PHILOSOPHICAL TRANSACTIONS B

rstb.royalsocietypublishing.org

Review

Cite this article: Matsuda Y, Hopkinson BM, Nakajima K, Dupont CL, Tsuji Y. 2017 Mechanisms of carbon dioxide acquisition and CO_2 sensing in marine diatoms: a gateway to carbon metabolism. *Phil. Trans. R. Soc. B* **372**: 20160403.
http://dx.doi.org/10.1098/rstb.2016.0403

Accepted: 31 January 2017

One contribution of 16 to a theme issue 'The peculiar carbon metabolism in diatoms'.

Subject Areas:
biochemistry, cellular biology, molecular biology, physiology

Keywords:
marine diatom, CO_2-concentrating mechanism, HCO_3^- transport, photosynthesis, carbonic anhydrase

Author for correspondence:
Yusuke Matsuda
e-mail: yusuke@kwansei.ac.jp

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3794785.

THE ROYAL SOCIETY PUBLISHING

Mechanisms of carbon dioxide acquisition and CO_2 sensing in marine diatoms: a gateway to carbon metabolism

Yusuke Matsuda[1], Brian M. Hopkinson[2], Kensuke Nakajima[1], Christopher L. Dupont[3] and Yoshinori Tsuji[1]

[1]Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, Hyogo 669-1337, Japan
[2]Department of Marine Sciences, University of Georgia, Athens, GA 30602, USA
[3]J. Craig Venter Institute, La Jolla, CA 92037, USA

YM, 0000-0002-1892-4397; KN, 0000-0002-6134-6676

Diatoms are one of the most successful marine eukaryotic algal groups, responsible for up to 20% of the annual global CO_2 fixation. The evolution of a CO_2-concentrating mechanism (CCM) allowed diatoms to overcome a number of serious constraints on photosynthesis in the marine environment, particularly low $[CO_2]_{aq}$ in seawater relative to concentrations required by the CO_2 fixing enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), which is partly due to the slow diffusion rate of CO_2 in water and a limited CO_2 formation rate from HCO_3^- in seawater. Diatoms use two alternative strategies to take up dissolved inorganic carbon (DIC) from the environment: one primarily relies on the direct uptake of HCO_3^- through plasma-membrane type solute carrier (SLC) 4 family HCO_3^- transporters and the other is more reliant on passive diffusion of CO_2 formed by an external carbonic anhydrase (CA). Bicarbonate taken up into the cytoplasm is most likely then actively transported into the chloroplast stroma by SLC4-type transporters on the chloroplast membrane system. Bicarbonate in the stroma is converted into CO_2 only in close proximity to RubisCO preventing unnecessary CO_2 leakage. CAs play significant roles in mobilizing DIC as it is progressively moved towards the site of fixation. However, the evolutionary types and subcellular locations of CAs are not conserved between different diatoms, strongly suggesting that this DIC mobilization strategy likely evolved multiple times with different origins. By contrast, the recent discovery of the thylakoid luminal θ-CA indicates that the strategy to supply CO_2 to RubisCO in the pyrenoid may be very similar to that of green algae, and strongly suggests convergent coevolution in CCM function of the thylakoid lumen not only among diatoms but among eukaryotic algae in general. In this review, both experimental and corresponding theoretical models of the diatom CCMs are discussed.

This article is part of the themed issue 'The peculiar carbon metabolism in diatoms'.

1. Introduction

The carbon-dioxide-concentrating mechanism (CCM) is a major evolutionary innovation for aquatic photosynthetic organisms that helps them to overcome constraints on acquisition of the photosynthetic substrate, CO_2. These systems are of particular relevance in the marine environment, where CO_2 availability is consistently low because of the aqueous chemistry of seawater, most notably its alkaline pH, high pH buffer capacity and high salinity. The solubility of CO_2 rarely exceeds 25 µM and the rate of dehydration of HCO_3^- to CO_2 is slow. Carbon dioxide as high as 25 µM is generally below the concentration needed to support fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) in most marine microalgae [1–4].

Marine diatoms are major primary producers in the oceans and are responsible for up to 20% of annual global primary production [5,6]. A long history of physiological experiments showed that diatoms take up both CO_2 and HCO_3^- and concentrate dissolved inorganic carbon (DIC) in their cells [7–12], strongly suggesting the occurrence of a CCM. However, only a select number of molecular components of diatom CCMs had been elucidated until very recently. The first breakthrough in identifying the molecular components of diatom CCMs was the isolation and characterization of two pyrenoidal β-carbonic anhydrases (CAs), PtCA1 and PtCA2, in the marine pennate diatom *Phaeodactylum tricornutum* [13–17]. Studies on CA in the context of the CCM had also revealed interesting new subsets of CAs in the marine centric species *Thalassiosira weissflogii* and *Thalassiosira pseudonana*, reinforcing the notion that CAs have evolved many times through convergent evolution and leading to new marine type CA classes denoted δ-CAs, ζ-CAs and θ-CAs [18–22]. In the mid to late 2000s, the sequencing of diatom genomes and development of molecular tools for diatoms including genetic transformation systems enabled more rapid identification of CCM components. CAs have been identified and localized in the model diatoms *P. tricornutum* and *T. pseudonana*, revealing a considerable diversity of origins and subcellular localizations of these CAs [15,23]. In contrast with such divergent features of diatom CCMs, convergent aspects in the function of the pyrenoid have recently begun to be recognized [22,24].

While molecular-level analysis of diatom CAs advanced, diatom DIC transport mechanisms were far less understood until recently. Nakajima *et al*. [25] reported the first discovery of a diatom DIC transport mechanism when they characterized a plasma membrane solute carrier (SLC) 4 type transporter, PtSLC4-2 in *P. tricornutum* which was shown to be a Na^+-dependent HCO_3^- transporter allowing this species to access the abundant extracellular HCO_3^- in seawater. Orthologous SLC4-type transporters are found in many diatom genomes and may also be located in chloroplast membranes and probably function as DIC transporters.

CCMs are highly responsive to environmental conditions, especially CO_2 levels, and the regulatory mechanisms that mediate these responses are of great interest. In *P. tricornutum*, transcriptional level regulation of the nuclear-encoded pyrenoidal β-CA genes, *Ptca1* and *Ptca2*, have been investigated extensively as representative genes of the diatom CCM. The role of the promoter regions of these genes (P*Ptca1* and P*Ptca2*) in responding to changes in CO_2 levels has been studied using the β-glucuronidase (GUS) reporter assay. These studies showed that transcriptional regulation of PtCA1 and PtCA2 are governed by the second messenger cAMP via interactions of a basic-ZIP (bZIP) type transcription factor, PtbZIP11, with its counterpart *cis*-element in the promoter regions, which were denoted CO_2–cAMP-responsive elements (CCREs) [26,27]. Through transcriptomic analysis, CCRE-bZIP was implicated in CO_2-based regulation of the CCM and photorespiration in *T. pseudonana* [28], strongly suggesting CCRE-bZIP CO_2 response systems are a general feature in marine diatoms. Recent work on the CCRE-bZIP-based regulation system revealed that light intensity signals are also integrated into this signalling pathway [29,30], indicating a pivotal function of the cAMP-mediated CO_2 signal transduction system as a point of cross-talk between CO_2 and light signals.

Taking this molecular information on CCM components and their regulation into account, mathematical modelling of the diatom CCM and its dynamics are also being refined [16,31,32]. However, the functions of the four-layered chloroplast envelopes of diatoms and the pyrenoid are still largely unknown at the molecular level, knowledge which is essential for further refinement of diatom CCM models.

In this review, recent progress in understanding the diatom CCM is updated and perspectives on future directions of the field are discussed. Finally, it should be noted that some diatom species are reported to incorporate a C_4-like biochemical CCM system. For brevity, however, this review focuses on the biophysical CCM.

2. Membrane dissolved inorganic carbon transport systems

(a) Plasma membrane HCO_3^- transport systems

The uptake of DIC across the plasma membrane represents the critical first step in supplying DIC for photosynthesis, and is especially challenging because environmental conditions are variable and in some cases unpredictable. Diatoms have been shown to actively take up HCO_3^- and/or CO_2, which has been demonstrated using a range of physiological approaches [7–12,33–35]. However, until recently, the molecular mechanisms responsible for DIC uptake and internal transport, and the regulatory mechanisms controlling these fluxes were not known.

In a recent study, it was demonstrated that a plasma membrane-localized transporter in the marine diatom *P. tricornutum* homologous to the mammalian SLC4 family, PtSLC4-2, functions as a major uptake mechanism for the acquisition of extracellular DIC under low-CO_2 conditions [25] (figure 1). PtSLC4-2 specifically transports HCO_3^- in the presence of a high concentration of sodium ions with a saturation level of about 100 mM Na^+. The HCO_3^- uptake rate of this protein reached its maximum level at pH 8.2, which is the typical pH of seawater [25]. Like PtSLC4-2, two other closely related putative HCO_3^- transporters, PtSLC4-1 and PtSLC4-4, are induced specifically under low CO_2 conditions, suggesting that these transporters also make a significant contribution to HCO_3^- influx into the cell in low CO_2 environments like seawater [25,31], although their localizations and functional details have yet to be determined.

