The role of PEP Carboxylases for carbon fixation
in the diatom *Phaeodactylum tricornutum*

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Zusammenfassung


In dieser Arbeit wurde zunächst eine Methode etabliert zur Kultivierung von *P. tricornutum* bei unterschiedlichen CO\textsubscript{2} Konzentrationen. Hierzu wurden Puffer mit unterschiedlichen Konzentrationen von HCO\textsubscript{3}/CO\textsubscript{3} in einer unteren Kammer eingesetzt, die verschiedene CO\textsubscript{2} Konzentrationen freisetzen, die wiederum die Algenkultur in der verbundenen oberen Kammer beeinflussen. *P. tricornutum* zeigte dabei bei hohen/niedrigen CO\textsubscript{2}-Konzentrationen vergleichbare PEPC-Transkription, Proteinmengen und Enzymaktivitäten. Wir haben zudem PEPCs rekombinant in *E. coli* exprimiert, aufgereinigt und enzymatische Parameter untersucht.

*P. tricornutum* PEPC Knockout-Mutanten sowie eine PEPC1/2-Doppelmutante, die im Rahmen dieser Arbeit erstellt wurden, zeigten unterschiedliche Enzymaktivitäten. So wiesen PEPC1-Mutanten ähnliche Aktivitäten auf wie Wildtyp-Zellen, während in PEPC2-Mutanten die Aktivität 60% vom Wildtyp betrug. Die Doppelmutanten wiesen hingegen gar keine PEPC-Aktivität auf. PEPC2 und PEPC1/2-Mutanten zeigten unter CO\textsubscript{2}-Mangel deutlich geringere Wachstumsraten, aber normale Raten bei hohen CO\textsubscript{2}-Konzentrationen. Diese beiden Mutanten-Linien zeigten unter CO\textsubscript{2}-Mangel zudem eine geringere Photosyntheserate. Eine Analyse der stabilen Isotope in den Algen zeigte ebenfalls bei PEPC1 keine Unterschiede zum Wildtyp, während PEPC2 und PEPC1/2 deutlich geringere \textsuperscript{13}C/\textsuperscript{12}C Verhältnisse (\(\delta^{13}\text{C}\)) aufwiesen. Diese Daten deuten zusammengenommen auf einen biochemischen Kohlenstoff-Konzentrierungsmechanismus in *P. tricornutum* hin, wobei offenbar nur die mitochondriale PEPC2 eine Rolle spielt. Dieser Mechanismus wird offenbar nicht durch externe CO\textsubscript{2}-Konzentrationen induziert.
Abstract

Marine diatoms represent an important group of phytoplankton, contributing 40% to the oceanic primary production. To circumvent limitations of dissolved inorganic carbon (DIC) in seawater, some diatoms possess carbon concentrating mechanisms (CCMs), which increase the CO₂ concentrations in the proximity of Rubisco, allowing more efficient photosynthesis in a carbon-limited environment. Previously published data indicates that a C4-type CCM exists in one diatom species *Thalassiosira weissflogii*, but it is controversially discussed whether this is also true for other diatoms. The enzyme Phosphoenolpyruvate carboxylase (PEPC) plays a crucial role in C4 photosynthesis. The model diatom *Phaeodactylum tricornutum* contains two PEPC isoforms, which are located in the periplastidic space (PEPC1) and in the mitochondria (PEPC2), respectively.

In this study, we established a culturing method that allows the supply of different CO₂ concentrations to *P. tricornutum*. This approach utilizes buffers with different ratios of HCO₃⁻/CO₃²⁻ in the lower chamber of a two-tier vessel, releasing different amounts of CO₂ to the upper chamber of the vessel, where the cell culture is suspended. We cultured *P. tricornutum* under low and high CO₂ concentrations via these vessels and found that the mRNA transcription, protein expression, and the total enzyme activity of both PEPCs were not affected by the CO₂ concentrations. We further have expressed both PEPCs from *P. tricornutum* as recombinant proteins in *Escherichia coli* and studied the kinetic parameters of PEPC1 and PEPC2, including pH optima and the *K_m* values for the substrates PEP and HCO₃⁻.

We generated individual knockout mutants of PEPC1 and PEPC2, as well as PEPC1/PEPC2 double-knockout cell lines via genome editing (TALEN). Enzymatic assays showed that PEPC activity is similar in PEPC1 knockout cell lines and wild type cells, while PEPC activity was reduced by 60% in PEPC2 knockout cell lines. As expected, no PEPC activity could be detected in a PEPC1/PEPC2 double-knockout cell line. We could not identify any significant phenotype in the PEPC1 knockout cell lines regarding growth rates and photosynthetic activity. However, PEPC2 and the PEPC1/PEPC2 double-knockout cell lines showed a significantly reduced growth under ambient air, but similar division rates as wild type cells under high CO₂ condition. PEPC2 and PEPC1/PEPC2 double-knockout cell lines further exhibited significantly less photosynthetic activity than wild type when incubated at low DIC concentrations. Finally, carbon stable isotope analyses showed no effects in PEPC1 knockout cell lines, but significantly lower $^{13}$C/$^{12}$C ratios ($\delta^{13}$C) in PEPC2 and PEPC1/PEPC2 double-knockout cell lines compared to wild type cells. The obtained data suggest that a biochemical CCM exists in *P. tricornutum*, in which the mitochondrial PEPC2
may play an important role in the inorganic carbon assimilation. The PEPC2 mediated C4-like photosynthesis apparently is not regulated by the DIC concentrations in *P. tricornutum*. This thesis presents new proofs for the existence of a biochemical CCM in *P. tricornutum*. 
Chapter 1

General Introduction
1. General Introduction

1.1 Biology of diatoms

Diatoms are eukaryotic, unicellular, photosynthetic organisms, belonging to the Stramenopiles. Diatoms are abundant in aquatic ecosystems, representing one of the most successful autotrophic communities (Falkowski et al., 2004). There are an estimated 200,000 different diatom species (Kooistra et al., 2007). The well-known characteristic of diatoms is their ability to generate cell walls (frustules) composed of silica (Figure 1.1). The diatom frustules are composed of two thecae of identical shape, which are arranged like a Petri dish, where the smaller theca (named hypotheca) fits into the larger one (named epitheca) (Figure 1.1). Each theca consists of a valve and a girdle band, and the two thecae are tightly connected together by the joint of their girdle bands (Figure 1.1) (Falciatore & Bowler, 2002). The name “diatom” originates from the Greek phrase ‘diatomos’, meaning ‘cut in half’, referring to the two parts of the silica cell wall (Armbrust, 2009). The frustules of diatoms show a nanoscale architecture of biosilica, these unique structural features of diatom frustules have inspired people to use the diatom biosilica as templates to explore novel functional materials, that can be utilized as natural and renewable nanomaterials for future nanotechnology industry (Gordon et al., 2009; Kröger & Brunner, 2014). Diatoms can be classified into two major groups according to the symmetry of their silica cell wall: the pennate diatoms are bilaterally symmetrical and the centric diatoms are radially symmetrical (Round et al., 2007). Pennate diatoms are mostly benthic, dominating in sediments. This group further includes raphid diatoms which contain a raphe slit on their frustules (Figure 1.1), which facilitates their movement on surfaces through secreted mucilage. Araphid diatoms, instead, lacking these slits, are not able to move. In comparison, centric diatoms are mostly planktonic, consisting of radial and multipolar centrics (Kooistra et al., 2007; Round et al., 2007).
Diatoms are diploid organisms, and vegetative mitotic division occurs by cleaving one cell into two cells (Falciatore & Bowler, 2002). After mitosis, the daughter cells generate new hypothecae and keep the valve from the mother cell as their own epithecae (shown in Figure 1.2) (Pickett-Heaps, 1990; Falciatore & Bowler, 2002). Therefore, one of the two daughter cells is smaller than the parental cell, and cells of different sizes can be found within one population after continued mitotic divisions. They can result in a cell size reduction of the whole population up to the point when cells reach a minimum size. The original cell size can only be recovered via sexual reproduction followed by auxospore formation (Falciatore & Bowler, 2002; Armbrust, 2009). The sexual reproduction mechanisms are different in centric and pennate diatoms. Centric diatoms are oogamous: male cells are converted to flagellated sperms which are released, and actively swim to an egg cell which remains between the valves. After the fusion of the two gametes, the auxospore is formed (Figure 1.3 a, b). The risk of oogamous reproduction in centric diatom is that if the sperms do not find any egg cells, the gametes will die (Armbrust, 2009). In pennate diatoms, the gametes will only be formed when two cells are paired (Armbrust, 2009). Pennate diatoms attract partner cells via pheromones (Sato et al., 2011), for example, diproline, which was identified as one of the sex pheromones in the pennate diatom *Seminavis robusta* (Gillard et al., 2013). Some diatoms can be induced to perform sexual reproduction by changes of environmental factors such as light, salinity, temperature, and nutrients (Vaulot et al., 1986; Vaulot et al., 1987; Armbrust et al., 1990).
Figure 1.2. Mitotic cell division and cell wall generation in a pennate diatom. After mitosis and cytokinesis in the diatom cell (1,2), the silica deposition vesicle (SDV) starts to form near the newly separated plasma membrane (3). The produced SDV gradually elongates into a tube and expands, finally spreading out on one side of the cell (4,5). Proteins, silica, and polysaccharides synthesized in the cell are transported into the SDV and form new hypovalves, which are exocytosed out of the cell (6). The daughter cells then are separated and grow individually. The girdle band formed in the SDV is also exocytosed from the cell, together with the exocytosed hypovalve to form a new hypotheca of the daughter cell (7). Adapted from (Falciatore & Bowler, 2002).
General Introduction

Centric diatom:

Figure 1.3. Examples of sexual reproduction in centric and pennate diatoms. (a,b). Sexual reproduction in centric diatoms. (a). Sperm is produced and released from the cell wall (white arrow); eggs are formed and remain within the cell wall (black arrow). (b). A newly produced auxospore via fusion of a sperm and egg forms a larger cell wall and restores the cell size. (c,d). Sexual reproduction in pennate diatoms. (c). Two gametes (black arrows) are produced after pairing of two pennate diatoms. Different from centric diatoms, the gametes are unable to swim but instead one move towards the other and they fuse; (d). A newly formed auxospore is released from the old cell wall (black arrow). Adapted from (Armbrust, 2009).

