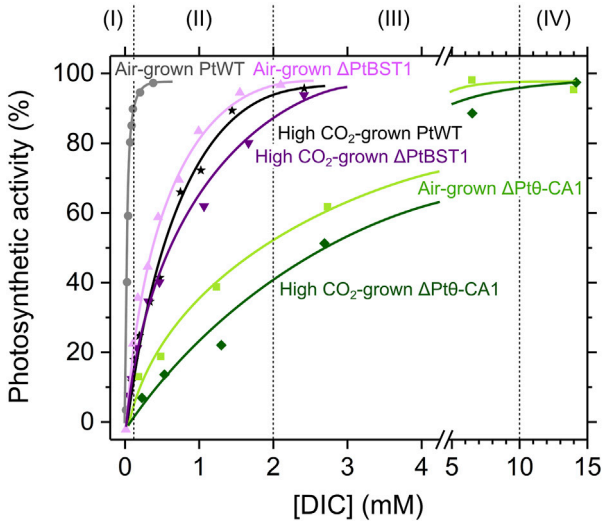

FIGURE 2
Photosynthetic affinity with dissolved inorganic carbon (DIC) in the wild type (PtWT) and the genome editing mutants (ΔPtBST1 and ΔPtθ-CA1) in the marine diatom *Phaeodactylum tricornutum*. The representative data in Shimakawa et al. (2023a) and Nigishi et al. (2024) are used after normalization of net O_2 evolution rates (μmol mg^{-1} Chl *a* h^{-1}) to P_{max} as 100%. Grey circles, atmospheric CO_2 (air)-grown PtWT; black stars, 1% (high) CO_2-grown PtWT; light purple triangles, air-grown ΔPtBST1; purple inverted triangles, high CO_2-grown ΔPtBST1; light green squares, air-grown ΔPtθ-CA1; green diamonds, high CO_2-grown ΔPtθ-CA1.

conserved in diatoms at the genetic level. Indeed, the centric diatom *T. pseudonana* also possesses stromal CA, thylakoid-luminal CA, thylakoid-localized BST, and PyShell proteins (Nawaly et al., 2023b; Nam et al., 2024; Nigishi et al., 2024). Overall, we speculate that the "CO_2-evolving machinery" we modeled in *P. tricornutum* and *T. pseudonana* is a central and evolutionary-conserved system of the diatom CCM.

Photosynthetic affinity for DIC in the genome editing mutants for CCM components

The efficiency of the algal CCM can be evaluated by measuring the dependency of photosynthetic activity (O_2 evolution rate) on total external DIC concentrations ([DIC]) reflected in the so-called "*P*-DIC curve" (Figure 2). The wild type *P. tricornutum* (PtWT) shows the maximum photosynthetic activity (P_{max}) even at very low [DIC], and the [DIC] giving half of P_{max} ($K_{0.5}$) is *ca.* 0.01–0.05 mM (Matsuda et al., 2001; Shimakawa et al., 2023a; Nigishi et al., 2024). This order of $K_{0.5}$ value is also observed in other marine diatoms fully acclimated to low CO_2 conditions (Tsuji et al., 2017a; Tsuji et al., 2021; Shimakawa et al., 2024a). In *P. tricornutum*, several CCM components, including plasma membrane-localized SLC4s, stromal β-CAs, and BST1, are highly downregulated at the transcript levels in 1–5% CO_2 conditions (high CO_2) (Ohno et al., 2011; Nakajima et al., 2013; Nigishi et al., 2024), resulting in the higher $K_{0.5}$ (approximately 0.2–0.8 mM) in PtWT when the cells are grown in high CO_2 bubbling (Matsuda et al., 2001; Shimakawa et al., 2023a; Nigishi et al., 2024). This indicates that CCM is repressed by high CO_2 growth conditions.