Interestingly, SLC4-type transporters in diatoms form a diatom-specific cluster and this group is phylogenetically close to members of the human SLC4 family with high bootstrap support [25]. In addition, HCO_3^- transporters already identified in cyanobacteria and the green alga *Chlamydomonas reinhardtii* do not share homology with the SLC4 transporters identified in diatoms. This strongly indicates that eukaryotic algae acquired HCO_3^- transporters independently from various ancestral eukaryotic hosts. Indeed, it has been suggested that diatom transporters share a common origin with those in human cells [25]. However, more detailed investigation is needed to substantiate this claim because several scenarios have been postulated for the evolution of the Heterokontphyta [36] and consequently, the origin of diatom SLCs may be complex. There has been no molecular work on plasma-membrane-type HCO_3^- transporters in freshwater diatoms. Physiological studies on the freshwater diatom *Navicula pelliculosa* have revealed that this species is able to take up HCO_3^- without external CA activity [37], suggesting it uses a specific HCO_3^- transporter. However, the freshwater environment does

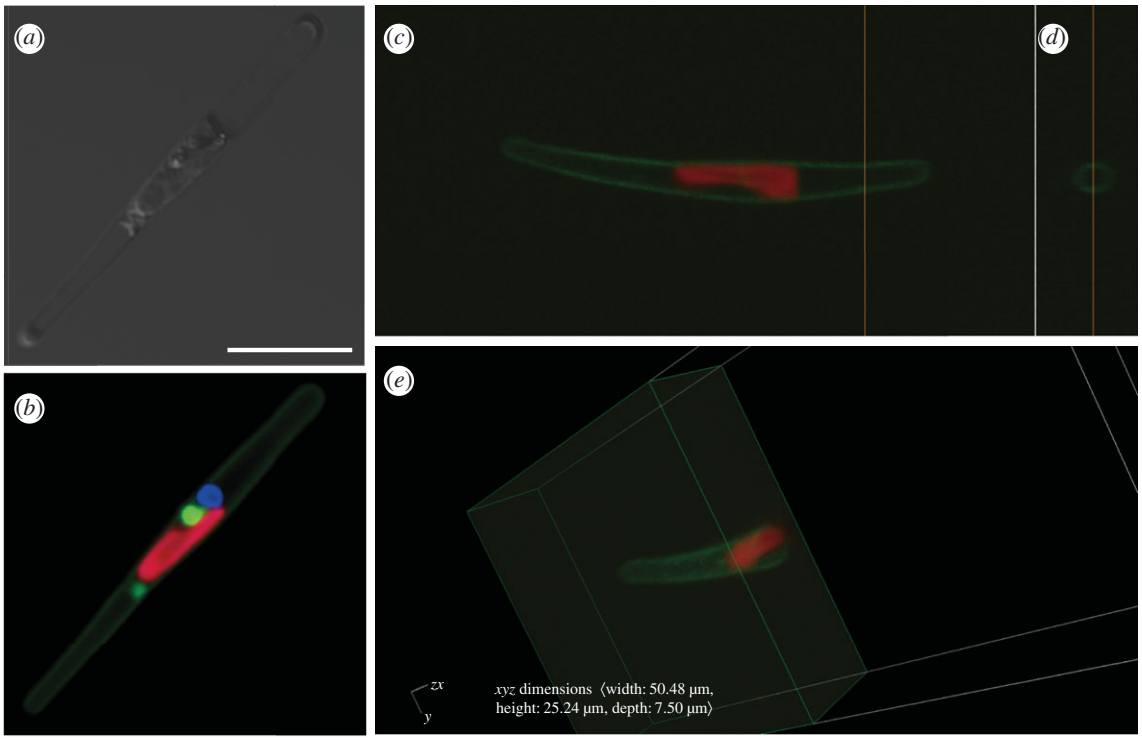

Figure 1. Localization of exogenously introduced PtSLC4-2:green fluorescent protein fusion in diatom cells. (*a*,*b*) Localization of PtSLC4-2:GFP fusion in *P. tricornutum*: (*a*) light image; (*b*) merged image of PtSLC4-2:GFP fusion (green), Hoechst-stained nucleus (blue) and auto-fluorescence of the chloroplast (red). (*c*–*e*) Z-stacked images with GFP signals (green) and chlorophyll auto-fluorescence. (*d*) The cross-section at the orange line in (*c*). (*e*) The cross-section of tiled image *c* at the middle part of the cell. Scale bar,10 µm.

not fulfil the Na^+ requirement needed by the SLC4 studied in the marine diatom *P. tricornutum*, strongly suggesting the occurrence of a different type of plasma membrane HCO_3^- transporter.

(b) Bicarbonate transport in the plastidic membrane system

The four-layered chloroplast membranes represent a series of barriers that prevent DIC imported into the cytosol from making its way to the chloroplast for fixation. Therefore, it has been suggested that HCO_3^- transporters are located at each four-layered chloroplast membrane [31] and that these transporters, in conjunction with CAs densely packed in the spaces between chloroplast membranes, control the permeation of DIC [15,23]. However, chloroplast-membrane-type HCO_3^- transporters have not yet been identified in diatoms.

There are seven PtSLC4 genes and three PtSLC26 genes in the genome of *P. tricornutum* [25]. A sequence alignment of seven PtSLC4s is given in the electronic supplementary material, figure S1, in comparison with human SLC4s, hsSLC4A1 and hsSLC4A4, and SLC4s in *T. pseudonana*, TpSLC4-1, TpSLC4-2 and TpSLC4-3. Eleven to 12 membrane-spanning helices of human SLC4A1 were highly conserved from PtSLC4-1 to PtSLC4-5 with the exception of the first transmembrane domain unique to PtSLC4-2 (electronic supplementary material, figure S1, TM2–TM13 of PtSLC4-1–PtSLC4-5). By contrast, TM2, 4, 5, 8 and 9 were relatively well conserved throughout SLCs as compared in the electronic supplementary material, figure S1, but there were significant variations in TM 6, 7 and 10–14 in PtSLC4-6, PtSLC4-7 and three TpSLC4s. Three of the *PtSLC4* genes, *PtSLC4-1*, *PtSLC4-2* and *PtSLC4-4*, are CO_2 responsive as mentioned earlier. Two other *PtSLC4* genes, *PtSLC4-6* and *PtSLC4-7*, encode proteins previously predicted to localize in the four-layered chloroplast membrane systems based on the presence of targeting sequences [38]. In addition, the N-terminal transit peptide sequence in PtSLC4-6 (GSA-FTS), PtSLC4-7 (SAA-FHT), TpSLC4-2 (SFS-FAP) and TpSLC4-3 (VNA-FPT) includes both an endoplasmic reticulum (ER) signal and a plastid-transit sequence at the predicted cleavage site of ER signal, corresponding to one of the variants of the ASA-FAP motif [39] (the upper N-terminal sequence including these transit sequences is not shown in the electronic supplementary material, figure S1). Interestingly, *PtSLC4-6* and *PtSLC4-7* cluster phylogentically with heterokont genes which are related to the human/diatom cluster, as mentioned above. The expression levels of *PtSLC4-6* and *PtSLC4-7* genes were constitutive under high CO_2 and low CO_2 conditions [25], suggesting that PtSLC4-6 and PtSLC4-7 perhaps constantly regulate DIC flow from the cytosol to the plastid regardless of ambient CO_2 concentrations. Currently, very little is known about the intracellular localizations and functions of either PtSLC4-6 and PtSLC4-7 as DIC transporters. The driving forces for transporters within the four-layered chloroplast membranes are also not known. Most probably, these transporters would work with pH and/or ionic gradients across these membranes. In fact, there are some studies reporting the periplastidal compartment (PPC) to be an acidified compartment [40,41]. However, pH and ion regulation systems within the four-layered chloroplast membranes in secondary endosymbionts have not been identified. These are clearly enticing targets for future research.

(c) Energy to drive HCO_3^- transport

Bicarbonate uptake is an energy-dependent active transport process, but how energy consumption is coupled with HCO_3^-

transport remains elusive. In *P. tricornutum*, HCO_3^- uptake across the plasma membrane is mediated by PtSLC4-2, and this activity is dependent on the Na^+ concentration. Most likely PtSLC4-2 is a secondary active transporter, co-transporting Na^+ and HCO_3^-, making use of a transmembrane Na^+ gradient [25], which can be made quite large due to the high salinity of seawater. As Na^+ continuously flows into the cell with HCO_3^-, an efflux of Na^+ back out of the cell is required to maintain a [Na^+] gradient across the plasma membrane. This would require an ATP-dependent primary transporter to export Na^+. While the molecular identity of this putative Na^+ efflux pump has not been identified, there is some suggestive evidence for the occurrence of Na^+/K^+-ATPase and involvement of K^+ in DIC acquisition in some diatoms [42–44]. However, orthologous genes encoding known Na^+/Ka^+-ATPases were not found in diatom genomes. Alternatively, there is a possibility that a secondary Na^+/H^+ antiporter maintains the Na^+ gradient as suggested in the cyanobacterial CCM [45]. Further study is required to reveal the molecular mechanism of Na^+-dependent HCO_3^- uptake. There are several possible mechanisms to generate the ATP ultimately required for HCO_3^- uptake including photophosphorylation, cyclic electron flow (CEF) around photosystem I (PSI) and respiration. In the case of cyanobacterial and green algal CCMs, the involvement of CEF in active DIC uptake has been suggested [46,47]. By contrast, in diatoms, the rate of CEF is reported to be negligibly low relative to total electron transport activity [48], making CEF an unlikely ATP source. Most probably, ATP is generated by linear electron transport through the photosynthetic electron transport chain or respiration, although further study is needed to distinguish between the two.

(d) Diffusive CO_2 uptake

In addition to the HCO_3^- uptake facilitated by plasma membrane SLC4, diatoms take up CO_2 from the external environment to support photosynthesis [7,10,11]. Because lipid bilayer membranes are permeable to CO_2 [49], CO_2 uptake cannot proceed through a typical membrane-embedded transporter mechanism. Instead, organisms take up CO_2 through a diffusive mechanism by generating a CO_2 deficit inside the cell, which then draws CO_2 in from the external environment. In cyanobacteria, this deficit is generated by active conversion of CO_2 to HCO_3^- in the cytoplasm through the action of NADPH dehydrogenase (NDH-1) complexes [50] with ferredoxin likely acting as the electron donor to NDH-1 [51,52]. In diatoms, this deficit was instead proposed to be generated by the active transport of HCO_3^- out of the cytoplasm and into the chloroplast resulting in a low HCO_3^- concentration in the cytoplasm [16,31]. The cytoplasmic CO_2 concentration is then lowered through the action of a cytoplasmic CA, which when the HCO_3^- concentration is below equilibrium with CO_2 will drive a net hydration of CO_2 to HCO_3^-. The CO_2 gradient passively draws CO_2 into the cell across the plasma membrane, and continued export of HCO_3^- from the cytoplasm maintains a constant cytoplasmic CO_2 deficit resulting in sustained CO_2 uptake. The activity of the transporter exporting HCO_3^- from the cytoplasm must match or exceed the rates of CO_2 and HCO_3^- influx in order to maintain the inward CO_2 gradient.