Pennate diatom:
1.2 Diatom endosymbiosis and gene transfer

Diatoms arose by secondary endosymbiosis, and their plastids contain four surrounding membranes. During primary endosymbiosis, a eukaryotic host cell engulfed a prokaryotic cyanobacterium. The resulting cells can be developed into green algae, red algae and land plants obtaining primary plastids surrounded by two membranes, which may be derived from the membranes of the engulfed cyanobacterium. Meanwhile, gene transfer could happen from the cyanobacterial genome to the nucleus of the host cell (Figure 1.4) (Cavalier-Smith, 1982; Delwiche & Palmer, 1997; Kroth & Strotmann, 1999). Secondary endosymbiosis is the engulfment of a eukaryotic endosymbiont by a eukaryotic host cell and the subsequent conversion of the endosymbiont into a secondary plastid. The resulting cells, which engulfed a red alga, gave rise to haptophytes, alveolates, cryptomonads, and stramenopiles; diatoms belong to stramenopiles (Kroth & Strotmann, 1999; Kroth, P, 2007; Armbrust, 2009; Keeling, 2013). The plastids of diatoms are surrounded by four membranes due to the secondary endosymbiosis, the two inner membranes are derived from the primary endosymbiont cyanobacteria, it is suggested that the third membrane (from inside to outside membranes) is originated from the cytoplasmic membrane of the secondary endosymbiont red alga (Keeling, 2013). The outermost membrane is connected with the endoplasmic reticulum (ER) of the host cell (Kroth & Strotmann, 1999). During the secondary endosymbiosis, gene transfer again occurred from the red algal nucleus and the plastid genomes to the host cell nucleus, and later the red algal nucleus and the mitochondria were lost (Figure 1.4) (Armbrust, 2009; Keeling, 2010). The genomes of several diatom species have been sequenced (Armbrust et al., 2004; Bowler et al., 2008; Curtis et al., 2012; Lommer et al., 2012; Mock et al., 2017), allowing a better understanding of how the genes function. The complete genome of *Thalassiosira pseudonana* (32.4 megabases (Mb)) is larger than the *P. tricornutum* genome size of approximately 27.4 Mb (Armbrust et al., 2004; Bowler et al., 2008). The genome analyses show that 7.5 % of the *P. tricornutum* genes were derived from bacteria, indicating horizontal gene transfer (HGT) from bacteria to the diatoms (Bowler et al., 2008) as shown in Figure 1.4. By accumulating genes from bacteria via HGT, diatoms could obtain novel metabolic capacities (for instance: the urea cycle) (Bowler et al., 2008). Horizontal gene transfer and its benefits may explain the diversity and ecological success of diatoms in the ocean (Bowler et al., 2008; Allen et al., 2011).
Figure 1.4. Secondary endosymbiosis and gene transfer in diatoms. Plastids of diatoms are surrounded by four membranes through (a) primary and (b) secondary endosymbiosis events. During primary endosymbiosis, a cyanobacterium was taken up by a heterotrophic host cell, followed by gene transfer from the cyanobacterial genome to the host nucleus (N1), as indicated by the blue arrow. The resulting cells developed into green algae, red algae and land plants with primary plastids containing two envelope membranes. In secondary endosymbiosis, another heterotrophic cell engulfed a red alga. The nucleus and the mitochondria of the engulfed red alga were lost, but again gene transfer occurred from the red algal nucleus and the plastid genome to the host cell nucleus (N2), labeled by blue arrows. This secondary endosymbiosis gave rise to diatoms with secondary plastids. Additionally, diatoms obtained bacterial genes by horizontal gene transfer during evolution indicated by the orange arrow. Adapted from (Armbrust, 2009).
1.3 The model diatom: *Phaeodactylum tricornutum*

The pennate diatom *Phaeodactylum tricornutum* has been used as a model organism for diatom genetic and physiological studies due to the availability of its genome sequences (Bowler et al., 2008) and nuclear genetic transformation via biolistic bombardment (Apt et al., 1996; Zaslavskaia et al., 2000; Kroth, PG, 2007), electroporation (Miyahara et al., 2013) and conjugation (Karas et al., 2015). The technique of RNAi-based silencing knock-down has been available since a couple of years (De Riso et al., 2009), while genome editing tools like TALEN (Transcription activator-like effector nuclease) and CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) have been established in *P. tricornutum* just recently, allowing the generation of targeted gene knockouts (Daboussi et al., 2014; Weyman et al., 2014; Nymark et al., 2016; Serif et al., 2017; Serif et al., 2018). Unlike most other diatoms, *P. tricornutum* can grow in the absence of silicon and shows three distinct morphotypes (oval, triradiate, and fusiform) as shown in Figure 1.5. A micrograph showing the cellular ultrastructure (transverse section) of *P. tricornutum* is shown in Figure 1.6.

![Figure 1.5](image_url)

Figure 1.5. The pennate diatom *Phaeodactylum tricornutum*. Light-microscopic images show the three morphotypes of *P. tricornutum*: (a) oval; (b) triradiate and (c) fusiform. The black bar indicates 10 μm. The image is used with permission of Ansgar Gruber (University of Konstanz, Germany).
1.4 Diatoms contribute to oceanic primary production

Diatoms are adapted to spatially variable aquatic habitats like coastal waters, open-ocean waters, upwelling regions, benthic habitats and sea ice (Falkowski et al., 2004; Reinfelder, 2010). Because of their dominant existence in the ocean, marine diatoms are responsible for a significant portion of primary oceanic production (Nelson et al., 1995; Falkowski & Raven, 2007). The maximum global diatom productivity is estimated as 26 gigatons (Gt) carbon each year (Nelson et al., 1995). The net primary productivity of the marine ecosystem is estimated as 45-55 Gt carbon, nearly as much as the net primary productivity of the terrestrial ecosystem, which is 55-70 Gt carbon per year (Field et al., 1998; Falkowski & Raven, 2007). Diatoms are highly involved in the global carbon cycle: by reducing the greenhouse gas carbon dioxide, they have influences on not only marine ecosystem but also the global climate (Denman, 2008; Armbrust, 2009). Because of their high photosynthetic activity and contributions to the oceanic primary production (Nelson et al., 1995; Field et al., 1998), diatom photosynthetic mechanisms have been intensively studied (Giordano et al., 2005; Reinfelder, 2010; Hopkinson et al., 2016; Clement et al., 2017; Matsuda et al., 2017; Shen et al., 2017; Young & Hopkinson, 2017).
1.5 Limitations of carbon utilization for diatoms

Plants, algae, as well as cyanobacteria, use CO$_2$ as the basic substrate for photosynthesis. There may be different problems though regarding their CO$_2$ supply among these photosynthetic organisms. Most land plants can take up CO$_2$ easily from the air by passive CO$_2$ diffusion via stomata, while for aquatic plant species, CO$_2$ supply can be limited due to the closure of stomata in the water environment (Meyer & Griffiths, 2013). To reduce the limitation of CO$_2$ supply and increase CO$_2$ fixation, some plants are using carbon concentrating mechanisms (CCMs), including the classical Kranz-type C4 pathway, single-cell C4 pathways, and the Crassulacean acid metabolism (CAM) (Edwards & Walker, 1983; Cushman & Bohnert, 1999; Sage, 2002; Edwards et al., 2004; Sage, 2004). Similarly, algae and cyanobacteria can become CO$_2$ limited because of their life in aquatic environments (Reinfelder, 2010).

In seawater, dissolved inorganic carbon (DIC) is present in the form of dissolved aqueous carbon dioxide (CO$_2$aq), carbonate (CO$_3^{2-}$), and bicarbonate (HCO$_3^-$) (Reinfelder, 2010). CO$_2$aq is used by the Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) of diatoms and other phytoplankton during photosynthesis (Cooper et al., 1969). The CO$_2$aq concentration can be very variable in the ocean. In the euphotic zone CO$_2$aq is low due to the photosynthetic consumptions by phytoplankton, while in deep water areas, CO$_2$aq is relatively higher because of the remineralization of organic matter by microbes. Similarly, CO$_2$aq can also be relatively higher in upwelling regions than the ocean surface as a result of the mixture of the CO$_2$ rich waters from the deep regions with low CO$_2$ waters from the surface (Reinfelder, 2010).

The CO$_2$aq concentrations can be limiting for marine phytoplankton because of slow CO$_2$ diffusion, and the slow dehydration kinetics of bicarbonate to CO$_2$aq in seawater (Reinfelder, 2010). Moreover, these limitations are accompanied by the low affinity of Rubisco to CO$_2$ as well as its oxygenase activity which inhibits CO$_2$ fixation (Badger et al., 1998) as shown in Figure 1.7. In seawater, the DIC corresponds to a CO$_2$aq concentration of 10-15 µM at pH 8.2 (Riebesell et al., 1993), which is much lower than the Michaelis–Menten constant ($K_m$) (20-60 µM) for CO$_2$ of the diatom Rubisco enzyme (Badger et al., 1998; Whitney et al., 2011). Thus Rubisco photorespiration is competing with the carboxylation activity, especially at low CO$_2$ concentrations. To overcome these limitations, diatoms, cyanobacteria and other marine phytoplankton have evolved carbon concentrating mechanisms (CCMs) for active uptake of inorganic carbon, in order to increase the CO$_2$ concentration for Rubisco to improve photosynthesis efficiencies in a carbon-limited
environment (Burkhardt et al., 2001; Matsuda et al., 2001; Giordano et al., 2005; Reinfelder, 2010; Hopkinson et al., 2011).

Figure 1.7. Limits of carbon acquisition in a diatom cell. (1) Slow diffusion of CO$_2$ from the air to seawater results in low CO$_2$aq concentration of 10-15 µM at pH 8.2 in seawater; (2) Slow dehydration kinetics of bicarbonate to CO$_2$aq in seawater; (3) Rubisco enzyme catalyzes CO$_2$ fixation with a high $K_m$(CO$_2$) of 20-60 µM; (4) Loss of CO$_2$ by Rubisco oxygenase activity. Abbreviations: RuBP, ribulose-1,5-bisphosphate; PGA, phosphoglycerate; GLL, glycolate; CO$_2$aq, dissolved aqueous CO$_2$; $K_m$(CO$_2$), Michaelis–Menten value of Rubisco to CO$_2$. Adapted from (Reinfelder, 2010).

1.6 Carbon concentrating mechanisms (CCMs) in cyanobacteria and eukaryotic microalgae

While different from plants, cyanobacteria, diatoms, and other eukaryotic microalgae have multiple types of CCMs including the utilization of membrane bicarbonate transporters and carbonic anhydrases and/or biochemical CCMs (Giordano et al., 2005; Raven, 2010; Reinfelder, 2010). The CCMs of cyanobacteria and the eukaryotic microalgae *Chlamydomonas reinhardtii* have been studied intensely in the last years (Badger et al., 2005; Moroney & Ynalvez, 2007; Price et al., 2007; Yamano et al., 2010; Wang et al., 2015). Cyanobacteria are prokaryotes, possess and only use few compartments to build up a simple CCM (Price et al., 2007). They generate a high concentration of HCO$_3^-$ in the cytosol by using five different membrane carbon transporters (Price et al., 2002; Badger et al., 2005). The highly concentrated HCO$_3^-$ then diffuses into the carboxysome, where the HCO$_3^-$ can be converted into CO$_2$ by carbonic anhydrases, the CO$_2$ can be captured by Rubisco for photosynthesis. The carboxysome is surrounded by a highly ordered protein shell (Kerfeld & Melnicki, 2016), which is the major component that prevents the leakage of CO$_2$, maintaining a
high concentration of CO$_2$ near Rubisco (Price et al., 2007). Although the cyanobacterial CCM genes which encode various HCO$_3^-$ transporters can be dramatically different among different cyanobacteria groups, the functional structure of the cyanobacteria CCM is basically the same among different groups (Badger et al., 2005). Eukaryotic algae C. reinhardtii and diatoms instead can build up CCMs using different types of CAs and various membrane HCO$_3^-$ transporters in various subcellular compartments (Young & Hopkinson, 2017). C. reinhardtii and diatoms do not have carboxysomes, but some of them utilize pyrenoids as a structure for CO$_2$ concentration. In C. reinhardtii, the LCIB/C protein complex is localized in the pyrenoids (Wang & Spalding, 2006), which possibly prevents the CO$_2$ leakage or recaptures leaking CO$_2$ (Mitra et al., 2004; Yamano et al., 2010).