Recent advances in molecular biology make it possible to isolate genome-edited mutants deficient in CCM components in *P. tricornutum* and *T. pseudonana*. We have found notable differences in *P*-DIC curve in two mutants defective of BST1 and PPT luminal θ-CA in *P. tricornutum*. The knock-out mutants of BST1 (ΔPtBST1) showed the higher $K_{0.5}$ (around 0.2 mM) than that in PtWT (Figure 2). This value is close to $K_{0.5}$ in high-CO_2 grown PtWT, suggesting that the BST is the important component that contributes to the induced-level DIC affinity in PtWT grown in sub-atmospheric CO_2 conditions. Meanwhile, the $K_{0.5}$ in ΔPtBST1 slightly increased when the cells were grown under high CO_2, indicating that other low CO_2 inducible CCM components such as plasma membrane-localized SLC4s and stromal β-CAs also contribute to the CCM induction (Nigishi et al., 2024). In the knock-out mutants of the PPT luminal θ-CA (ΔPtθ-CA1), we observed the extremely low photosynthetic affinity for DIC than that in PtWT, giving $K_{0.5}$ exceeding 2 mM in both air- and high CO_2-grown cells of ΔPtθ-CA1 (Figure 2) (Shimakawa et al., 2023a), indicating that low CO_2 inducible CCM components cannot rescue the deficit of the CA localized in the PPT lumen. Importantly, a very similar null CCM phenotype was also observed in the genome edited knock-out mutant of PyShells in *T. pseudonana*, a strain that fails to construct a proper pyrenoid structure with PPT at the core (Shimakawa et al., 2024a). We thus model that pyrenoid structure harboring the right arrangement of PPT is a critical factor for the CO_2 evolving machinery and that PPT luminal CA is a pivotal component in this machinery operation.

Based on the [DIC] required for P_{max} in *P*-DIC curves shown in Figure 2, we defined several phases of CO_2-dependent photosynthesis in diatoms: Phase I, less than 0.1 mM; Phase II, 0.1–2 mM; Phase III, 2–10 mM; and Phase IV, more than 10 mM of [DIC], which differ in their rate-determining steps of CO_2 utilization for photosynthesis (Figure 1).

Phase I: photosynthesis is limited even in the presence of fully functional CCM

At Phase I, photosynthesis is limited by very low levels concentrations of DIC (~0.1 mM), which are much smaller than the abundance of DIC in natural seawater (*ca.* 2 mM). Such a strong CO_2 limitation may arise in natural tide pools and artificial culture tanks containing heavily concentrated cells. Less than 0.1 mM DIC in natural seawater contains less than 0.001 mM CO_2 which is far lower than the Michaelis constant for the carboxylation reaction of Rubisco (0.052 mM and 0.036 mM with and without atmospheric O_2) (Young et al., 2016). This indicates that CO_2 in the pyrenoid matrix is higher than the external one. Thus, CO_2 is literally "concentrated" by a factor way exceeding 50, perhaps approaching 100 times. In this situation, HCO_3^- is actively pumped first into the chloroplasts by chloroplast membrane transporters and finally to the thylakoid lumen, while CO_2 leakage is strictly prevented in the chloroplast stroma. During the operation of the chloroplastic CCM, a steep drawdown of cytosolic DIC occurs, but it is replenished quickly by efficient HCO_3^- transport across plasma membrane from the environment.

Overall, maximum photosynthesis is fulfilled by CCM components in PtWT grown in atmospheric CO_2 conditions (Figure 1), and the range of [DIC] giving Phase I defines the exact limit of CCM. The rate-determining step of photosynthesis in diatom cells at Phase I is not yet evidenced, but it is presumably the same as that at Phase II (described below). We note that *P. tricornutum* has relatively higher photosynthetic affinity for DIC (lower $K_{0.5}$) even among diatom species (Tsuji et al., 2017a), which could be due to the high ability to prevent CO_2 leakage from chloroplasts.

Phase II: photosynthesis is limited by restoration of stromal DIC into the thylakoid lumen