New evidence suggests this model for CO_2 uptake needs to be modified because there is no known CA localized at the cytoplasm in *P. tricornutum* [15,23] and cytoplasmic CA localized in *T. pseudonana* is a γ type [23], which is confirmed to be a CA enzyme only in bacteria and archaea [53,54] but not in eukaryotes [55]. Even under alkaline pH of diatom cytoplasm at around 7.6 [8,56], it would not be possible without CA activity to maintain an inward CO_2 gradient between the cytoplasm and the external environment, because the uncatalysed hydration rate of CO_2 is slow. Instead, the CO_2 deficit may be generated in one of the membrane-bound compartments surrounding the chloroplast (the chloroplastic ER or the periplastidal space) where CAs are definitely present rather than the cytoplasm. Modelling studies of this modified CO_2 uptake mechanism indicate that it would be functionally equivalent to the older mechanism involving the cytoplasm [31].

An additional emerging complexity in the CO_2 uptake pathway involves the possible role that aquaporins play in enhancing membrane permeability to CO_2. While aquaporins were originally identified as water channels, they are also believed to facilitate CO_2 diffusion in cyanobacteria [47,57], higher plant mesophyll cells [57,58] and red blood cells [59,60] and are found in diatom genomes. While lipid bilayers are inherently permeable to CO_2, they do present some resistance to diffusion that could be reduced by the presence of aquaporins. CO_2 permeation through diatom membranes is very rapid [16], and this high permeability may be in part due to the presence of channels such as aquaporins.

3. Carbonic anhydrase as a mobilizer and an insulator for dissolved inorganic carbon movement

(a) Carbonic anhydrases and their locations in diatom cells

CA is typically an extremely fast enzyme and catalyses CO_2 hydration and HCO_3^- dehydration moving the CO_2/HCO_3^- system towards equilibrium [61]. As CA interconverts CO_2 and HCO_3^-, and these inorganic carbon species have contrasting membrane permeability, CA must have significant roles in controlling the direction and magnitude of DIC fluxes, as will be described in the next section. In the marine diatom *P. tricornutum*, at least 10 putative CA genes from four families have been identified in its genome. The most prominent CA family is the α-CA family with five members, but there are also two β-CAs, two γ-CAs and one CA from the newly discovered θ family [15,22]. These 10 CAs have all been localized and interestingly, they display specific localizations based upon subtype: α-CAs are located at the four-layered chloroplastic membrane, β-CAs in the pyrenoid, γ-CAs in the mitochondria and the θ-CA in the thylakoid lumen [15,22]. No cytosolic CAs have been identified so far in *P. tricornutum*. Of these 10 CAs, CA activity has only been confirmed with two β-CAs and one θ-CA, but the Zn binding sites in most of the other CAs are intact, suggesting they are functional [17,22]. Most of the CAs are not regulated transcriptionally by CO_2, but the two pyrenoidal β-CAs, PtCA1 and PtCA2, are highly CO_2 responsive at the transcriptional level via signal transduction involving a second messenger cAMP [26,27,29]. Transcription of *Ptca1* and *Ptca2* is also controlled by light using the same signal transduction pathway as that used to respond to CO_2 [29],

illustrating that there is a cross-talk between light and CO_2 signals, two factors that have a major effect on the need to generate CO_2 for photosynthesis [29]. Interestingly, these two CAs are also regulated at the post-translational level by the redox state of the chloroplast through the counteraction of thioredoxins (Trxs) and molecular oxygen [17]. The reduced forms of PtCA1 and PtCA2 showed higher activity than oxidized forms [17]. Oxygen from PSII and reduced Trxs would competitively modulate CA activity, suggesting the occurrence of a system to fine tune pyrenoidal CA activities during photosynthesis. In *P. tricornutum*, there are no external, cytosolic nor free stromal CAs.

In *T. pseudonana*, at least 13 putative CA genes have been identified [23]. In sharp contrast with the case of *P. tricornutum*, subcellular locations of *T. pseudonana* CAs are not related to the CA subtypes [15,23]. Also in contrast with *P. tricornutum*, there is no identified pyrenoidal CA, but there is a stromal α-CA, a cytosolic γ-CA, and two external CAs, one δ-CA and one ζ-CA [23]. There are also three γ-CAs and one δ-CA in the mitochondria, and one δ-CA in the four-layered chloroplast membrane system [23]. Of these CAs, the activity of δ-CA is confirmed [62,63] and also ζ-CA activity has been confirmed in *T. weissflogii* [21]. Among these putative CAs in *T. pseudonana*, transcript levels of two external CAs (Tpδ-CA1 and Tpζ-CA1) and a CA in the PPC (Tpδ-CA1) were greatly increased in air-grown cells relative to those in high CO_2-grown cell [23]. The result strongly suggests contributions of these putative CAs to DIC acquisition and/or recapturing leaked CO_2 under CO_2-limited conditions.

Interestingly, in addition to these 13 potential CAs, the occurrence of the new subtype θ-CA family with chloroplast and thylakoid targeting motifs at its N-terminus was discovered recently in the *T. pseudonana* genome [20], suggesting the generality of occurrence of this type of CA in the lumen of diatom thylakoid.

The localization of CAs in these two model diatoms are updated in figure 2. Even within diatoms, the subtype of CAs found in each species and their localizations are extremely diverse, strongly suggesting that CA genes were acquired by diatoms from diverse origins after they had undergone substantial diversification and that these CAs may be involved in diverse strategies for DIC flux control depending upon the diatom species.

(b) Function of carbonic anhydrases in diatom CO_2-concentrating mechanisms

CAs may have multiple critical functions in diatom CCMs, including roles in CO_2 uptake, generation of CO_2 in the chloroplast for fixation and recovery of CO_2 leaking out of the chloroplast. As discussed above, CO_2 uptake proceeds by the generation of an internal CO_2 deficit, proximally generated by the CA-catalysed hydration of CO_2 to HCO_3^- in the cytoplasm or other compartment [16]. External CAs, located in the periplasmic space [15,23], facilitate CO_2 uptake by generating CO_2 from HCO_3^- at the cell surface [64]. These external CAs are apparently very important for diatom CCMs as they occur in a number of diatoms species and are expressed at very high levels, in contrast with other microalgae where they are less commonly found [11,65,66].

Bicarbonate imported into the cell must be converted to CO_2 for fixation by RubisCO and CAs are necessary to catalyse this conversion, because the intrinsic dehydration rate of bicarbonate is slow. In *P. tricornutum*, a newly discovered route to produce CO_2 involves import of HCO_3^- into the pyrenoid-penetrating thylakoid lumen where the low pH and action of a θ-CA rapidly converts HCO_3^- to CO_2 for RubisCO [22]. β-CAs inside the pyrenoid also convert bicarbonate that diffuses in the stroma into CO_2, elevating the CO_2 concentration around RubisCO [14,15]. A portion of the CO_2 supplied to RubisCO is fixed, but the CCM is not perfectly efficient, and a significant fraction of the CO_2 leaks out of the chloroplast [16]. This leaked CO_2 is recovered by CA-catalysed conversion to HCO_3^- back in the cytoplasm or other outlying compartment, and the regenerated HCO_3^- can be transported back into the chloroplast. Like many other aspects of the diatom CCM, such CA-based CO_2 recovery systems seem to be highly diverse. In *T. pseudonana*, either the cytoplasmic, chloroplast envelope or stromal CAs could function in CO_2 recovery. In sharp contrast, *P. tricornutum* has numerous chloroplast envelope CAs but lacks cytosolic and stromal CAs [15,23], strongly suggesting that the main recovery points are in the four-layered chloroplastic envelope. It should also be noted that pyrenoidal β-CAs in *P. tricornutum* may be a part of such a recovery system of leaking CO_2, which will be discussed in the next section. The detailed molecular role of these CAs in diatoms however has yet to be determined and await further reverse genetics approaches to be confirmed.

(c) Pyrenoid functions with carbonic anhydrase

Apart from the significant divergence in the origins of chloroplast-based CAs, examples of convergence can also be found in the chloroplast and pyrenoid. The recent discovery of θ-CA (Pt43233) in the lumen of the thylakoid membrane suggests dramatic convergent coevolution of CCM function in eukaryotes. In the green alga *C. reinhardtii*, it has long been known that the final conversion of HCO_3^- to CO_2 occurs within a portion of the thylakoid that penetrates the pyrenoid. HCO_3^- is imported into the thylakoid lumen where the low pH and action of CA convert it to CO_2, which then diffuses out into the pyrenoid [67]. The newly discovered θ-CA in *P. tricornutum* is also localized specifically to the region of the thylakoid that passes through the pyrenoid and it is critical for CCM function, suggesting this final CO_2 generation step works quite similarly in the disparate diatom and green algal groups. Notably, the θ-CA sequence is not related to that of the *C. reinhardtii* thylakoid CA (an α-CA).