1.7 Carbon concentrating mechanisms (CCMs) in diatoms

The physiology and biochemistry of CCMs in diatoms have been studied intensively in recent years (Giordano et al., 2005; Reinfelder, 2010; Hopkinson et al., 2013; Nakajima et al., 2013; Kustka et al., 2014; Tanaka et al., 2014; Hopkinson et al., 2016; Kikutani et al., 2016; Clement et al., 2017; Matsuda et al., 2017; Shen et al., 2017; Young & Hopkinson, 2017; Ewe et al., 2018). Diatom CCMs are genetically and functionally diverse among different species (Shen et al., 2017). Physiological data indicates that diatoms not only take up CO$_2$ but also HCO$_3^-$ from seawater, and concentrate dissolved inorganic carbon (DIC) for efficient photosynthesis (Colman & Rotatore, 1995; Rotatore et al., 1995; Burkhardt et al., 2001; Clement et al., 2016). There are two types of CCMs suggested in diatoms, biophysical CCMs, and biochemical CCMs.

1.7.1 Biophysical CCMs in diatoms

The functional components of biophysical CCMs in diatoms comprise bicarbonate transporters (Rotatore et al., 1995; Burkhardt et al., 2001; Nakajima et al., 2013) and carbonic anhydrases (CAs) (Morel et al., 1994; Colman & Rotatore, 1995; Burkhardt et al., 2001; Morel et al., 2002; Hopkinson et al., 2013; Kikutani et al., 2016). The plasma membrane bicarbonate transporter SLC4-2 was characterized in P. tricornutum, which plays a crucial role in DIC uptake (Nakajima et al., 2013). It has been suggested that there should also be HCO$_3^-$ transporters in the plastid membrane, which could cooperate with the plastid CA, controlling the DIC influx into the chloroplast (Hopkinson, 2014), however, such plastid membrane located HCO$_3^-$ transporters have not been identified yet. As shown in Figure 1.8, HCO$_3^-$ could be accumulated in the plastid via bicarbonate transporters in the cytoplasmic membrane and the potential plastid membrane, which
would enable the accumulated HCO$_3^-$ to be subsequently converted into CO$_2$ in close proximity to Rubisco by plastidic CAs (Hopkinson, 2014).

CAs catalyze CO$_2$ hydration and HCO$_3^-$ dehydration (Badger & Price, 1994). There are various CA subtypes in diatoms, which can be distributed among different intercellular compartments depending on different diatom species. For instance, *P. tricornutum* shows distinct intercellular localization of CAs from *T. pseudonana*. In *P. tricornutum*, as shown in Figure 1.8, five CAs are located in the periplastidic space (PPS), two CAs in pyrenoid and one CA in thylakoid lumen (Tachibana et al., 2011; Hopkinson et al., 2016; Kikutani et al., 2016). In *T. pseudonana*, two external CAs, one cytosolic and one stromal CA were identified, but the respective pyrenoidal CA is not found in this species (Figure 1.8) (Hopkinson et al., 2016). The different intercellular localization of subtypes of CAs in these two diatom species causes a distinct carbon flow in their CCMs (Figure 1.8).

CAs play multiple functions depending on their intracellular location during DIC uptake: 1) external CAs convert HCO$_3^-$ to CO$_2$ leading to increasing CO$_2$ concentration outside of the cell; 2) PPS-located or cytosolic CAs may convert CO$_2$ to HCO$_3^-$ in the cytosol or PPS to reduce CO$_2$ in those compartments, resulting in a CO$_2$ gradient between the inside and outside of the cell, which drives the CO$_2$ diffusion from environment into the cell; 3) the pyrenoidal/stromal CAs prevent the leakage of CO$_2$, if it is not used directly by Rubisco, from the chloroplast by converting it to HCO$_3^-$, while HCO$_3^-$ can be transported back to the chloroplast; 4) lumenal CA may generate CO$_2$ for Rubisco as shown in Figure 1.8 (Matsuda et al., 2017; Young & Hopkinson, 2017). In summary, the biophysical CCM in diatoms functions due to the proper spatial arrangement of bicarbonate transporters and carbonic anhydrases in the subcellular compartments (Young & Hopkinson, 2017).
General Introduction

Figure 1.8. Model of biophysical CCMs in (a) *P. tricornutum* and (b) *T. pseudonana*. The functional components of a biophysical CCM in diatoms comprise of bicarbonate transporters and carbonic anhydrases (CAs). The plasma membrane bicarbonate transporters are labeled as light blue arrows surrounded by grey ovals. The potential plastid membrane bicarbonate transporters are shown with light blue dashed arrows surrounded by grey ovals. The different subtypes of CAs are marked by circles of different colors and Greek letters. The CAs are located in different intercellular compartments of these two diatoms, resulting in distinct carbon flows in the CCM pathways. The inter-conversion of HCO$_3^-$ and CO$_2$ is marked as dark blue arrows. Black dashed arrows indicate the passive diffusion of carbon. Adapted from (Young & Hopkinson, 2017).

1.7.2 The current state of knowledge on biochemical CCMs in diatoms

Except showing some functional similarities, the CCMs in diatoms also show a distinct feature from *C. reinhardtii* and cyanobacteria that some species, for instance, *T. weissflogii* has been shown to perform a biochemical CCM, or at least produce C4 intermediates during photosynthesis (Reinfelder *et al.*, 2000; Roberts *et al.*, 2007b). Such biochemical CCM has not yet been reported.
in *C. reinhardtii* and cyanobacteria. In biochemical CCMs, HCO$_3^-$ and phosphoenolpyruvate (PEP) are pre-fixed by carboxylating enzymes, usually generating C4 acids; thus this mechanism is also known as C4 photosynthesis. These C4 acids are then transported into the chloroplast and decarboxylated by a decarboxylase to produce CO$_2$ in close proximity to Rubisco (Sage, 2004). In most terrestrial C4 plants, carboxylation and decarboxylation are accomplished in different cells: the fixation of atmospheric CO$_2$ into C4 acids happens in mesophyll cells, while the release of CO$_2$ from C4 acids takes place in bundle sheath cells (Sage *et al.*, 2012). Single-Cell C4 photosynthesis has also been described for some terrestrial and aquatic plants (Salvucci & Bowes, 1981; Ascencio & Bowes, 1983; Magnin *et al.*, 1997; Voznesenskaya *et al.*, 2001; Edwards *et al.*, 2004; Edwards & Voznesenskaya, 2010). However, there are only a few reports on C4 photosynthesis in algae, for instance, the marine, macroscopic green alga *Udotea flabellum* (Reiskind *et al.*, 1988; Reiskind & Bowes, 1991). In marine diatom *Thalassiosira weissflogii*, observations of principal intracellular C4 compounds (malate) after $^{14}$CO$_2$ labeling and $^{14}$C transfer from malate to phosphoglyceric acid (PGA) in CO$_2$ stressed cells; the higher activity of phosphoenolpyruvate carboxylase (PEPC) at low CO$_2$ than high CO$_2$ concentrations suggest the existence of a biochemical CCM in this species (Reinfelder *et al.*, 2000). Indications of C4 photosynthesis in diatoms is not only limited to *T. weissflogii*, some biochemical and molecular data also suggest C4-assisted photosynthesis in *T. pseudonana* and *P. tricornutum* (Beardall *et al.*, 1976; McGinn & Morel, 2008; Kustka *et al.*, 2014). The availability of genome sequences and genome editing tools for *P. tricornutum* and *T. pseudonana* has promoted the molecular and physiological investigation into the existence of biochemical CCMs in marine diatoms. However, other studies indicate the absence of a biochemical CCM in *P. tricornutum* and *T. pseudonana* (Cassar & Laws, 2007; Roberts *et al.*, 2007b; Granum *et al.*, 2009; Nunn *et al.*, 2009; Trimborn *et al.*, 2009; Haimovich-Dayan *et al.*, 2013; Tanaka *et al.*, 2014; Clement *et al.*, 2016; Clement *et al.*, 2017; Ewe *et al.*, 2018). Genomic analysis of *T. pseudonana* (Armbrust *et al.*, 2004) and *P. tricornutum* (Bowler *et al.*, 2008) showed that genes required for the C4 pathway exist in both of these diatom species (Kroth *et al.*, 2008). However, none of the known decarboxylating enzymes like malic enzyme (ME) and phosphoenolpyruvate carboxykinase (PEPCK) are located in the plastid (Tanaka *et al.*, 2014; Ewe *et al.*, 2018).
PEPC in land plants, green algae, and diatoms

PEPC catalyzes the irreversible reaction of phosphoenolpyruvate (PEP) and bicarbonate to form oxaloacetate and phosphate using Mg$^{2+}$ as a cofactor. PEPC is widely distributed in land plants, algae, bacteria, but absent in animals and fungi (Chollet et al., 1996; Izui et al., 2004). The PEPCs from land plants have been largely investigated, but little is known about PEPC from algae. The physiological function of PEPC in plants and green algae is shown in Figure 1.9. PEPCs involved in photosynthesis are responsible for carbon assimilation in C4 and CAM plants. Non-photosynthetic PEPCs play roles in other pathways that supply carbon skeletons for intermediates used in the tricarboxylic acid cycle (TCA) cycle, seed development and germination, supply of energy for symbiotic bacteria, and other (O'Leary et al., 2011) (Figure1.9). PEPCs in green algae have been shown to be involved in non-photosynthetic anaplerotic functions (Schuller et al., 1990; Happe & Turpin, 1994; Norici et al., 2002; Mamedov et al., 2005). In diatoms, the PEPCs were proposed to be involved in photosynthesis (Reinfelder et al., 2000; Reinfelder et al., 2004). C4 photosynthesis has evolved many times independently during the evolution of land plants (Sage, 2004), and photosynthetic PEPCs have independently evolved from non-photosynthetic PEPCs (Sage, 2004). Phylogenetic analysis by (Gehrig et al., 1998; Gehrig et al., 2001) indicates that the C4 PEPCs are not closely grouped with the non-photosynthetic PEPCs from the same or closely related species, indicating that the C4 PEPC have evolved independently from a non-photosynthetic ancestor. Thus, photosynthetic PEPCs acquired distinct properties, which can be distinguished from the other PEPCs of land plants.
Figure 1.9. The physiological function of PEPC in land plants and green algae. The physiological functions of PEPCs in land plants are indicated by dark green arrows: photosynthetic PEPCs are involved in atmospheric CO$_2$ fixation in C4 and CAM plants. Non-photosynthetic PEPCs may supply carbon skeleton for intermediates used in the tricarboxylic acid cycle (TCA) cycle, seed development, and germination, and supply energy for symbiotic bacteria. PEPCs in green algae are involved in anaplerotic functions, indicated by the light green arrow. Adapted from (O'Leary et al., 2011).
1.8 Aims of this thesis

The existence of a biochemical CCM is controversially discussed in different diatom species. Although the putative C4 enzymes are found in the model diatom *P. tricornutum*, the function of these enzymes is not yet clear. The main purpose of this study was to examine the presence of a biochemical CCM in *P. tricornutum*. For this goal, firstly, a culture method that allows a supply of different CO₂ concentrations for *P. tricornutum* need to be established. Secondly, we targeted investigating the functions of the two PEPCs in *P. tricornutum*. Therefore, the enzymatic characteristics of recombinant PEPCs from *P. tricornutum* expressed in *Escherichia coli* should be determined in enzymatic assays. Furthermore, we aimed to show whether the two PEPCs from *P. tricornutum* are involved in biochemical CCMs via generating PEPC knockout mutants and physiological characterization on the obtained PEPC knockouts.
Chapter 2

Controlled supply of CO₂ to batch cultures of the diatom *Phaeodactylum tricornutum*

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2. Controlled supply of CO₂ to batch cultures of the diatom *Phaeodactylum tricornutum*

2.1 Abstract

For the growth of photosynthetic organisms, the supply of CO₂ is essential. Experimental work on the uptake and utilization of inorganic carbon, requires that CO₂ concentrations can be adjusted and kept stable. Here we tested the suitability of a culture method that allows the supply of CO₂ to a cell suspension, without the need of continuous external gas supply for experimental work with the diatom *Phaeodactylum tricornutum*. This approach utilizes buffers with different ratios of HCO₃⁻/CO₃²⁻ in one chamber of a two-tier vessel, releasing different amounts of CO₂ to the gas phase of the vessel, which is shared with the cell culture in the other chamber of the vessel. We cultured *P. tricornutum* under three different CO₂ concentrations, while monitoring cell density, CO₂ concentration in the gas phase, and pH within the cultures. We found the method very useful for work with *P. tricornutum* and found that the method also allows the creation of CO₂ deplete conditions. This culturing system, while not as precise as a chemostat culture with a supply of gas mixtures containing CO₂, is simple to use and offers the possibility to adjust CO₂ growth conditions.