The *P*-DIC curve in ΔPtBST1 (Figure 2) suggests that the transport of HCO_3^- from the chloroplast stroma into the lumen of thylakoid membranes is a major rate-determining step for cells in a DIC range between 0.1 and 2 mM. The HCO_3^- transport by BST should depend on (i) the permeability of HCO_3^-, (ii) the gradient of HCO_3^- concentrations, and (iii) ΔΨ across thylakoid membranes. Thylakoid HCO_3^- permeability is determined by the abundance of channels, corresponding to the balance of synthesis and degradation of BST. Indeed, PtWT grown under 1% CO_2 showed little expression of BST1 and exhibited a similar affinity for DIC during CO_2 assimilation to that in the ΔPtBST1 mutants (Nigishi et al., 2024). In *P. tricornutum*, the active DIC pumping into chloroplasts would be powerful enough to supply the chloroplast stroma with enough HCO_3^- to provide a saturating concentration of CO_2 for Rubisco, at least, where the external [DIC] is more than 0.1 mM. Additionally, stromal CA is expected to play an important role in preventing CO_2 leakage and maintaining adequate levels of stromal DIC. Without the leakage barrier, CO_2 could quickly diffuse out of the chloroplast, reducing the efficiency of the CCM. In the CCM model in the green alga *Chlamydomonas reinhardtii*, the occurrence of a leakage barrier at the pyrenoid could account for their role of CCM in the cells grown in the atmospheric CO_2 (Fei et al., 2022). Perhaps, the leakage barrier model could be applicable to the CCM at Phase II in diatoms. In these contexts, stromal CA would mainly play a CO_2-leakage resistant role in diatom chloroplasts at Phase II. Furthermore, PyShell and the stroma-thylakoid membranes surrounding the pyrenoid could be a physical barrier against the diffusion of CO_2. It should be noted that such physical barrier could also prevent O_2 influx into the pyrenoid. In diatoms, there is also an additional system that could work as a leakage barrier of CO_2 from the chloroplast; that is, the four-layered membrane system of chloroplast. This complicated membrane system comprises two additional matrices specific to chloroplasts derived from secondary endosymbiotic events (like diatoms) but absent in chloroplasts of primary endosymbionts, that is the CER lumen and PPC. Indeed, there are numerous CAs located in these membrane matrices even though the exact matrix that each CA is present in has not yet been determined (Tachibana et al., 2011; Samukawa et al., 2014; Jensen et al., 2019). The active HCO_3^- transporters on the plasma membrane and the chloroplast membranes are less critical in Phase II relative to Phase I, but still important to maintain DIC levels against the drawdown in the cytosol and the stroma. In thylakoid membranes, photosynthetic electron transport is driven by light energy to generate ΔΨ as a component of the *proton motive force*, implying that the HCO_3^- transport *via* PtBST1 is promoted under illumination. In nature, marine diatoms could be easily exposed to the low [DIC] characteristic of Phase II, either where the cell concentrations are high or where the DIC supply is limited.

Phase III: photosynthesis is limited by CO_2 evolution inside the pyrenoid

Here, we defined the [DIC] at Phase III as 2–10 mM, which is equal to or more than the [DIC] in natural seawater. In Phase III, ΔPtθ-CA1 does not achieve P_{max} (Figure 2), indicating that the CO_2-evolving machinery in PPT membranes is a rate-determining step for photosynthesis at these levels. In other words, CO_2 is not supplied quickly enough to Rubisco in the pyrenoid matrix without the CO_2-evolving machinery even at mM-order stromal [DIC]. The DIC equilibration should be largely shifted to HCO_3^- in the stroma (pH 8.0), resulting in 0.077 mM CO_2 even for 10 mM total DIC at 20°C, in which the Rubisco carboxylation would show only 60–70% activity of V_{max} (Young et al., 2016). Additionally, Rubisco is densely packed in the pyrenoid. Because of this, in the absence of the CO_2 evolving machinery, CO_2 diffusing into the pyrenoid from the surrounding stroma is quickly fixed by Rubisco on the periphery of the pyrenoid. This may be especially true in the case of *P. tricornutum*, where the pyrenoid is expected to be immobile (Oh et al., 2023). Nevertheless, the CO_2-dependent photosynthesis in ΔPtθ-CA1 almost reaches P_{max} at 10 mM DIC, indicating that the stromal [DIC] should be maintained much higher than extracellular [DIC] in *P. tricornutum* also at Phase III owing to the function of CO_2-evolving machinery. The difference in $K_{0.5}$ between atmospheric and high CO_2 grown ΔPtθ-CA1 (Figure 2) (Shimakawa et al., 2023a) would be derived from low CO_2 inducible components for the HCO_3^- transport (Nakajima et al., 2013). We mention again that neither the PPT luminal CA nor the proper pyrenoid structure with PPT are dispensable for marine diatoms to achieve P_{max} within 10 mM of [DIC] (Shimakawa et al., 2023a; Shimakawa et al., 2024a). Overall, under DIC conditions generally found in nature, the CO_2 substrate for diatom Rubisco is dominantly supplied from the lumen of PPT membranes at the pyrenoid core.