The *P. tricornutum* θ-CA possesses a Cys-Gly-His rich (CGHR) domain with an N-terminal chloroplast-thylakoid targeting motif and similar putative thylakoid targeted CGHR family proteins also exist in *T. pseudonana*, strongly suggesting this new class θ-CA occurs throughout the diatoms [22]. The RNAi suppression of this protein in *P. tricornutum* resulted in inhibition of growth and reduced photosynthetic DIC affinity [22], indicating the pivotal function of the thylakoid luminal CA for the diatom CCM. Interestingly, CGHR-containing proteins also occur in *C. reinhardtii* (denoted as LCIB, C, D and E). An LCIB/C hexamer complex was localized at the peripheral pyrenoid and is critical for CCM function [64,65]. CA activity of the LCIB/C complex in *C. reinhardtii* has not yet been tested, but the occurrence of proteins homologous to diatom luminal θ-CA as a pyrenoid-localized component critical to the CCM in the distant green algae [68,69] indicates that this

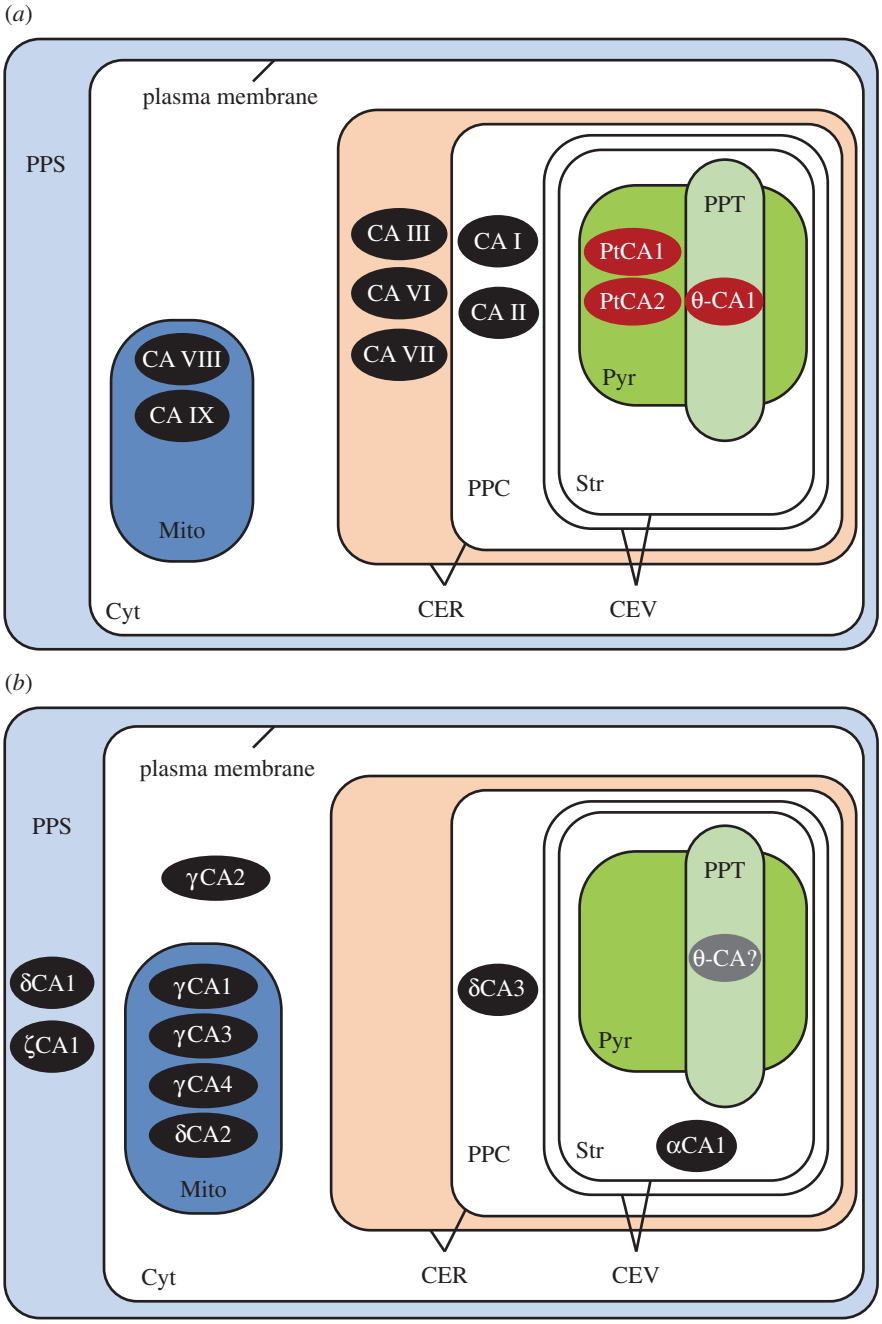

Figure 2. Subtypes and localization of CAs in *P. tricornutum* (*a*) and *T. pseudonana* (*b*). Black colouring indicates that the CA has been localized, but activity has not been confirmed, whereas red colouring indicates the CA has been both localized and confirmed to have CA activity. CAs shown in grey have been identified in the genome, but have not been localized or tested for CA activity. The compartmental abbreviations are: periplasmic space (PPS), cytoplasm (Cyt), chloroplastic endoplasmic reticulum (CER), mitochondria (Mito), periplastidal compartment (PPC), chloroplast envelope (CEV), stroma (Str), pyrenoid (Pyr) and pyrenoid-penetrating thylakoid (PPT).

CA class is commonly involved in CCM functions associated with the pyrenoid.

It is hypothesized that the LCIB/C complex in *C. reinhardtii* functions as a part of the system for recapturing CO_2 leaking out of the pyrenoid by hydrating it to HCO_3^- [69–71]. If *C. reinhardtii* LCIB/C serves as CA, CO_2 hydration occurs via LCIB/C itself in the pyrenoid, which potentially competes with the reaction of RubisCO for CO_2. This consideration also applies to the case of *P. tricornutum* pyrenoid, which possesses pyrenoidal PtCAs outside the thylakoid membrane and θ-CA at the lumen [22]. It is noteworthy that, in *C. reinhardtii* and *P. tricornutum*, the LCIB/C complex and PtCAs revealed a clumped distribution to highly localized parts in the pyrenoid, while RubisCO disperses over the pyrenoid [15,70,72], suggesting that pyrenoidal CAs localize differentially from RubisCO. Detailed localization of these components is needed to clarify their functions. Recently, a novel pyrenoidal protein EPYC1/LCI5 was identified in the green alga *C. reinharditii*. EPYC1/LCI5 is essential to the formation of a dense aggregation of RubisCO in the pyrenoid, suggesting EPYC1/LCI5 is an essential structural component for pyrenoid formation [24]. A putative structural analogue of EPYC1/LCI5 occurs in the diatom genome [24], suggesting the involvement of such structural proteins in the arrangement of diatom pyrenoidal proteins.

4. CO_2 sensing at the ocean surface

(a) CO_2/light response

Like most algal CCMs, diatom CCMs are highly responsive to the ambient CO_2 concentration [73]. In general, components are more highly expressed at low CO_2 when the CCM is

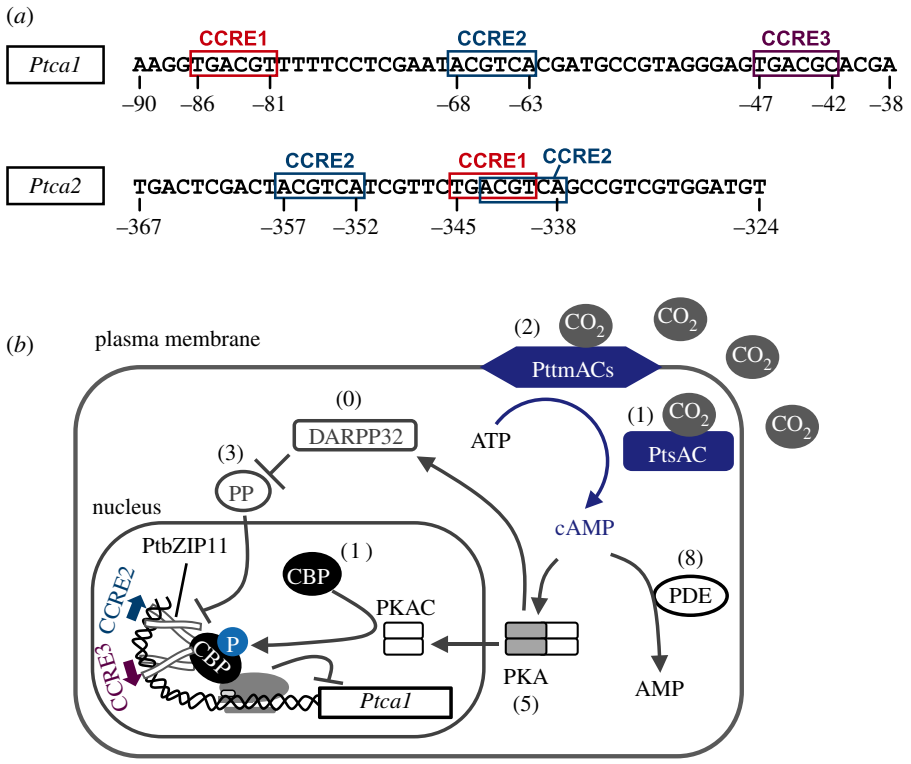

Figure 3. The CO_2-responsive elements in the *Ptca1* and *Ptca2* promoters and the putative CO_2 signalling pathway. (*a*) Structures of the core-regulatory region of the *Ptca1* and the *Ptca2* promoter. (*b*) A suggested model of the cAMP-mediated CO_2 signalling pathway based upon the mammalian cAMP signalling cascade. (Redrawn from Matsuda & Kroth [82]). This model describes the signalling route under high CO_2 conditions. PDE, cAMP phosphodiesterase; PKA, protein kinase A; PKAC, C subunit of PKA; PP, protein phosphatase; CBP, CREB (cAMP binding protein) binding protein; DARPP, dopamine- and cAMP-regulated phosphoprotein. Parentheses indicate the number of candidate genes in the *P. tricornutum* genome. No functional analogue of DARPP has been identified so far.

most needed to maintain high CO_2 concentrations around RubisCO [15,23,25,26,74,75]. Changes in the partial CO_2 pressure in the atmosphere result in a suite of changes in the concentrations of aqueous DIC species, including increased CO_2 and HCO_3^- and decreased concentrations of CO_3^{2-} in the range of pH relevant to marine systems. Typically, it is unclear which DIC species are eliciting the observed modifications of the algal CCM. However, a few quantitative investigations have been carried out on this topic. In cyanobacteria, the critical factor governing CCM expression is known to be the total DIC concentration, and photorespiratory metabolism is involved in the CO_2 signal transduction process [76–78]. By contrast, in freshwater green algae, *Chlorella* and *Chlamydomonas*, dissolved CO_2 is known to be a critical DIC species controlling CCM expression [79–81], strongly suggesting the involvement of some direct sensing systems in this process in eukaryotic algae. Similarly in diatoms, several physiological studies have shown that CO_2 is the critical determinant of the extent of CCM expression [8,10,11,34,73].