*Keywords*: diatom, *Phaeodactylum tricornutum*, carbon concentrating mechanism, carbon dioxide, photosynthesis, batch culture
2.2 Introduction

Diatoms are an important part of the phytoplankton and contribute significantly to the global oceanic organic carbon production each year (Nelson et al., 1995). In addition, diatoms are also important for biotechnological applications (see the themed issue of Philosophical Transactions of the Royal Society B) (Schoefs et al., 2017). Carbon fixation in diatoms is particularly interesting because some diatoms use carbon concentrating mechanisms (CCMs), which allow more efficient photosynthesis in carbon-limited environments. These mechanisms are mainly of biophysical nature and combine the activities of carbonic anhydrases (Morel et al., 1994; Colman & Rotatore, 1995; Kikutani et al., 2016) and of specific HCO$_3^-$ transporters (Burkhardt et al., 2001; Nakajima et al., 2013). There is also some evidence for a biochemical pre-fixation of inorganic carbon in Thalassiosira weissflogii (Reinfelder et al., 2000), however, the existence of a biochemical CCM in other diatom species is quite controversial (McGinn & Morel, 2008; Haimovich-Dayan et al., 2013; Kustka et al., 2014; Clement et al., 2016).

For basic research, as well as for biotechnology, liquid phase cell cultures are universally employed systems. There is a range of culturing systems, which offer various levels of control over the culture (Table 2.1). While batch cultures are the simplest in the application, they are problematic because the growth conditions change during cultivation time. These changes are very difficult to quantify and to account for in the analyses of the results. For investigating diatom CCMs in the lab, a possibility to culture the cells at a variety of different CO$_2$ conditions is desirable. Bubbling algal cultures with gas mixtures is currently a popular method to achieve this (King et al., 2015; Clement et al., 2016), however, this method can be problematic due to the constant release of CO$_2$ to the culture chamber, which also requires continuous monitoring of the gas flow.

In this study, we tested HCO$_3^-$/CO$_2^2-$ buffers as an alternative CO$_2$ source or sink. Historically CO$_2$ has been depleted from reaction chambers of Warburg apparatuses using KOH solution (Oesper, 1964). With the advance of manometrical methods in biochemistry, Warburg and Kippahl (1960) also developed a system that allows adjustment of the CO$_2$ content of a gas phase reaction chamber to measure photosynthetic oxygen production of leaves. The ability of the HCO$_3^-$/CO$_2^2-$ buffer mixtures to regulate the CO$_2$ content of the corresponding gas phase was subsequently used for cultivation of photosynthetic organisms in two-tier culture vessels that can be illuminated and placed on horizontal shakers (Hüsemann & Barz, 1977; Tripathi et al., 2001; Ranga Rao et al., 2007; Aikawa et al., 2014; Bähr et al., 2016). Such two-tier vessels consist of two Erlenmeyer flasks that are connected via a ground glass joint (Figure 2.1). The lower compartment contains the HCO$_3^-$
buffer solution, which exchanges CO$_2$ with the corresponding gas phase. CO$_2$ in this gas phase then freely diffuses through the joint into the upper compartment where the cells are suspended in culture medium. In this work, we present our results on the suitability of the two-tier vessel system for cultivating the diatom *P. tricornutum* in CO$_2$ deplete or replete conditions.

Table 2.1. Change of parameters during photosynthetic cell growth in the widely employed culture systems and the CO$_2$ supplied batch cultures tested in this study.

<table>
<thead>
<tr>
<th></th>
<th>Nutrients</th>
<th>CO$_2$</th>
<th>O$_2$</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Batch culture</strong></td>
<td>Decreasing</td>
<td>Decreasing unquantifiably</td>
<td>Increasing unquantifiably</td>
<td>Increasing</td>
</tr>
<tr>
<td><strong>Semi continuous</strong></td>
<td>Changing</td>
<td>Decreasing unquantifiably</td>
<td>Increasing unquantifiably</td>
<td>Increasing</td>
</tr>
<tr>
<td><strong>culture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fed batch culture)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Batch culture with</strong></td>
<td>Decreasing</td>
<td>Constant</td>
<td>Constant</td>
<td>Increasing</td>
</tr>
<tr>
<td><strong>constant gas supply</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(by bubbling)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Continuous culture</strong></td>
<td>Constant</td>
<td>Constant</td>
<td>Constant</td>
<td>Constant</td>
</tr>
<tr>
<td>(chemostat with gas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exchange/supply)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Batch culture with</strong></td>
<td>Decreasing</td>
<td>Stable within the</td>
<td>Increasing unquantifiably</td>
<td>Increasing</td>
</tr>
<tr>
<td><strong>CO$_2$ repletion</strong></td>
<td></td>
<td>targeted range</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Batch culture with</strong></td>
<td>Decreasing</td>
<td>Stable within the</td>
<td>Increasing unquantifiably</td>
<td>Increasing</td>
</tr>
<tr>
<td><strong>CO$_2$ depletion</strong></td>
<td></td>
<td>targeted range</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3 Materials and methods

2.3.1 Two-tier vessel and CO$_2$ supplying buffer

The two-tier culture vessel (shown in Figure 2.1-B) was designed to be sealed with a rubber plug and parafilm to prevent gas exchange between the vessel and the surrounding air. Figure 2.1-C shows the model of the two-tier culture vessel, three silicon tubes were inserted into the plug to allow sampling. One tube was placed in the upper chamber to allow taking gas samples for CO$_2$ concentration measurements; the second tube facilitates the exchange of the HCO$_3^-$/CO$_3^{2-}$ buffer by freshly made buffer if needed. The third tube allows sampling of the algal culture. The HCO$_3^-$/CO$_3^{2-}$ buffer was made by mixing different ratios of KHCO$_3$ and K$_2$CO$_3$ solutions to adjust different CO$_2$
concentration in the corresponding gas phase. A good starting point for choosing HCO$_3^-$/CO$_3^{2-}$ buffer compositions are provided in Table 2.2 of the publication by (Warburg & Krippahl, 1960). Starting with these suggestions, we chose three different HCO$_3^-$/CO$_3^{2-}$ buffers to supply defined CO$_2$ concentrations to $P. tricornutum$ cultures as shown in Table 2.2.

2.3.2 Strains and culture conditions

$P. tricornutum$ strain UTEX 646, obtained from Culture Collection of Algae, University of Texas (https://utex.org/), was used in this study. The culture was grown in modified f/2 medium with artificial half-concentrated sea salts (16.6 g l$^{-1}$) and other supplements (Guillard & Ryther, 1962; Guillard, 1975), at 20 °C, illuminated with a light intensity of 65-70 μmol photons m$^{-2}$s$^{-1}$ (16h light: 8h dark cycle). All cultures were grown in two-tier culture vessels on a horizontal shaker (shown in Figure 2.1-A) and were inoculated from exponential phase cultures.

Figure 2.1. The two-tier vessel culture method for photoautotrophic growth. (A) Cultures in two-tier vessels on a horizontal shaker with illumination from the top. (B) Loaded two-tier vessel with 50 ml of diatom culture in the upper chamber and 100 ml of KHCO$_3$/K$_2$CO$_3$ buffer solution in the lower chamber, sealed with a rubber plug and parafilm, and the three tubes are closed by clips. (C) Schematic of the two-tier vessel. (1) lower chamber; (2) upper chamber; (3) KHCO$_3$/K$_2$CO$_3$ buffer mixture; (4) $P. tricornutum$ culture; (5) gas sample tube; (6) buffer tube; (7) algal sample tube; (8) rubber plug.
Table 2.2. The composition of the three different KHCO$_3$/K$_2$CO$_3$ buffers.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>buffer A</th>
<th>buffer B</th>
<th>buffer C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of KHCO$_3$/K$_2$CO$_3$</td>
<td>0.2 M (30 ml/70 ml)</td>
<td>0.2 M (45 ml/55 ml)</td>
<td>2 M (60 ml/40 ml)</td>
</tr>
<tr>
<td>KHCO$_3$</td>
<td>0.6 g</td>
<td>0.9 g</td>
<td>12 g</td>
</tr>
<tr>
<td>K$_2$CO$_3$</td>
<td>1.9348 g</td>
<td>1.518 g</td>
<td>11.056 g</td>
</tr>
<tr>
<td>Dissolve in ddH$_2$O</td>
<td>in 100 ml ddH$_2$O</td>
<td>100 ml ddH$_2$O</td>
<td>100 ml ddH$_2$O</td>
</tr>
</tbody>
</table>

2.3.3 Carbon dioxide measurement

The CO$_2$ concentration in the gas phase of the upper chamber was measured using a Gas Chromatograph (GC) (SRI 8610C, Bad Honnef, Germany). For measurements of high CO$_2$ concentrations, 0.5 %, 1 % and 2 % CO$_2$ standards were prepared from pure CO$_2$ and N$_2$. These standards were measured and plotted to a standard gas curve before the samples were measured. For low CO$_2$ concentration measurements, 0.04 % CO$_2$ was used as a standard CO$_2$ concentration before sample measurements. A 0.04 % standard CO$_2$ gas, consisting of 0.04 % CO$_2$, 0.0002 % CH$_4$ and 99.9598 % N$_2$, was obtained from AIR LIQUIDE (Düsseldorf, Germany). Settings on the GC were as follows: N$_2$: -25 psi; H$_2$: -20 psi, air: -5 psi, methanizer (detector 2) temperature: 335 °C, column oven temperature: 60 °C, and 1.5 ml standard CO$_2$ gas or sample gas were injected into the sample loop for measurement.

2.3.4 Culture growth curve and pH measurement

The growth curve of *P. tricornutum* and the pH of the culture medium were determined during growth. The cell number was monitored daily with help of a Coulter Counter Multisizer 3 (Beckman Coulter, Krefeld, Germany). The pH of the culture was measured with a pH electrode EL2 (Mettler-Toledo Group, Switzerland).