Phase IV: photosynthesis is limited by DIC transport to the chloroplast stroma

At Phase IV (more than 10 mM external [DIC]), extracellular CO_2 should be directly taken up to the cells and passively diffused to the chloroplast stroma. Additionally, external HCO_3^- is actively pumped into chloroplasts to keep the stromal [DIC] higher than the outside of cells. We note that the CO_2-evolving machinery is dispensable at Phase IV, and CO_2 directly delivered from the stroma to Rubisco in the pyrenoid matrix is enough to produce P_{max} in *P. tricornutum*. Such extremely high DIC concentration likely does not occur in much of the present ocean environment, but it does describe some unique situations such as an artificial culture tank bubbled with high CO_2 gas and (potentially) a future high CO_2

world of more than 5 times current pCO_2. Approximately 0.3 mM CO_2 can fulfill the maximum turnover of Rubisco carboxylation (Young et al., 2016), which corresponds to 40 mM total DIC at pH 8.0. In such an absolutely unnatural situation, CO_2 could be supplied *via* passive diffusion, and marine diatoms would not require the use of a CCM. Nevertheless, the pyrenoid structure is maintained even when bubbling in 1% CO_2 in *P. tricornutum* (Kikutani et al., 2016).

Conclusion and perspectives

Here, we defined multiple phases of CO_2-dependent photosynthesis in marine diatoms, based on the phenotype of genome-edited mutants deficient in pyrenoid structure and CO_2-evolving machinery. In natural marine environments containing 2 mM DIC, Phase II and Phase III are, respectively, assumed to be the conditions with and without the limitation of CO_2 availability, in which the incorporation of HCO_3^- into the thylakoid lumen and the PPT architecture mainly support photosynthetic CO_2 assimilation. The CO_2-evolving machinery should be functionally coupled with photosynthetic electron transport on thylakoid membranes, because lumen acidification is theoretically essential for the conversion of HCO_3^- to CO_2. Indeed, it has been demonstrated that alternative electron transports promote the formation of ΔpH to support CCM in *C. reinhardtii* (Burlacot et al., 2022), although it is unclear if this occurs in diatoms. On the contrary, the HCO_3^- transport into the thylakoid lumen may indirectly decrease ΔpH to relieve heat dissipation of excess light energy (the so-called qE quenching), which is a possible combination of light and CO_2 utilization by diatom cells (Nigishi et al., 2024). It should be also noted that diatom cells evolve CO_2 also at tricarboxylic acid cycle and urea cycle in mitochondria (Allen et al., 2011). However, restoration of DIC from mitochondria to chloroplasts is still elusive in diatoms (Shimakawa et al., 2024b).

The green alga *C. reinhardtii*, another well studied algal species in CCM research, shows some similarities to the diatom CCM in its DIC utilization strategy for photosynthesis, which suggests convergent evolution of the pyrenoid-based CCM in a variety of algae. For example, the LCIB-LCIC complex, which is a most probably θ-type CA complex, and BST function in blocking leakage and recycling the unfixed CO_2 in chloroplasts (Yamano et al., 2010; Mukherjee et al., 2019), and the α-type CA evolves CO_2 in the lumen of pyrenoid tubules (Raven, 1997), which can be defined as functional analogs to the essential components for Phase II and Phase III in the photosynthesis of *P. tricornutum*. In *C. reinhardtii*, the pyrenoid disperses at Phase IV, which may have the advantage for Rubisco to utilize CO_2 in the stroma. Importantly, the chloroplast and pyrenoid architecture are likely to be largely different among different eukaryotic algal species (He et al., 2023). Pyrenoids lacking thylakoid-penetrating membranes are observed in many algae, and the existence of genes encoding CA and BST is also varied. Although the multiphase model of CCM fits to *P. tricornutum*, *T. pseudonana*, and presumably *C. reinhardtii*, it still remains to be seen if it accurately describes the CO_2-concentrating and -evolving strategies of other phytoplankton on the earth.

Author contributions

GS: Writing–original draft, Writing–review and editing. YM: Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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