The molecular mechanisms mediating responses to CO_2 in diatoms are most well studied with regard to the transcriptional control system of two pyrenoidal β-CAs in *P. tricornutum*. It has been clearly demonstrated that transcription of both *Ptca1* and *Ptca2* are CO_2 and light responsive; i.e. the expression of these genes are stimulated by low (at least atmospheric level) CO_2 and largely repressed in enriched (1–5%) CO_2, and this expressional control requires light [26,74,75]. Interestingly, such stimulation (or de-repression) of these CA genes under low CO_2 conditions are efficiently suppressed by cAMP analogues and/or cAMP phosphodiesterase inhibitors [26,27,29], indicating an involvement of the cAMP second messenger system downstream of the CO_2 sensing mechanism (figure 3). A detailed GUS reporter assay targeting the region 1.3 kbp upstream of the transcription-start sites of both *Ptca1* and *Ptca2* revealed new *cis*-elements critical for the CO_2 response, termed CO_2/cAMP-responsive elements (CCREs: ACGTCA/G) [27,29]. Three CCRE sequences in the *Ptca1* promoter region reside within −90 bp relative to the transcription-start site and these sequences run in opposite directions to each other with 15–18 bp intervals [27]. Gel shift assays showed that these CCREs are a target of a group 4 basic-zipper (bZIP) transcription factor, PtbZIP11 in *P. tricornutum* [27,83]. The promoter region of the *Ptca2* gene also possesses three CCRE sequences with a different arrangement from that of the *Ptca1* gene promoter, and these sequences were also shown to be critical *cis*-elements mediating the CO_2 response of this gene [29]. A further transcriptomics study using *T. pseudonana* demonstrated that the transcriptional response of the CCM and photorespiratory gene clusters are responsive to CO_2 using CCRE sequences for their regulation [28], indicating a common mechanism for CO_2 signalling in diatoms.

A recent study further demonstrated that *Ptca1* and *Ptca2* genes are also light responsive and this response is governed by the same set of CCREs that are involved in the CO_2 response [29] (figure 3), strongly suggesting that the light signal is integrated with the CO_2/cAMP signal by some type of cross-talk mechanism [48]. Interestingly, a very weak dose of 2,6-dichlorophenol-indophenol, which oxidizes the acceptor side of PSI, efficiently suppressed the transcriptions of *Ptca1* and *Ptca2* under low-CO_2 and illuminated conditions [29]. This strongly suggests that light may generate a retrograde signal at the acceptor side of PSI as a part of an electron sorting system from ferredoxin, which is one of the

Table 1. CCM-associated proteins encoded in the genome of *P. tricornutum* and *T. pseudonana*, and role of each protein shown in this review. PM and CM indicate plasma membrane and chloroplast membrane, respectively.

annotation name	Protein ID	role
PtbZIP11	49205[a]	regulating
PtCA1	45433[a]	pyrenoidal carbonic anhydrase
PtCA2	51305[a]	pyrenoidal carbonic anhydrase
θ-CA	43233[a]	pyrenoid-penetrating thylakoidal lumen carbonic anhydrase
PtSLC4-1	1677[a]	PM-type bicarbonate transporter?
PtSLC4-2	bd1806[b]	PM-type bicarbonate transporter
PtSLC4-3	bd1743[b]	PM-type bicarbonate transporter?
PtSLC4-4	bd714[b]	PM-type bicarbonate transporter?
PtSLC4-5	54405[a]	PM-type bicarbonate transporter?
PtSLC4-52bd	bd52[b]	PM-type bicarbonate transporter?
PtSLC4-6	43194[a]	CM-type bicarbonate transporter?
PtSLC4-7	45656[a]	CM-type bicarbonate transporter?
PtSLC26-1	42555[a]	PM-type bicarbonate transporter?
PtSLC26-2	42556[a]	PM-type bicarbonate transporter?
PtSLC26-3	50075[a]	PM-type bicarbonate transporter?
TpSLC4-1	266801[c]	PM-type bicarbonate transporter?
TpSLC4-2	1403[c]	CM-type bicarbonate transporter?
TpSLC4-3	267979[c]	CM-type bicarbonate transporter?

[a]Protein IDs refer to JGI genome database (http://genome.jgi.doe.gov/Phatr2/Phatr2.home.html).
[b]Protein IDs refer to JGI genome database (http://genome.jgi.doe.gov/Phatr2_bd/Phatr2_bd.home.html).
[c]Protein IDs refer to JGI genome database (http://genome.jgi.doe.gov/Thaps3/Thaps3.home.html).

first examples of an involvement of PSI in a CO_2/light retrograde signal which ultimately manipulates nuclear gene expression.

(b) CO_2 with diel light and Fe signals

The micronutrient iron has a major role in diatom metabolism, particularly in the light reaction of photosynthesis and in nitrate assimilation. For both of these metabolic pathways, light levels greatly influence the regulation, and thus potentially Fe requirements. A recently published study examined the global transcriptional response of *P. tricornutum* to diel light cycles at three different Fe concentrations [84], and we have examined the expression of the CCM components detailed in this review. As part of that study, individual gene expression profiles were determined to be statistically responsive to either light or iron levels using multiple methods.

Of the various CCM components, PtCA2 is by far the most highly expressed component, with PtCA1 being expressed below statistical significance. PtCA2 had a statistically similar expression profile to PtSLC4-1, PtSLC4-5, the θ-CA and PtbZIP11. Each of these components are upregulated in light conditions relative to dark conditions and are downregulated at lower Fe concentrations. PtbZIP11 is induced by both light conditions but also by low Fe concentrations. Curiously, both PtSLC4-3 and PtSLC4-52bd are upregulated in the dark relative to the light, suggesting different functional roles to the other putative outermembrane-localized PtSLC4. PtSLC4-7 shows constitutive expression, while PtSLC4-6 is mildly upregulated in light conditions. The names of the CCM components in *P. tricornutum* referred to in this paper are listed with their corresponding Protein ID and updated annotations in table 1.

5. Models of dissolved inorganic carbon flux and its dynamic control

The basic components used to create CCMs are few in number—HCO_3^- transporters, CAs, RubisCO—and it is the careful spatial arrangement of these components and manipulation of CO_2/HCO_3^- equilibrium and gradients that combine to form efficient CCMs. Consequently, numerical models of CCMs have been especially helpful in establishing the essential functional characteristics of CCMs in diverse microalgae [1,85,86]. In the case of diatoms, the most extensive numerical modelling of the CCM has involved the diatom *P. tricornutum*. Here, model-data comparisons were used to infer quantitative rates of inorganic carbon fluxes into the cell and within the major cellular compartments [16,31]. These models suggested that a high rate of active HCO_3^- transport out of the cytoplasm and into the chloroplast (the 'chloroplast pump') was the major driver of the CCM in *P. tricornutum* leading to HCO_3^- accumulation in the chloroplast stroma and the CO_2 deficit in the cytoplasm. Furthermore, according to the transcriptional analysis of some CCM components in response to CO_2 (and partially to light), acclimation responses of the CCM to CO_2 concentration seemed to take place primarily in the DIC acquisition system from the bulk medium, while by contrast, putative components of a 'chloroplast pump' moving DIC

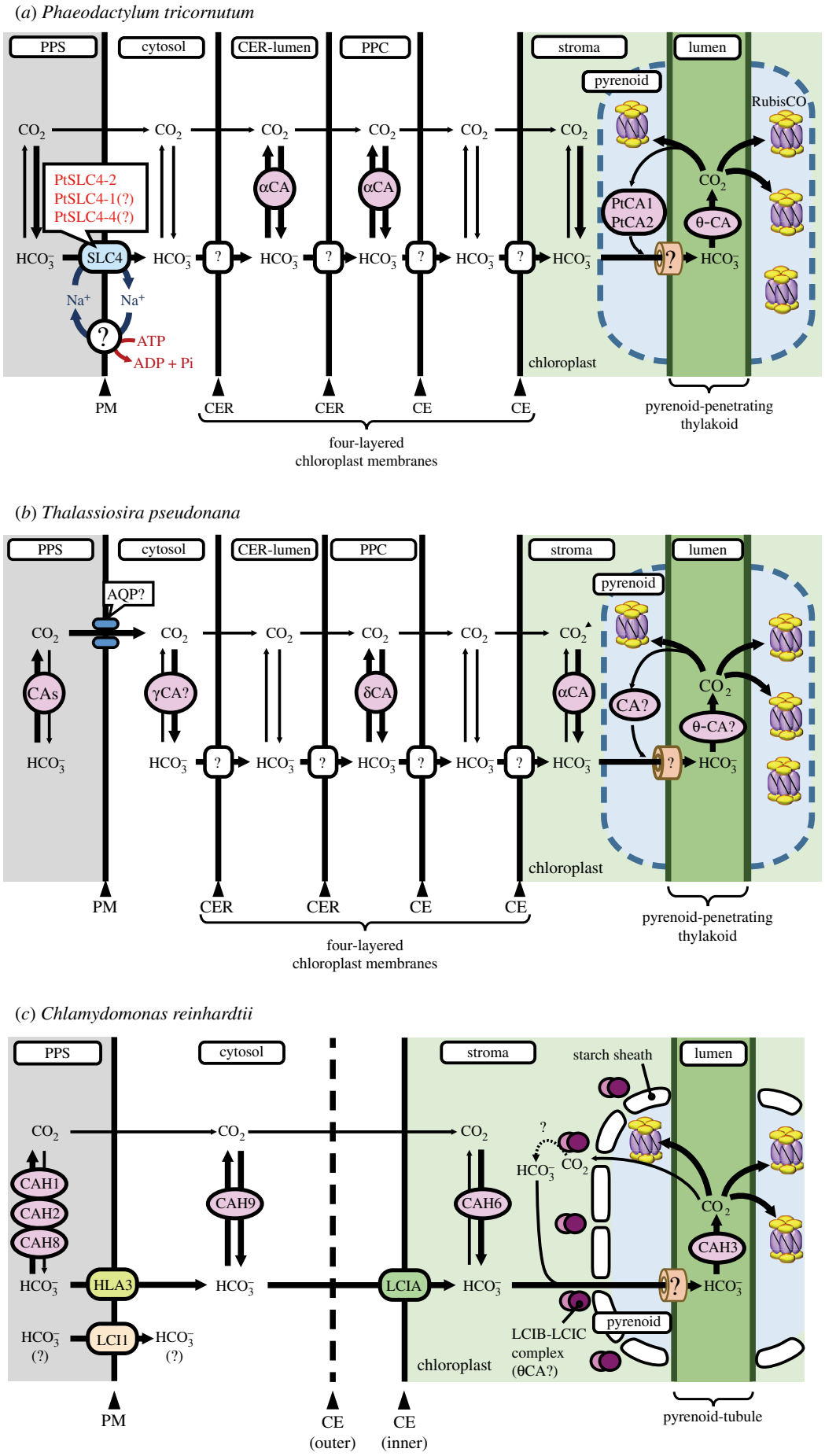

Figure 4. (*Caption overleaf.*)