2.3.5 Statistical analysis

Every culture experiment was carried out with four replicates in parallel. Data are given as arithmetic means with standard deviations. The Student’s t-test was applied to evaluate the difference between mean values.
2.4 Results

According to the experiments by Warburg and Krippahl (1960), different mixing ratios of HCO$_3^-$ /CO$_3^{2-}$ buffers can supply different CO$_2$ concentrations in the gas phase of a closed system. Accordingly, three different HCO$_3$/CO$_3^{2-}$ buffer mixtures were used here to obtain low, near-ambient, and high CO$_2$ concentrations for *P. tricornutum* using the two-tier vessels. For exact determination of the actual CO$_2$ concentrations in the vessels, the HCO$_3$/CO$_3^{2-}$ buffers and the culture were added, the vessels were sealed and subsequently incubated overnight on a shaker. Then a gas sample from each vessel’s gas phase was taken and analyzed via GC. The results showed that buffer A can generate a concentration of 0.0146 % ± 0.0014 % CO$_2$, while buffer B can supply 0.0303 % ± 0.0003 % CO$_2$, respectively. Accordingly, these buffers were used to generate low and near-ambient CO$_2$ concentrations. Buffer C resulted in a CO$_2$ concentration of 0.64 % ± 0.022 % CO$_2$, which was later used to create a high CO$_2$ concentration. From the gas phase measurements, we calculated dissolved CO$_2$ concentrations in the f/2 medium using the Henry's law constant at the temperature 20°C and standard atmosphere pressure by this formula: 

\[
C \text{ (dissolved CO}_2\text{)} = K_{\text{Henry's law constant at 20°C}} \text{ (M/atm)} \times \text{CO}_2 \text{ concentration (percentage, v/v)} \times \text{standard atmosphere pressure (101325 Pa)}
\]

Table 2.3. CO$_2$ concentrations in the gas and liquid phase adjusted in the culture medium via different KHCO$_3$/K$_2$CO$_3$ buffers.

<table>
<thead>
<tr>
<th></th>
<th>Buffer A</th>
<th>Buffer B</th>
<th>Buffer C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$ concentration in the gas phase (%)</td>
<td>0.0146%±0.0014%</td>
<td>0.0303%±0.0003%</td>
<td>0.64%±0.022%</td>
</tr>
<tr>
<td>Corresponding CO$_2$ concentration in the liquid culture phase (µM)</td>
<td>5.33±0.51</td>
<td>11.05±0.11</td>
<td>233.45±8.02</td>
</tr>
</tbody>
</table>

The three different mixing ratios of the HCO$_3$/CO$_3^{2-}$ buffers mentioned above were tested by incubating a *P. tricornutum* wild type strain over a period of 9 days. Growth curves were recorded, and the CO$_2$ concentrations in the upper chamber, the cell number, and the pH of the medium were measured daily during the growth period.

The results showed that with buffer C, the CO$_2$ concentration in the culture gas phase increased from that of ambient air to 0.64 % ± 0.022 % within a single day (Figure 2.2-A). On the seventh day of growth, the CO$_2$ concentration decreased approximately 34 % compared to the value on the
second day because of the large consumption of CO₂ during the exponential phase of the cultures. Afterward, the CO₂ concentration stabilized around 0.4 % until the end of the growth curve. During the whole cultivation period, the CO₂ concentrations stayed within the range of 0.42 % ± 0.03 % to 64 % ± 0.022 %. The cultures showed a doubling time of 12.51 hours (calculated from the growth of 96 to 144 hours) during the exponential phase under the CO₂ saturated condition (Figure 2.2-B). As the concentration of CO₂ may also affect the pH value, we have also followed the pH value in the culture medium during cultivation, which dropped from 8.1975 ± 0.001 to 6.875 ± 0.017 after one day (Figure 2.2-C). However, due to the buffer capacity of the culture medium, the pH did not change strongly during the exponential phase. This was because the CO₂ supply was able to compensate for the rate of CO₂ consumption.

For generating low and near-ambient CO₂ conditions, we used buffers A and B respectively. Here, on the seventh day, the CO₂ in the culture medium decreased considerably by up to 50 % (Figure 2.2-A). This might be because CO₂ consumption by the culture in the closed system exceeded the capacity of the CO₂ generating buffers. In such a low CO₂ condition, the cultures grew significantly slower than in high CO₂ condition supplied by buffer C (Figure 2.2-B), with doubling time of 15.89 and 14.17 hours respectively (calculated from the growth of 72 to 122 hours). The maximum cell densities of the low and near-ambient CO₂ cultures are smaller than at the high CO₂ conditions, which means that cell growth is limited by the available CO₂. The pH within the low and near-ambient CO₂ cultures developed differently than at high CO₂ concentrations. During the first four days the pH was only weakly affected, then the pH increased because of the consumption of residual CO₂ and the corresponding alkalinization of the medium Figure 2.2-C.
Figure 2.2. (A) CO$_2$ concentrations supplied by different KHCO$_3$/K$_2$CO$_3$ buffers measured using a GC during the growth curve. (B) Growth curves of *P. tricornutum* wild type strain under three different CO$_2$ concentrations. Doubling time of wild type from buffer A and B were calculated from 72 to 120 hours and from buffer C 96 to 144 hours. (C) The pH of the culture during the growth curve.
2.5 Discussion

Our experiments showed that different mixing ratios of HCO$_3^-$/CO$_3^{2-}$ buffers in the two-tier vessel can successfully supply low and high concentrations of CO$_2$ to *P. tricornutum*. Using this buffer system, also other than the described concentrations can be used by applying different mixing ratios of HCO$_3^-$/CO$_3^{2-}$ buffers. Using this two-tier vessel system is an easy-to-use alternative to bubbling cultures with CO$_2$ gas mixtures. Advantages of the two-tier vessels include easy handling as well as the fact that there is no need for gas bottles, mass flow pressure regulators or CO$_2$ warning systems. Furthermore, two-tier vessels are very convenient to use for axenic cultures under lab conditions and allow for more replicates in parallel. Finally, the components required here are not very costly. Compared to bubbling with CO$_2$, there are also some disadvantages. The two-tier vessels can supply constant high ranges of CO$_2$ concentrations, while for low CO$_2$ concentrations, the CO$_2$ will be consumed gradually by the culture in the closed system. Thus the CO$_2$ concentration cannot be as stable as in a continuous gas supply system. In order to increase stability, it is possible to change the depleted HCO$_3^-$/CO$_3^{2-}$ buffer with a freshly prepared buffer during the growth regularly, either at fixed intervals or according to the observed CO$_2$ concentrations. Furthermore, the two-tier vessels cannot easily be applied to large culture volumes because of reduced convection and diffusion within larger culture flasks. Thus, if there is no CO$_2$ gas or mass flow pressure regulator available in the lab, using two-tier vessels is a good and economic method to supply different CO$_2$ concentrations to cultured cells. This approach may also easily be applied to other diatom species or other eukaryotic algae.

2.6 Acknowledgments

We like to thank R. Miller for sharing her expertise on the two-tier vessels, as well as M. Pester for helpful discussions and for access to the GC. This work was supported by the University of Konstanz, the Graduate School Biological Sciences (GBS) and the China Scholarship Council (CSC).
Chapter 3

Overexpression and kinetic analyses of recombinant PEPCs of Phaeodactylum tricornutum

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3. Overexpression and kinetic analyses of recombinant PEPCs of *Phaeodactylum tricornutum*

3.1 Abstract

Phosphoenolpyruvate carboxylase (PEPC) is a key enzyme in the C4 photosynthesis. Genes for two isoforms of PEPC were identified in the diatom *Phaeodactylum tricornutum* (Pt). PEPC1 is located in the periplastidic space, and PEPC2 inside the mitochondria. Comparing the amino acid sequences of the PEPCs of *P. tricornutum* and *Thalassiosira pseudonana* with plant-type and bacterial-type PEPCs shows that diatom PEPCs contain the typical motifs and binding sites, but lack the N-terminal serine phosphorylation site which is a specific characterization of plant-type PEPCs. Kinetic analyses showed that recombinant PtPEPC1 and PtPEPC2 showed different pH optima. Compared to the C4 and C3-type PEPCs in land plants, the $K_m$ values of both recombinant PtPEPCs to PEP and HCO$_3^-$ are higher than in C4 or C3-type PEPCs of land plants. The $K_m$ value of PtPEPC2 for PEP (1.6 mM) is higher than that of PtPEPC1 (1.0 mM), while the $K_m$ value of PtPEPC2 for HCO$_3^-$ (0.1mM) is lower than that of PtPEPC1 (0.8mM).

3.2 Introduction

Phosphoenolpyruvate carboxylase (PEPC) catalyzes the irreversible reaction of phosphoenolpyruvate (PEP) and bicarbonate to form oxaloacetate (OAA) and phosphate. This enzyme is widely distributed in plants, algae, and bacteria, but not found in animals and fungi (Chollet et al., 1996; Izui et al., 2004). PEPC was also recently identified in several archaea species (Ettena et al., 2004; Izui et al., 2004; Matsumura et al., 2006). Photosynthetic PEPCs play a crucial role in primary CO$_2$ assimilation in C4 metabolism, crassulacean acid metabolism (CAM) plants, macroscopic green alga and unicellular microalgae (Salvucci & Bowes, 1981; Ascencio & Bowes, 1983; Reiskind et al., 1988; Reiskind & Bowes, 1991; Magnin et al., 1997; Reinfelder et al., 2000; Sage, 2004; Sage et al., 2012). Non-photosynthetic PEPCs are involved in carbon skeleton supply for the tricarboxylic acid (TCA) cycle, regulation of carbon and nitrogen metabolism, maintenance of cellular pH, carbon supply for N$_2$ fixation in legume root nodules, seed formation and others (Schuller et al., 1990; Huppe & Turpin, 1994; Rivoal et al., 1998; O'Leary et al., 2011). PEPCs use PEP and bicarbonate as substrates to produce oxaloacetate and phosphate, Mg$^{2+}$ serves as a cofactor, the active form of this enzyme is usually a homotetramer (Izui et al., 2004). PEPC activity is regulated by different factors, most of them are allosteric enzymes with a wide variety of allosteric effectors depending on the species (Izui et al., 2004). For instance, PEPCs are activated by glucose-6-phosphate, and feedback inhibited by malate and aspartate (Bauwe & Chollet, 1986). The
photosynthetic PEPCs acquired distinct properties from non-photosynthetic PEPCs during the evolution in terrestrial plants regarding gene expression and the kinetic properties (Sheen, 1999; Gowik & Westhoff, 2011). The C4 photosynthesis of terrestrial plants takes place in two different cell types, the PEPC carboxylation functions in mesophyll cells and decarboxylation takes place in bundle sheath cells (Sage, 2004). The C4 PEPCs are only expressed in mesophyll cells and are highly expressed to ensure the carbon flow for C4 photosynthetic function, while the C3 PEPCs are expressed at a lower level (Sheen, 1999). The photosynthetic PEPCs bind to its substrate PEP with a higher $K_m$ value than the non-photosynthetic PEPCs (Dong et al., 1998; Gowik et al., 2006; Lara et al., 2006), in contrast, the $K_m$ value of photosynthetic PEPCs for bicarbonate is lower (Gutierrez et al., 1974). On the other hand, the photosynthetic PEPCs are more tolerant to the allosteric inhibitor malate and are strongly regulated by the allosteric activator glucose-6-phosphate (Dong et al., 1998; Blä sing et al., 2002).