from the cytoplasm to RubisCO were not affected by CO_2 availability [15,22,23,25]. The latest iteration of these *P. tricornutum* CCM models [31] emphasized the coherence between molecular and physiological data when incorporated into a modelling framework, and suggested that the system was largely understood. However, the recent discovery of the role of

Figure 4. (*Overleaf*.) Models of DIC transport in marine diatoms. (*a*) In *P. tricornutum*, plasma-membrane-located-SLC4s (PtSLC4-2, possibly PtSLC4-1 and PtSLC4-4) transport HCO_3^- from seawater in a Na^+-dependent manner, most probably $Na^+ - HCO_3^-$ cotransport. This would require an unidentified ATP-dependent primary Na^+ pump or a proton-dependent secondary pump to maintain a $[Na^+]$ gradient across the plasma membrane. The mechanism of DIC transport from the cytosol to the stroma is unknown. Unidentified transporters on CER and CE might be involved in this process in combination with CAs in intermembrane spaces. In the pyrenoid, CO_2 is generated by luminal θ-CA in the pyrenoid-penetrating thylakoid (PPT). CO_2 released from the lumen is fixed by RubisCO, or otherwise recaptured at specialized pyrenoidal loci by β-type CAs, PtCA1 and/or PtCA2 by which HCO_3^- is regenerated to be transported back to the thylakoid lumen. (*b*) In *T. pseudonana*, extracellular CA accelerates the dehydration of HCO_3^-, resulting in high diffusive permeation of CO_2. Active HCO_3^- transport into the chloroplast would lower cytoplasmic $[HCO_3^-]$, and then CO_2 that diffuses into the cell is readily converted into HCO_3^- by cytosolic CA (alternatively this could occur in the PPC). As in *P. tricornutum*, transporters on CER and CE have not been identified. In *T. pseudonana*, a putative θ-CA is predicted in the PPT, suggesting a role for θ-CA in CO_2 generation as proposed in *P. tricornutum*. There is no known CA localized in the pyrenoid. (*c*) In *C. reinharditii*, HCO_3^- is delivered into the stroma by plasma-membrane-located HLA3 and chloroplast-envelope-located LCIA. LCIA is shown to be on the chloroplast envelope, but its exact localization, whether inner or outer membrane, is unknown. Here, we assumed that LCIA is on the inner envelope because the outer membrane is generally permeable to low molecular weight compounds which most probably allows spontaneous HCO_3^- permeation. LCI1 on plasma membrane was strongly suggested to be a DIC transporter, but the inorganic carbon species transported by LCI1 have not been determined. CAH3, a luminal CA in the thylakoid tubule, generates CO_2 from HCO_3^-. Unlike *P. tricornutum*, the luminal CAH3 is α-type CA, suggesting convergent evolution of a mechanism to supply CO_2 by using luminal acidification. Similarly, the LCIB-C complex is a putative θ-CA, another example of convergent evolution of pyrenoidal CA which may play a role in recapturing leaking out CO_2. CE, chloroplast envelopes; CER, chloroplast ER; PM, plasma membrane; PPC, periplastidal compartment; PPS, periplasmic space.

the thylakoid and its associated θ-CA in CO_2 supply require a reassessment of this conclusion. There is also the need to develop CCM models for other diatoms, and this should be feasible for additional species such as *T. pseudonana* as more molecular and physiological data on these species become available.

6. Conclusion

The molecular components, their origin, their localization and some of their putative functions in the CCM described in this review illustrate the diversity of diatom CCMs. In particular, putative DIC flux control systems between the external medium and the cytosol seem to be divided into at least two low-CO_2 inducible strategies: one uses active HCO_3^- pumps across plasma membranes and the other relies more significantly on passive CO_2 entry aided by external CAs, and these likely use contrasting diffusion barrier systems against CO_2 leakage (figure 4). However, in both cases, as a convergent aspect, we assume a strong constitutive HCO_3^- pumping action at the chloroplast envelope, which allows an efficient removal of CO_2 from the cytosol and an efficient accumulation of HCO_3^- in the stroma. Furthermore, both systems most likely use the pyrenoid as a hub to generate ample CO_2 flux from accumulated HCO_3^- aided by thylakoid luminal θ-CA to support fixation by RubisCO. In this process, pyrenoidal CA activity may function as a critical part of the CCM in concert with luminal CA, which appears to be a general feature of algal CCM, but details of this mechanism in diatoms requires further fine structural analysis of the pyrenoid.

Data accessibility. This article has no data.

Competing interests. We declare we have no competing interests.

Funding. This work was supported by Grant-in-Aid for Scientific Research B (grant no. 24310015 to Y.M.), by Grant-in-Aid for Scientific Research on Innovative Areas (grant no. 16H06557 to Y.M.), by Grant-in-Aid for Young Scientists B (grant no. 26870750 to K.N., and grant nos 15K16156 and 17K15326 to Y.T.) from Japan Society for the Promotion of Science (JSPS), by MEXT-Supported program for the Strategic Research Foundation for the Advancement of Environmental Protection Technology and for Development of Intelligent Self-Organized Biomaterials and by the Science Research Promotion Fund of the Promotion and Mutual Aid Corporation for Private Schools of Japan (to Y.M.). B.M.H. was supported by the US National Science Foundation (EF 1041023, MCB 1129326) and the Alfred P. Sloan Foundation (BR2014-049).

References

1. Badger MR, Andrews TJ, Whitney SM, Ludwig M, Yellowlees DC, Leggat W, Price GD. 1998 The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO_2-concentrating mechanisms in algae. *Can. J. Bot.* **76**, 1052–1071. (doi:10.1139/b98-074)
2. Losh JL, Young JN, Morel FMM. 2013 Rubisco is a small fraction of total protein in marine phytoplankton. *New Phytol.* **198**, 52–58. (doi:10.1111/nph.12143)
3. Young JN, Goldman JAL, Kranz SA, Tortell PD, Morel FMM. 2015 Slow carboxylation of Rubisco constrains the rate of carbon fixation during Antarctic phytoplankton blooms. *New Phytol.* **205**, 172–181. (doi:10.1111/nph.13021)
4. Young JN, Heureux AMC, Sharwood RE, Rickaby REM, Morel FMM, Whitney SM. 2016 Large variation in the Rubisco kinetics of diatoms reveals diversity among their carbon-concentrating mechanisms. *J. Exp. Bot.* **67**, 3445–3456. (doi:10.1093/jxb/erw163)
5. Tréguer P, Nelson DM, Van Bennekom AJ, Demaster DJ, Leynaert A, Quéguiner B. 1995 The silica balance in the world ocean: a reestimate. *Science* **268**, 375–379. (doi:10.1126/science.268.5209.375)
6. Falkowski P *et al.* 2000 The global carbon cycle: a test of our knowledge of earth as a system. *Science* **290**, 291–296. (doi:10.1126/science.290.5490.291)
7. Rotatore C, Colman B, Kuzma M. 1995 The active uptake of carbon dioxide by the marine diatoms *Phaeodactylum ticornutum* and *Cyclotella* sp. *Plant Cell Environ.* **18**, 913–918. (doi:10.1111/j.1365-3040.1995.tb00600.x)
8. Colman B, Rotatore C. 1995 Photosynthetic inorganic carbon uptake and accumulation in two marine diatoms. *Plant Cell Environ.* **18**, 919–924. (doi:10.1111/j.1365-3040.1995.tb00601.x)
9. Korb RE, Saville PJ, Johnston AM, Raven JA. 1997 Sources of inorganic carbon for photosynthesis by three species of marine diatom. *J. Phycol.* **33**, 433–440. (doi:10.1111/j.0022-3646.1997.00433.x)
10. Burkhardt S, Amoroso G, Riebesell U. 2001 CO_2 and HCO_3^- uptake in marine diatoms acclimated to different CO_2 concentrations. *Limnol. Oceanogr.* **46**, 1378–1391. (doi:10.4319/lo.2001.46.6.1378)
11. Rost B, Burkhardt S, Sültemeyer D, Riebesell U, Burkhardt S, Sültemeyer D. 2003 Carbon acquisition of bloom-forming marine phytoplankton. *Limnol.*

Oceanogr. **48**, 55–67. (doi:10.4319/lo.2003.48.1.0055)