While details of PEPC gene expression, enzyme biochemical regulation, evolution and physiologic functions in plans are well studied (Sheen, 1999; Izui et al., 2004; Gowik & Westhoff, 2011); PEPCs in algae are poorly investigated. The physiologic role of PEPC in Chlorophyceae is limited to some green algae species (Schuller et al., 1990; Rivoal et al., 1998; Rivoal et al., 2001; Norici et al., 2002; Mamedov et al., 2005). PEPCs from green algae have been reported to be related to non-photosynthetic PEPC, (Schuller et al., 1990; Huppe & Turpin, 1994; Norici et al., 2002; Mamedov et al., 2005), the PEPC in the diatom *T. weissflogii* interestingly has been shown to be involved in a C4-type photosynthesis (Reinfelder et al., 2000). Unlike plants, but similar to green algae, two PEPC isoforms were found in the marine diatoms *P. tricornutum* and *T. pseudonana* (Kroth et al., 2008). Although few physiological data of PEPC is available (Reinfelder et al., 2000; Reinfelder et al., 2004; Cassar & Laws, 2007; McGinn & Morel, 2008; Clement et al., 2017; Kroth et al., 2018), there is no study of kinetic properties and the regulation of both isoforms of PEPCs in diatoms. Therefore, the aim of this study was the investigation of the kinetic properties of the recombinant PEPC proteins of *P. tricornutum*. The two PEPC isoforms of *P. tricornutum* were overexpressed in *Escherichia coli*, the recombinant PtPEPC proteins were purified and enzyme activities were studied, allowing a comparison of the kinetic properties to C4 and C3-type PEPCs of terrestrial plants.
3.3 Materials and Methods

3.3.1 PEPC protein sequences alignment of diatoms and other organisms

*Phaeodactylum tricornutum* PEPC1 (JGI Protein ID 56026) and PEPC2 (JGI Protein ID 20853) protein sequences annotated by (Kroth et al., 2008) were used in this study. The PEPC protein sequences of *P. tricornutum* and *T. pseudonana* were downloaded from the JGI database, PEPC protein sequences of other organisms used for alignment were downloaded from the Genebank database. The JGI and Genebank accession number of PEPC protein sequences are listed in Table S3.1. PEPC sequences were aligned using a multiple sequence alignment online program (Clustal Omega) (https://www.ebi.ac.uk/Tools/msa/clustalo/), using the default values.

3.3.2 Pre-sequence prediction of *P. tricornutum* PEPCs

Protein sequences of *P. tricornutum* PEPCs were analyzed to predict the presence of N-terminal targeting peptides using the prediction program Signal P server 3.0 (www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al., 2007), and ASAFind (http://rocaplab.ocean.washington.edu/tools/asafind) (Gruber et al., 2015). The program TargetP 1.1 server (www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2000) was used to predict transit peptide of mitochondria-localized protein.

3.3.3 Generation of PtPEPC1 and PtPEPC2 overexpression constructs

The pre-sequences of PtPEPCs were removed according to predictions. The DNA fragment encoding the putative mature PEPC1 protein was amplified by PCR from genomic DNA using primers PEPC1-gy38 for and PEPC1-gy39 rev, and the PCR product was ligated into the PCR\textsuperscript{script}-MssI vector. The intron was deleted by PCR using the primers PEPC1-gy41 for and PEPC1-gy42 rev. Primers PEPC1-gy43 for and PEPC1-gy44 rev were used to correct one site mutation caused by PCR. The resulting plasmid PCR\textsuperscript{script}-MssI-PEPC1 was confirmed by sequencing. The PCR\textsuperscript{script}-MssI-PEPC1 vector and overexpression vector pET28a(+) (Novagen part of Merck KGaA, Darmstadt, Germany) were both digested with NdeI and BamHI, and the purified *PEPC1* DNA fragments were ligated into pET28a(+) vector at the NdeI and BamHI position to generate the PtPEPC1 overexpression vector pET28a-His-PEPC1. The gene fragments of PtPEPC2 were amplified from genomic DNA with primers PEPC2- gy34 for and PEPC2- gy35 rev. The PCR products were ligated into the PCR\textsuperscript{script}-MssI vector, primers PEPC2-gy36 for and PEPC2-gy37 rev were used to correct one site mutation caused by PCR. The PtPEPC2 sequence was confirmed by sequencing, both PCR\textsuperscript{script}-MssI-PEPC2 and pET28a(+) vectors were digested with NdeI and
HindIII, and the purified PEPC2 DNA fragments were ligated into pET28a(+) vector in the NdeI and HindIII position to generate the PEPC2 overexpression vector pET28a-His-PEPC2. Primers used in this study are listed in Table S3.2.

3.3.4 Cell culture

Competent cell *Escherichia coli* XL1 blue and protein overexpression strains *E. coli* BL21 and BL21 (DE3 Rosetta) were cultivated in LB medium (10 g NaCl, 10 g Pepton (Roth), 5 g yeast extract (Roth) in 1 L distilled water) at 37 °C. A small volume of the cell cultures was cultivated together with larger volumes of cell cultures (500 mL) on a rotary shaker with a speed of 180 rpm (INFORS, Bottmingen, Switzerland). For solid media, 1.2 g L⁻¹ agar (Roth) was added to the LB medium. The antibiotics: ampicillin (100 μg/mL), kanamycin (50 μg/mL), and Chloramphenicol (34 μg/mL) were used for selection.

3.3.5 Overexpression and purification of recombinant PtPEPCs

The overexpression vectors pET28a-His-PEPC1 and pET28a-His-PEPC2 were respectively transformed into *E. coli* BL21 and BL21 (DE3 Rosetta) cells by electroporation (MicroPulser™, BIO-RAD, USA). Then single colonies were cultivated in LB medium at 37 °C until the OD600 reached 0.5-0.6. Thereafter, cells were induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Roth) at a final concentration of 0.5 mM, and the cultures incubated at 20 °C overnight in a rotary shaker at a rotation speed of 180 rpm. The control cells were not induced with 0.5 mM IPTG but cultured at the same condition. Two milliliter *E. coli* cells from 1 L of IPTG induced culture were harvested by centrifugation at 5,000 g for 3 min and analyzed by SDS PAGE. The residual *E. coli* cells cultures were harvested and resuspended in lysis buffer (50 mM Hepes-NaOH pH 8.0, 100 mM NaCl, 25 mM imidazole, and proteinase inhibitor). Cells were disrupted with a French Press (FA-079, SLM Aminco) at a pressure of 1000 Psi twice at 4 °C. The cell lysate was centrifuged at maximum speed for 30 min at 4 °C, and the supernatant was filtered through 0.22 μm filters to remove big particles then was applied to His Trap FF crude column (GE Healthcare, Uppsala, Sweden) by ÄKTA Fast Protein Liquid Chromatography (FPLC) (P900, Amersham Biosciences). The column was washed first with 20% Ethanol and water, then was equilibrated with 5% B1 buffer (50 mM Hepes-NaOH pH 8.0, 100 mM NaCl, 500 mM imidazole) and 95% A1 buffer (50 mM Hepes-NaOH pH 8.0, 100 mM NaCl). Afterward, the filtered supernatant was injected into the sample loop and the flow-through was collected and the column was washed with 5% B1 buffer and 95% A1 buffer. Lastly, the His tag-PtPEPC fusion protein was eluted with a gradient elution
buffer (5% B1 - 100% B1) to determine the optimal concentration of B1 buffer for protein elution, all the eluted proteins were collected and analyzed by SDS PAGE. All solutions used in the purification process were filtered through a 0.22 µm filter to remove big particles. One liter of IPTG induced culture was used for recombinant PEPC purification, 20% buffer B1 and 80% buffer A1 was used for washing step; 50% buffer B1 and 50% buffer A1 was used for recombinant protein elution.

### 3.3.6 Immunoblotting analysis and measurement of recombinant PtPEPC activity

The concentration of the purified recombinant PtPEPC protein was determined by using the Pierce™ 660nm Protein Assay Reagent (Thermo Scientific, Rockford, USA) according to the manufacturer’s protocol. The defined amount of purified proteins was separated on 8% acrylamide SDS PAGE, and transferred to a nitrocellulose membrane (Amersham Protran 0.1 NC, GE Healthcare) via a Trans-Blot Turbo cassettes (Bio-Rad) at 1.3 mA and 25 V for 20 mins. The membrane was first probed with the commercially available antiserum against PEPC (Agrisera AS09 458, Vännas, Sweden, 1:1000 dilution), and then probed with a second antibody Goat anti-Rabbit IgG (Invitrogen, 1:20000 dilution). For His tag detection, the membrane was probed with anti-His antibodies conjugated to horseradish peroxidase (HRP) using the Penta·His HRP Conjugate Kit (Cat No: 34460, QIAGEN). Roti-Block, Roti-Lumin plus (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and InstantBlue™ (Expedeon, San Diego, CA, USA) were used according to manufacturer’s instructions. Immunodetection was performed with the Odyssey® Fc Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The PEPC enzymatic activity was determined by spectrophotometrical monitoring of the decrease of NADH. In a two-step reaction, PEPC converts PEP and bicarbonate into oxaloacetate and phosphate, then the malate dehydrogenase (MDH) catalyzes the NADH-dependent reduction of oxaloacetate to malate. The consumption of NADH is monitored spectrometrically at 340 nm, the reactions were shown in Figure 3.1. The recombinant PtPEPC activity was measured in enzymatic buffer (50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, 15 mM MgCl₂, 10 vol % glycerol), 20 mM bicarbonate, 5 units of MDH (Roche, Karlsruhe, Germany), 0.2 mM NADH (Sigma, Mannheim, Germany). One unit of PEPC activity is defined as the amount of enzyme required to produce one µmol of OAA per minute, 1 µg purified recombinant PtPEPC protein was used for each assay in 1 mL reaction volume. The reaction was initiated by the addition of 4 mM PEP (Applichem, Darmstadt, Germany), the activity was continuously followed by recording absorbance by a Ultrospec™ 8000 photometer (GE Healthcare Life Sciences, Cambridge, UK), the temperature was maintained at 25 °C using a water
bath (JULABO, Seelbach, Germany). The absorption was recorded at least 5 min. For PEPC activity at different pH conditions, enzymatic buffers with different pHs were prepared as shown in Table 3.1. For PEP or HCO$_3^-$ saturation curves, increasing PEP or HCO$_3^-$ concentrations were added to the assay while the other substrates and MDH were in excess. The obtained kinetic data were plotted and calculated using nonlinear regression analysis software SigmaPlot (Version 12.5). Data depending on various substrate concentrations were fitted to Michaelis-Menten equation (1) (Michaelis et al., 2011) for hyperbolic kinetics and to the Hill equation (Hill, 1910) (2) for sigmoidal kinetic.

$$\text{equation (1)} \quad V = \frac{V_{\text{max}} \times [S]}{(K_m + [S])}$$

$$\text{equation (2)} \quad V = \frac{V_{\text{max}} \times [S]^n}{(K_m^n + [S]^n)}$$

In the equation, $V$ is the experimentally determined velocity; $V_{\text{max}}$ is the determined maximum velocity; $[S]$ is the concentration of substrate; $K_m$ is the concentration of substrate when reaching half $V_{\text{max}}$; $n$ is the Hill coefficient.
Figure 3.1. The reaction of PEPC coupled with the catalyzation of MDH to produce NAD$^+$.