12. Trimborn S, Lundholm N, Thoms S, Richter KU, Krock B, Hansen PJ, Rost B. 2008 Inorganic carbon acquisition in potentially toxic and non-toxic diatoms: the effect of pH-induced changes in seawater carbonate chemistry. *Physiol. Plant.* **133**, 92–105. (doi:10.1111/j.1399-3054.2007.01038.x)
13. Satoh D, Hiraoka Y, Colman B, Matsuda Y. 2001 Physiological and molecular biological characterization of intracellular carbonic anhydrase from the marine diatom *Phaeodactylum tricornutum*. *Plant Physiol.* **126**, 1459–1470. (doi:10.1104/pp.126.4.1459)
14. Tanaka Y, Nakatsuma D, Harada H, Ishida M, Matsuda Y. 2005 Localization of soluble β-carbonic anhydrase in the marine diatom *Phaeodactylum tricornutum*. Sorting to the chloroplast and cluster formation on the girdle lamellae. *Plant Physiol.* **138**, 207–217. (doi:10.1104/pp.104.058982)
15. Tachibana M, Allen AE, Kikutani S, Endo Y, Bowler C, Matsuda Y. 2011 Localization of putative carbonic anhydrases in two marine diatoms, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*. *Photosynth. Res.* **109**, 205–221. (doi:10.1007/s11120-011-9634-4)
16. Hopkinson BM, Dupont CL, Allen AE, Morel FMM. 2011 Efficiency of the CO_2-concentrating mechanism of diatoms. *Proc. Natl Acad. Sci. USA* **108**, 3830–3837. (doi:10.1073/pnas.1018062108)
17. Kikutani S, Tanaka R, Yamazaki Y, Hara S, Hisabori T, Kroth PG, Matsuda Y. 2012 Redox regulation of carbonic anhydrases via thioredoxin in chloroplast of the marine diatom *Phaeodactylum tricornutum*. *J. Biol. Chem.* **287**, 20 689–20 700. (doi:10.1074/jbc.M111.322743)
18. Roberts SB, Lane TW, Morel FMM. 1997 Carbonic anhydrase in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae). *J. Phycol.* **33**, 845–850. (doi:10.1111/j.0022-3646.1997.00845.x)
19. Lane TW, Morel FM. 2000 A biological function for cadmium in marine diatoms. *Proc. Natl Acad. Sci. USA* **97**, 4627–4631. (doi:10.1073/pnas.090091397)
20. Lane TW, Saito MA, George GN, Pickering IJ, Prince RC, Morel FMM. 2005 Biochemistry: a cadmium enzyme from a marine diatom. *Nature* **435**, 42. (doi:10.1038/435042a)
21. Xu Y, Feng L, Jeffrey PD, Shi Y, Morel FMM. 2008 Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. *Nature* **452**, 56–61. (doi:10.1038/nature06636)
22. Kikutani S, Nakajima K, Nagasato C, Tsuji Y, Miyatake A, Matsuda Y. 2016 Thylakoid luminal θ-carbonic anhydrase critical for growth and photosynthesis in the marine diatom *Phaeodactylum tricornutum*. *Proc. Natl Acad. Sci. USA* **113**, 9828–9833. (doi:10.1073/pnas.1603112113)
23. Samukawa M, Shen C, Hopkinson BM, Matsuda Y. 2014 Localization of putative carbonic anhydrases in the marine diatom, *Thalassiosira pseudonana*. *Photosynth. Res.* **121**, 235–249. (doi:10.1007/s11120-014-9967-x)
24. Mackinder LCM *et al.* 2016 A repeat protein links Rubisco to form the eukaryotic carbon-concentrating organelle. *Proc. Natl Acad. Sci. USA* **113**, 5958–5963. (doi:10.1073/pnas.1522866113)
25. Nakajima K, Tanaka A, Matsuda Y. 2013 SLC4 family transporters in a marine diatom directly pump bicarbonate from seawater. *Proc. Natl Acad. Sci. USA* **110**, 1767–1772. (doi:10.1073/pnas.1216234110)
26. Harada H, Nakajima K, Sakaue K, Matsuda Y. 2006 CO_2 sensing at ocean surface mediated by cAMP in a marine diatom. *Plant Physiol.* **142**, 1318–1328. (doi:10.1104/pp.106.086561)
27. Ohno N, Inoue T, Yamashiki R, Nakajima K, Kitahara Y, Ishibashi M, Matsuda Y. 2012 CO_2-cAMP-responsive cis-elements targeted by a transcription factor with CREB/ATF-like basic zipper domain in the marine diatom *Phaeodactylum tricornutum*. *Plant Physiol.* **158**, 499–513. (doi:10.1104/pp.111.190249)
28. Hennon GMM, Ashworth J, Groussman RD, Berthiaume C, Morales RL, Baliga NS, Orellana MV, Armbrust EV. 2015 Diatom acclimation to elevated CO_2 via cAMP signalling and coordinated gene expression. *Nat. Clim. Chang.* **5**, 761–765. (doi:10.1038/nclimate2683)
29. Tanaka A, Ohno N, Nakajima K, Matsuda Y. 2016 Light and CO_2/cAMP signal cross talk on the promoter elements of chloroplastic β-carbonic anhydrase genes in the marine diatom *Phaeodactylum tricornutum*. *Plant Physiol.* **170**, 1105–1116. (doi:10.1104/pp.15.01738)
30. Huysman MJJ *et al.* 2013 AUREOCHROME1a-mediated induction of the diatom-specific cyclin dsCYC2 controls the onset of cell division in diatoms (*Phaeodactylum tricornutum*). *Plant Cell* **25**, 215–228. (doi:10.1105/tpc.112.106377)
31. Hopkinson BM. 2014 A chloroplast pump model for the CO_2 concentrating mechanism in the diatom *Phaeodactylum tricornutum*. *Photosynth. Res.* **121**, 223–233. (doi:10.1007/s11120-013-9954-7)
32. Hopkinson BM, Dupont CL, Matsuda Y. 2016 The physiology and genetics of CO_2 concentrating mechanisms in model diatoms. *Curr. Opin. Plant Biol.* **31**, 51–57. (doi:10.1016/j.pbi.2016.03.013)
33. Patel BN, Merrett MJ. 1986 Inorganic-carbon uptake by the marine diatom *Phaeodactylum tricornutum*. *Planta* **169**, 222–227. (doi:10.1007/BF00392318)
34. Johnston AM, Raven JA. 1996 Inorganic carbon accumulation by the marine diatom *Phaeodactylum tricornutum*. *Eur. J. Phycol.* **31**, 285–290. (doi:10.1080/09670269600651491)
35. Mitchell C, Beardall J. 1996 Inorganic carbon uptake by an Antarctic sea-ice diatom, *Nitzschia frigida*. *Polar Biol.* **16**, 95–99. (doi:10.1007/BF02390429)
36. Keeling PJ. 2013 The number, speed, and impact of plastid endosymbioses in eukaryotic evolution. *Annu. Rev. Plant Biol.* **64**, 583–607. (doi:10.1146/annurev-arplant-050312-120144)
37. Rotatore C, Colman B. 1992 Active uptake of CO_2 by the diatom *Navicula pelliculosa*. *J. Exp. Bot.* **43**, 571–576. (doi:10.1093/jxb/43.4.571)
38. Kroth PG *et al.* 2008 A model for carbohydrate metabolism in the diatom *Phaeodactylum tricornutum* deduced from comparative whole genome analysis. *PLoS ONE* **3**, e1426. (doi:10.1371/journal.pone.0001426)
39. Gruber A, Vugrinec S, Hempel F, Gould SB, Maier UG, Kroth PG. 2007 Protein targeting into complex diatom plastids: functional characterisation of a specific targeting motif. *Plant Mol. Biol.* **64**, 519–530. (doi:10.1007/s11103-007-9171-x)
40. Lee RE, Kugrens P. 1998 Hypothesis: the ecological advantage of chloroplast ER—the ability to outcompete at low dissolved CO_2 concentrations. *Protist* **149**, 341–345. (doi:10.1016/S1434-4610(98)70040-9)
41. Lee RE, Kugrens P. 2000 Ancient atmospheric CO_2 and the timing of evolution of secondary endosymbioses. *Phycologia* **39**, 167–172. (doi:10.2216/i0031-8884-39-2-167.1)
42. Sullivan CW, Volcani BE. 1974 Synergistically stimulated (Na^+,K^+)-adenosine triphosphatase from plasma membrane of a marine diatom. *Proc. Natl Acad. Sci. USA* **71**, 4376–4380. (doi:10.1073/pnas.71.11.4376)
43. Rees TAV. 1984 Sodium dependent photosynthetic oxygen evolution in a marine diatom. *J. Exp. Bot.* **35**, 332–337. (doi:10.1093/jxb/35.3.332)
44. Chen X, Qiu CE, Shao JZ. 2006 Evidence for K^+-dependent HCO_3^- utilization in the marine diatom *Phaeodactylum tricornutum*. *Plant Physiol.* **141**, 731–736. (doi:10.1104/pp.106.079616)
45. Kaplan A, Scherer S, Lerner M. 1989 Nature of the light-induced H^+ efflux and Na^+ uptake in cyanobacteria. *Plant Physiol.* **89**, 1220–1225. (doi:10.1104/PP.89.4.1220)
46. Sültemeyer DF, Fock HP, Canvin DT. 1991 Active uptake of inorganic carbon by *Chlamydomonas reinhardtii*: evidence for simultaneous transport of HCO_3^- and CO_2 and characterization of active CO_2 transport. *Can. J. Bot.* **69**, 995–1002. (doi:10.1139/b91-128)
47. Tchernov D, Helman Y, Keren N, Luz B, Ohad I, Reinhold L, Ogawa T, Kaplan A. 2001 Passive entry of CO_2 and its energy-dependent intracellular conversion to HCO_3^- in cyanobacteria are driven by a photosystem I-generated $\Delta\mu H^+$. *J. Biol. Chem.* **276**, 23 450–23 455. (doi:10.1074/jbc.M101973200)
48. Bailleul B *et al.* 2015 Energetic coupling between plastids and mitochondria drives CO_2 assimilation in diatoms. *Nature* **524**, 366–369. (doi:10.1038/nature14599)
49. Gutknecht J, Bisson MA, Tosteson FC. 1977 Diffusion of carbon dioxide through lipid bilayer membranes: effects of carbonic anhydrase, bicarbonate, and unstirred layers. *J. Gen. Physiol.* **69**, 779–794. (doi:10.1085/jgp.69.6.779)
50. Ogawa T, Mi H. 2007 Cyanobacterial NADPH dehydrogenase complexes. *Photosynth. Res.* **93**, 69–77. (doi:10.1007/s11120-006-9128-y)
51. Mi H, Endo T, Ogawa T, Asada K. 1995 Thylakoid membrane-bound, NADPH-specific pyridine nucleotide dehydrogenase complex mediates cyclic electron transport in the cyanobacterium

Synechocystis sp. PCC 6803. *Plant Cell Physiol.* **36**, 661–668.

52. He Z, Zheng F, Wu Y, Li Q, Lv J, Fu P, Mi H. 2015 NDH-1 L interacts with ferredoxin via the subunit NdhS in *Thermosynechococcus elongatus*. *Photosynth. Res.* **126**, 341–349. (doi:10.1007/s11120-015-0090-4)
53. Peña KL, Castel SE, de Araujo C, Espie GS, Kimber MS. 2001 Structural basis of the oxidative activation of the carboxysomal γ-carbonic anhydrase, CcmM. *Proc. Natl Acad. Sci. USA* **107**, 2455–2460. (doi:10.1073/pnas.0910866107)
54. Alber BE, Ferry JG. 1994 A carbonic anhydrase from the archaeon *Methanosarcina thermophila*. *Proc. Natl Acad. Sci. USA* **91**, 6909–6913. (doi:10.1073/pnas.91.15.6909)
55. DiMario RJ, Clayton H, Mukherjee A, Ludwig M, Moroney JV. 2016 Plant carbonic anhydrases: structures, locations, evolution, and physiological roles. *Mol. Plant* **10**, 30–46. (doi:10.1016/j.molp.2016.09.001)
56. Burns BD, Beardall J. 1987 Utilization of inorganic carbon by marine microalgae. *J. Exp. Mar. Biol. Ecol.* **107**, 75–86. (doi:10.1016/0022-0981(87)90125-0)
57. Kaldenhoff R, Kai L, Uehlein N. 2014 Aquaporins and membrane diffusion of CO_2 in living organisms. *Biochim. Biophys. Acta* **1840**, 1592–1595. (doi:10.1016/j.bbagen.2013.09.037)
58. Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R. 2003 The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. *Nature* **425**, 734–737. (doi:10.1038/nature02027)
59. Tu C, Wynns GC, McMurray RE, Silverman DN. 1978 CO_2 kinetics in red cell suspensions measured by ^{18}O exchange. *J. Biol. Chem.* **253**, 8178–8184.
60. Endeward V *et al.* 2006 Evidence that aquaporin 1 is a major pathway for CO_2 transport across the human erythrocyte membrane. *FASEB J.* **20**, 1974–1981. (doi:10.1096/fj.04-3300com)
61. Chegwidden WR, Carter ND. 2000 Introduction to the carbonic anhydrases. In *The carbonic anhydrases: new horizons,* vol. 9 (eds WR Chegwidden, ND Carter, YH Edwards), pp. 13–28. Basel, Switzerland: Birkhaeuser. (doi:10.1007/978-3-0348-8446-4_2)
62. Lee RBY, Smith JAC, Rickaby REM. 2013 Cloning, expression and characterization of the δ-carbonic anhydrase of *Thalassiosira weissflogii* (Bacillariophyceae). *J. Phycol.* **49**, 170–177. (doi:10.1111/j.1529-8817.2012.01226.x)
63. Del Prete S, Vullo D, Scozzafava A, Capasso C, Supuran CT. 2014 Cloning, characterization and anion inhibition study of the δ-class carbonic anhydrase (TweCA) from the marine diatom *Thalassiosira weissflogii*. *Bioorg. Med. Chem.* **22**, 531–537. (doi:10.1016/j.bmc.2013.10.045)
64. Hopkinson BM, Meile C, Shen C. 2013 Quantification of extracellular carbonic anhydrase activity in two marine diatoms and investigation of its role. *Plant Physiol.* **162**, 1142–1152. (doi:10.1104/pp.113.217737)
65. Nimer NA, Iglesias-Rodriguez MD, Merrett MJ. 1997 Bicarbonate utilization by marine phytoplankton species. *J. Phycol.* **33**, 625–631. (doi:10.1111/j.0022-3646.1997.00625.x)
66. Shen C, Hopkinson BM. 2015 Size scaling of extracellular carbonic anhydrase activity in centric marine diatoms. *J. Phycol.* **51**, 255–263. (doi:10.1111/jpy.12269)
67. Moroney JV, Ma Y, Frey WD, Fusilier KA, Pham TT, Simms TA, DiMario RJ, Yang J, Mukherjee B. 2011 The carbonic anhydrase isoforms of *Chlamydomonas reinhardtii*: intracellular location, expression, and physiological roles. *Photosynth. Res.* **109**, 133–149. (doi:10.1007/s11120-011-9635-3)
68. Wang Y, Spalding MH. 2006 An inorganic carbon transport system responsible for acclimation specific to air levels of CO_2 in *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA* **103**, 10 110–10 115. (doi:10.1073/pnas.0603402103)
69. Mitra M, Lato SM, Ynalvez RA, Xiao Y, Moroney JV. 2004 Identification of a new chloroplast carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiol.* **135**, 173–182. (doi:10.1104/pp.103.037283)
70. Yamano T, Tsujikawa T, Hatano K, Ozawa S, Takahashi Y, Fukuzawa H. 2010 Light and low-CO_2-dependent LCIB-LCIC complex localization in the chloroplast supports the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* **51**, 1453–1468. (doi:10.1093/pcp/pcq105)
71. Wang Y, Spalding MH. 2014 Acclimation to very low CO_2: contribution of limiting CO_2 inducible proteins, LCIB and LCIA, to inorganic carbon uptake in *Chlamydomonas reinhardtii*. *Plant Physiol.* **166**, 2040–2050. (doi:10.1104/pp.114.248294)
72. Jenks A, Gibbs SP. 2000 Immunolocalization and distribution of form II Rubisco in the pyrenoid and chloroplast stroma of *Amphidinium carterae* and form I Rubisco in the symbiont-derived plastids of *Peridinium foliaceum* (Dinophyceae). *J. Phycol.* **36**, 127–138. (doi:10.1046/j.1529-8817.2000.99114.x)
73. Matsuda Y, Hara T, Colman B. 2001 Regulation of the induction of bicarbonate uptake by dissolved CO_2 in the marine diatom, *Phaeodactylum tricornutum*. *Plant Cell Environ.* **24**, 611–620. (doi:10.1046/j.1365-3040.2001.00702.x)
74. Harada H, Matsuda Y. 2005 Identification and characterization of a new carbonic anhydrase in the marine diatom *Phaeodactylum tricornutum*. *Can. J. Bot.* **83**, 909–916. (doi:10.1139/b05-078)
75. Harada H, Nakatsuma D, Ishida M, Matsuda Y. 2005 Regulation of the expression of intracellular β-carbonic anhydrase in response to CO_2 and light in the marine diatom *Phaeodactylum tricornutum*. *Plant Physiol.* **139**, 1041–1050. (doi:10.1104/pp.105.065185)
76. Mayo WP, Williams TG, Birch DG, Turpin DH. 1986 Photosynthetic adaptation by *Synechococcus leopoliensis* in response to exogenous dissolved inorganic carbon. *Plant Physiol.* **80**, 1038–1040. (doi:10.1104/PP.80.4.1038)
77. Marcus Y, Harel E, Kaplan A. 1983 Adaptation of the cyanobacterium *Anabaena variabilis* to low CO_2 concentration in their environment. *Plant Physiol.* **71**, 208–210. (doi:10.1104/PP.71.1.208)
78. Nishimura T, Takahashi Y, Yamaguchi O, Suzuki H, Maeda S, Omata T. 2008 Mechanism of low CO_2-induced activation of the cmp bicarbonate transporter operon by a LysR family protein in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. *Mol. Microbiol.* **68**, 98–109. (doi:10.1111/j.1365-2958.2008.06137.x)
79. Shiraiwa Y, Satoh H, Hirokawa T. 1988 Factors controlling induction of carbonic anhydrase in *Chlorella vulgaris* 11h: effects of CO_2 and O_2. *Plant Cell Physiol.* **29**, 731–734. (doi:10.1093/oxfordjournals.pcp.a077554)
80. Matsuda Y, Colman B. 1995 Induction of CO_2 and bicarbonate transport in the green alga *Chlorella ellipsoidea* (II. Evidence for induction in response to external CO_2 concentration). *Plant Physiol.* **108**, 253–260. (doi:10.1104/PP.108.1.253)
81. Vance P, Spalding MH. 2005 Growth, photosynthesis, and gene expression in *Chlamydomonas* over a range of CO_2 concentrations and CO_2/O_2 ratios: CO_2 regulates multiple acclimation states. *Can. J. Bot.* **83**, 796–809. (doi:10.1139/b05-064)
82. Matsuda Y, Kroth PG. 2014 Carbon fixation in diatoms. In *The structural basis of biological energy generation*, vol. 39 (ed. MF Hohmann-Marriott), pp. 335–362. Dordrecht, The Netherlands: Springer. (doi:10.1007/978-94-017-8742-0_18)
83. Bowler C *et al.* 2008 The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature* **456**, 239–244. (doi:10.1038/nature07410)
84. Smith SR *et al.* 2016 Transcriptional orchestration of the global cellular response of a model pennate diatom to diel light cycling under iron limitation. *PLoS Genet.* **12**, e1006490. (doi:10.1371/journal.pgen.1006490)
85. Aizawa K, Miyachi S. 1986 Carbonic anhydrase and CO_2 concentrating mechanisms in microalgae and cyanobacteria. *FEMS Microbiol. Lett.* **39**, 215–233. (doi:10.1016/0378-1097(86)90447-7)
86. Reinhold L, Zviman M, Kaplan A. 1987 Inorganic carbon fluxes and photosynthesis in cyanobacteria—a quantitative model. In *Progress in photosynthesis research*, vol. 4 (ed. J Biggins), pp. 289–296. Dordrecht, The Netherlands: Springer. (doi:10.1007/978-94-017-0519-6_62)