Table 3.1. Enzymatic buffers with different pH used in this study.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
</tr>
<tr>
<td>Hepes-NaOH</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>9</td>
</tr>
<tr>
<td>CAPS-NaOH</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>

3.4 Results

3.4.1 Protein sequences of PEPCs from *P. tricornutum* compared to other organisms

Two isoforms of PEPC protein sequences from *P. tricornutum* (Pt) were aligned with PEPC protein sequences from other organisms. Results showed PtPEPC1 (JGI Protein ID 56026) and PtPEPC2 (JGI Protein ID 20853) are 40.54% and 40.02% identity to *Zea mays* PEPC-C4, 41.06% and 40.50% identity to *Zea mays* PEPC-C3, 40.98% and 42.94% identity to *E. coli* PEPC. The alignment of the PEPC protein sequences of plants, green algae, red algae, bacteria, and diatoms shows the catalytically critical residues, PEP and HCO$_3^-$ binding sites, glucose-6-phosphate and aspartate
binding sites are generally found among those species (Figure 3.2). The conserved phosphorylation domain was only found in Zea mays PEPC; Zea mays PEPC; Sorghum bicolor PEPC; Arabidopsis thaliana plant-type PEPC (PTPC), not in any of the diatom PEPCs, green algae PEPC, red algae PEPC, Arabidopsis thaliana bacteria-type PEPC (BTPC), and Escherichia coli PEPC. Characteristic serine (S) residue of C4 PEPCs only presents in Zea mays and Sorghum bicolor, but not in other organisms.

**Phosphorylation**

- Arabidopsis thaliana
- Zea mays
- Sorghum bicolor
- C. reinhardtii
- E. coli
- T. pseudonana

**Catalytic Base**

- Arabidopsis thaliana
- Zea mays
- Sorghum bicolor
- C. reinhardtii
- E. coli
- T. pseudonana

**Tetramer Formation**

- Arabidopsis thaliana
- Zea mays
- Sorghum bicolor
- C. reinhardtii
- E. coli
- T. pseudonana

**G6P Binding**

- Arabidopsis thaliana
- Zea mays
- Sorghum bicolor
- C. reinhardtii
- E. coli
- T. pseudonana
Figure 3.2. Alignment of partial PEPC protein sequences from plants (Arabidopsis thaliana, Sorghum bicolor, Zea mays), green algae (Chlamydomonas reinhardtii), red algae (Cyanidioschyzon merolae, Phaeodactylum tricornutum, Thalassiosira pseudonana), and diatoms (Phaeodactylum tricornutum, Thalassiosira pseudonana). The experimentally proven functions of amino acid residues (labeled with different colors) were referred to (Kai et al., 2003). [E/DR/KxS/DAQL/MLR]: phosphorylation Ser reside in a plant-invariant motif; x: phosphorylation; X: catalytically critical residue; X: glucose-6-phosphate binding; X: hydrophobic pocket; X: PEP binding; X: tetramer formation; X: Mg$^{2+}$ binding; X: HCO$_3$ binding; X: characteristic Ser of C4 PEPC; X: aspartate binding. RNTG: bacterial-type PEPC C-terminal (R/K) NTG tetrapeptide; QNTG: plant-type PEPC C-terminal QNTG tetrapeptide.
### 3.4.2 Generation of PtPEPC1 and PtPEPC2 overexpression constructs

In order to express the mature protein domains (without potential pre-sequences), we have studied the N-terminal protein sequence of the PtPEPCs. SignalP indicates an endoplasmic reticulum (ER) targeting signal peptide in PtPEPC1, but neither TargetP1.1 and nor ASAFind (Gruber et al., 2015) indicated a subsequent signal peptide. Instead, a mitochondrial transit peptide was detected in PtPEPC2. The signal peptide from the N-terminal was deleted from PtPEPC1 and the transit peptide was deleted from PtPEPC2 as shown in Figure 3.3. The gene fragments encoding the putative mature PtPEPC1 and PtPEPC2 proteins were ligated into the pET28a(+) vector introducing the His tag at the N-terminal of the PtPEPCs.

**PEPC1**

**Signal peptide**

<table>
<thead>
<tr>
<th>MKLLWST SILLTVVLAVPSSASPLFQVPSIVKN</th>
<th>QGMRNSG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>His Tag</strong> SPLFQVPSIVKN</td>
<td>QGMRNSG</td>
</tr>
</tbody>
</table>

**PEPC2**

**Mitochondrial transit peptide**

<table>
<thead>
<tr>
<th>MLSSSCRRSFL AAKTRLRSCVT STL GST GPWS</th>
<th>NGMGTG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>His Tag</strong> RSCV T STL GST GPWS</td>
<td>NGMGTG</td>
</tr>
</tbody>
</table>

Figure 3.3. Deletion of N-terminal targeting sequences of PtPEPC1 and PtPEPC2 proteins. The amino acid sequences in the long boxes show the respective full PtPEPC protein sequences, while in the shorter boxes show the sequences of the truncated protein and after addition of a His tag. The dashed lines in the middle of amino acid sequences indicate the abridged sequences, which are not shown. The signal peptide of the PtPEPC1 N-terminal sequence and the mitochondrial transit peptide of the PtPEPC2 N-terminal sequence are underlined in the long boxes.
3.4.3 Overexpression, purification and immunoblotting analysis of recombinant PtPEPC protein

PtPEPC1 and PtPEPC2 overexpression constructs were respectively transformed into *E. coli* BL21 and BL21 (DE3 Rosetta). The soluble protein and inclusion body fractions were separated by SDS PAGE and stained with Coomassie blue (Figure 3.4a). Both PtPEPC1 and PtPEPC2 recombinant proteins were successfully overexpressed in both *E. coli* strains after induction by 0.5 mM IPTG at 20 °C overnight. The recombinant PtPEPC1 was expressed mostly as soluble proteins, while recombinant PtPEPC2 mostly accumulated as inclusion bodies (Figure 3.4a). The PtPEPC2 recombinant protein was better expressed in *E. coli* BL21 (DE3 Rosetta) than in BL21, therefore, *E. coli* BL21 (DE3 Rosetta) strain was used for PtPEPC2 and PtPEPC1 overexpression in the following work. The PtPEPC1 and PtPEPC2 recombinant proteins were successfully purified via a His-Trap FF crude column by FPLC under native conditions. The crude protein extracts and purified recombinant protein were separated by SDS PAGE and stained with Coomassie Brilliant blue (Figure 3.4b). The purified recombinant PtPEPC proteins were loaded on SDS PAGE for western blot. One blot was probed using a commercial antiserum specific against PtPEPCs on the left side (Figure 3.4c), the other blot was probed using a His-tag antiserum against His tag on the right (Figure 3.4c). The two blots show bands containing recombinant PtPEPC proteins that are larger than 110KDa. The left blot confirms that the PEPC antibody can recognize both PtPEPC1 and PtPEPC2 isoforms, these two PtPEPC sizes in SDS gel are similar, and that the binding affinity of the antiserum to both PEPCs is similar.
Figure 3.4. Overexpression, purification and western blot of recombinant PtPEPC1 and PtPEPC2 proteins.  
(a) Overexpression of recombinant PtPEPC1 and PtPEPC2 proteins. The recombinant PtPEPC1 and PtPEPC2 proteins were both expressed in *E. coli* BL21 and BL21 (DE3 Rosetta). The soluble protein and inclusion bodies were separated by SDS PAGE and stained with Coomassie blue. “-” indicates the control sample without IPTG induction, “+” indicates the IPTG overnight induced samples. (b) Purification of recombinant PtPEPC1 and PtPEPC2 proteins. The recombinant PtPEPC1 and PtPEPC2 proteins were purified by His Trap purification. The crude soluble proteins and the elution fraction were both separated by
SDS PAGE and stained with Coomassie blue. (c) Western Blot of purified recombinant PtPEPC1 and PtPEPC2 proteins. The purified recombinant PtPEPC1 and PtPEPC2 protein were incubated with PEPC antibody and His tag antibody respectively. The membrane was stained with Ponceau S after blotting to show an equal amount of PEPC protein was loaded for western blots.

3.4.4 The kinetic properties of recombinant PtPEPCs

The enzymatic assays were performed directly after the purification of the recombinant PtPEPC proteins. The purified protein stored in a final concentration of 17 vol % glycerol, was fast frozen in liquid N₂ and be stored at -80 ° C. The stored protein was stable under this storage condition for at least one week.

pH Optima of recombinant PtPEPC enzymatic activities

Since two PtPEPC isoforms are localized in two different compartments that may have different pH values, we have estimated the pH conditions, at which the recombinant PtPEPCs have the highest activities. The results are shown in Figure3.5, demonstrating that under very acidic conditions, pH at 5.8 or 6.4, both recombinant PtPEPCs showed very low activity. Optimal conditions for PtPEPC1 activity is at pH 7.5 to 9 and for PEPC2 activity between at 8.5 and 9.

Recombinant PtPEPC kinetics

To investigate the affinities of recombinant PtPEPC proteins to the substrates PEP and HCO₃⁻, the PEPC activity was measured with different PEP or HCO₃⁻ concentrations. The PEP saturation curves of both PEPCs are shown in Figure 3.6a. Both recombinant PtPEPC proteins showed a sigmoidal saturation curve for the substrate PEP, which indicates a cooperative PEP binding. The calculated $K_m$ value of PtPEPC1 for PEP is 1 mM, while the $K_m$ value of PtPEPC2 is 1.6 mM, indicating that the affinity of PtPEPC1 for PEP is higher than that of PtPEPC2 (Table 3.2). The HCO₃⁻ saturation curves of both PEPC are shown in Figure 3.6b. Here, both PEPCs showed a hyperbolic saturation curve. The $K_m$ value of PtPEPC1 to HCO₃⁻ is 0.85 mM, while that of PtPEPC2 is 0.11 mM, indicating that the affinity of PtPEPC1 to HCO₃⁻ is 8 times lower than the affinity of PtPEPC2 to HCO₃⁻ (Table 3.3). Lastly, PtPEPC2 showed higher maximum PEPC activity than PtPEPC1 at the optimal conditions (Table 3.2 and Table 3.3).
Figure 3.5. Impact of pH to the purified recombinant PtPEPC enzyme. PEPC activity was measured in different pH buffer range from 5.8 to 11 at 25 °C.
Figure 3.6. Plots of the affinity of recombinant PEPC protein to substrates PEP and HCO$_3^-$: (a) The saturation curve of recombinant PtPEPCs to PEP plotted according to Hill equation. (b) Saturation curve of recombinant PtPEPCs to HCO$_3^-$ plotted according to Michaelis-Menten equation.
Table 3.2: Kinetic properties of PtPEPCs to PEP.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Assay pH</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (µM min$^{-1}$ mg$^{-1}$)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>PtPEPC1</td>
<td>8</td>
<td>1.04 ± 0.04</td>
<td>41.25 ± 0.95</td>
<td>2.62 ± 0.26</td>
</tr>
<tr>
<td>PtPEPC2</td>
<td>8.5</td>
<td>1.61 ± 0.03</td>
<td>68.56 ± 0.80</td>
<td>2.33 ± 0.07</td>
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Table 3.3: Kinetic properties of PtPEPCs to HCO$_3^-$.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Assay pH</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (µM min$^{-1}$ mg$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtPEPC1</td>
<td>8</td>
<td>0.85 ± 0.15</td>
<td>42.00 ± 1.15</td>
<td>1</td>
</tr>
<tr>
<td>PtPEPC2</td>
<td>8.5</td>
<td>0.11 ± 0.01</td>
<td>68.75 ± 1.14</td>
<td>1</td>
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</tbody>
</table>

3.5 Discussion and outlook

In plants, the plant-type PEPC that is encoded by 100–110-kDa polypeptides, contains the N-terminal serine phosphorylation domain and critical C-terminal tetrapeptide QNTG. These are the distinguishing features of plant-type PEPCs (PTPCs) (Izui et al., 2004; Xu et al., 2006). However, bacterial-type PEPCs (BTBCs) encoded by a larger 116–118-kDa polypeptide was also found in many plants, lacking N-terminal serine phosphorylation domain, but containing a prokaryotic-like (R/K) NTG tetrapeptide at its C terminus, they usually are weakly expressed (Sánchez & Cejudo, 2003; Sullivan et al., 2004; Sánchez et al., 2006; Gennidakis et al., 2007; O’Leary et al., 2009). Plant-type PEPCs are regulated by reversible phosphorylation of the N-terminal serine residue, which results in activation of the enzymes by reducing the inhibition by the allosteric inhibitors aspartate and malate (Vidal & Chollet, 1997; Nimmo, 2000). This target Ser residue is in a plant-invariant motif [E/DR/KxxSIDAQL/MR] (Chollet et al., 1996), also see Figure 3.2. The N-terminal serine residue phosphorylation site is only conserved in eukaryotic plant-type PEPCs (PTPCs), but not found in the bacteria-type PEPCs (BTBCs) of eukaryotes and bacteria. As shown in Figure 3.2, diatom PEPCs, green algae PEPCs, red algae PEPCs, Arabidopsis thaliana BTBC, and Escherichia coli PEPC do not possess the conserved phosphorylation domain, indicating these PEPCs may not able to do N-terminal phosphorylation. Although a serine residue at the same position was found in mitochondrial PtPEPC2 in diatoms, it does not fit to the plant-invariant motif.
This serine residue could be the phosphorylation site in diatoms, but this needs to be proven experimentally. The mature PtPEPC1 and PtPEPC2 from *P. tricornutum* have a calculated molecular of 110 kDa, which is in the range of PTPCs. Neither PtPEPC1 nor PtPEPC2 contains a C-terminal tetrapeptide of PTPCs or BTPCs, indicating that the diatom PEPCs are different from BTPCs and PTPCs in plants. However, the green algal *C. reinhardtii* PEPC1 shows the C-terminal tetrapeptide of RNTG of BTPCs, *C. reinhardtii* PEPC2, and the red algal PEPCs possess the C-terminal tetrapeptide of QNTG of PTPCs. Plant and bacterial PEPCs form a homotetrameric “dimer of dimer” structure, composed of four identical subunits, depending on two critical residues glutamate and arginine residues shown in Figure 3.2 (Kai *et al.*, 1999; O’Leary *et al.*, 2009). Interestingly, these two residues were also found in PtPEPC1, but not PtPEPC2 and in PEPCs from red algae (Figure 3.2), indicating that PEPC1 in *P. tricornutum* might form homotetramers.

PtPEPC1 has been located in the periplastidic space between the two innermost and two outermost envelope membranes of the diatom plastids (Ewe *et al.*, 2018), this compartment is likely the meanwhile strongly reduced former cytosol of the red algal endosymbiont. Although several proteins were found to be targeted to this compartment, the role of it is still unknown (Gruber & Kroth, 2017). In early works, this compartment was proposed to be acidic, eventually playing a role in concentrating CO2 (Lee & Kugrens, 1998), but this was not experimentally proven yet. The optimal pH of recombinant PtPEPC1 was 7.5 to 9, which would not fit the hypothetically acidic compartment. PtPEPC2 is a mitochondrial protein, the pH of mitochondrial matrix in diatoms is unknown, but likely similar to other mitochondria, for which a pH of 7.8 has been determined in the mitochondrial matrix of human cell (Porcelli *et al.*, 2005). PtPEPC1 showed a wider optimum pH range than mitochondria PtPEPC2 in this study. Compared to the optimum pH range (8-8.5) of mitochondrial recombinant PEPC by Chang *et al.* (2014), mitochondria recombinant PtPEPC2 in this study showed higher optimum pH of 8.5-9 shown in Figure 3.5. The optimal pH range of PEPC in diatom is similar to that in land plants and green algae (Table 3.4), but quite different from bacteria and archaea (Hoban & Lyric, 1975; Sadaie *et al.*, 1997; Chen *et al.*, 2002; Patel *et al.*, 2004), the optimum pH of bacteria PEPC showed more variability.

For most land plant PEPCs, the PEP substrate saturation curves follow a Michaelis–Menten kinetics, including the C3 and C4 PEPCs of *Zea mays*. However, the photosynthetic PEPCs (none of C3 PEPCs) of the genera *Flaveria* and *Alternanthera* all show a sigmoidal saturation curve for the substrate PEP, indicating a cooperative PEP binding. After being activated by glucose-6-phosphate, the cooperativity disappears and the PEP saturation curve becomes hyperbolic (Svensson *et al.*, ...
1997; Gowik et al., 2006). Similarly, both recombinant PtPEPCs showed a sigmoidal saturation curve with PEP as substrate (Figure 3.6a), indicating a cooperative PEP binding. Furthermore, the comparison of the affinity of PtPEPCs to substrate PEP and HCO$_3^-$ among land plants, green algae, and diatoms are shown in Table 3.5 and Table 3.6. Both PtPEPC1 and PtPEPC2 from *P. tricornutum* showed much lower affinities to PEP and HCO$_3^-$ compared to C3 and C4 PEPCs in plants, especially the mitochondrial PtPEPC2. In general, C4 PEPCs have shown a lower affinity to PEP than the non-photosynthetic PEPCs (Dong et al., 1998; Gowik et al., 2006; Lara et al., 2006), but a higher affinity to bicarbonate than C3 PEPCs in land plants (Gutierrez et al., 1974). Similarly, the recombinant PtPEPC2 has a lower affinity to PEP, but a more than 8 times higher affinity to HCO$_3^-$, thus PtPEPC2 shows similar kinetic properties as C4 PEPCs in land plants. In contrast, PtPEPC1 from *P. tricornutum* in this study showed a similar affinity to both substrates PEP and HCO$_3^-$.

Thus the mitochondrial PtPEPC2 has properties of photosynthetic PEPCs, while the plastidic PtPEPC1 seems to be a non-photosynthetic PEPC. In summary, PEPCs from diatom not only showed some similarity but also indicate distinct features from the plant or bacteria PEPCs. Further characterization of PEPC in diatoms should be done to better understand the function and regulation of this enzyme.
Table 3.4. Optimum pH for PEPC of different organisms.

<table>
<thead>
<tr>
<th>Class</th>
<th>Organisms</th>
<th>PEPC localization</th>
<th>Optimum pH</th>
<th>PEPC protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4 Land plant</td>
<td><em>Zea mays</em></td>
<td></td>
<td>7.9 - 8.3</td>
<td>native protein</td>
<td>(Mareš et al., 1979)</td>
</tr>
<tr>
<td>C3 Land plant</td>
<td><em>Alternanthera sessilis</em></td>
<td></td>
<td></td>
<td>recombinant protein</td>
<td>(Gowik et al., 2006)</td>
</tr>
<tr>
<td>C3-C4 Land plant</td>
<td><em>Alternanthera tenella</em></td>
<td>cytosol</td>
<td>8</td>
<td>recombinant protein</td>
<td>(Gowik et al., 2006)</td>
</tr>
<tr>
<td>C4 Land plant</td>
<td><em>Alternanthera pungens</em></td>
<td></td>
<td></td>
<td>recombinant protein</td>
<td>(Gowik et al., 2006)</td>
</tr>
<tr>
<td>C3 Land plant</td>
<td><em>Brassica napus</em></td>
<td></td>
<td>8-9.5</td>
<td>native protein</td>
<td>(Moraes &amp; Plaxton, 2000)</td>
</tr>
<tr>
<td></td>
<td><em>Monoraphidium minutum</em></td>
<td></td>
<td>7-9</td>
<td>native protein</td>
<td>(Rivoal et al., 2002)</td>
</tr>
<tr>
<td>Green algae</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>cytosol</td>
<td>8.8</td>
<td>native protein PEPC1</td>
<td>(Rivoal et al., 1998)</td>
</tr>
<tr>
<td></td>
<td><em>Selenastrum minutum</em></td>
<td></td>
<td>8-9.5</td>
<td>native protein</td>
<td>(Schuller et al., 1990)</td>
</tr>
<tr>
<td>Archaea</td>
<td><em>Methanothermobacter thermautotrophicus</em></td>
<td></td>
<td>6.8</td>
<td>native protein</td>
<td>(Patel et al., 2004)</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td><em>Thermosynechococcus vulcanus</em></td>
<td>cytosol</td>
<td>9</td>
<td>recombinant protein</td>
<td>(Chen et al., 2002)</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td><em>Rhodopseudomonas sp.</em></td>
<td></td>
<td>9</td>
<td>recombinant protein</td>
<td>(Sadaie et al., 1997)</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td><em>Thiobacillus thioaratus</em></td>
<td></td>
<td>5.3</td>
<td>native protein</td>
<td>(Hoban et al., 1975)</td>
</tr>
<tr>
<td>Diatom</td>
<td><em>Phaeodactylum tricornutum</em></td>
<td>mitochondria</td>
<td>8-8.5</td>
<td>recombinant protein</td>
<td>(Chang et al., 2014)</td>
</tr>
<tr>
<td>Diatom</td>
<td><em>Phaeodactylum tricornutum</em></td>
<td>plastidic space</td>
<td>7.5-9</td>
<td>recombinant protein</td>
<td>This study</td>
</tr>
<tr>
<td>Diatom</td>
<td><em>Phaeodactylum tricornutum</em></td>
<td>mitochondria</td>
<td>8.5-9</td>
<td>recombinant protein</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 3.5. Comparison of $K_m$ (PEP) in land plants, green algae, and a diatom.

<table>
<thead>
<tr>
<th>Photosynthesis</th>
<th>Species</th>
<th>C3 or C4 PEPC</th>
<th>$K_m$ (PEP) (µM)</th>
<th>Assay pH</th>
<th>PEPC Protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kranz C4</td>
<td><em>Zea mays</em></td>
<td>C4</td>
<td>590</td>
<td>8</td>
<td>recombinant protein</td>
<td>(Dong <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kranz C4</td>
<td><em>Flaveria trinervia</em></td>
<td>C4</td>
<td>652</td>
<td>8</td>
<td>recombinant protein</td>
<td>(Svensson <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td>C3</td>
<td><em>Flaveria pringlei</em></td>
<td>C3</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td><em>Flaveria pubescens</em></td>
<td>C3</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3-C4</td>
<td><em>Flaveria pringlei</em></td>
<td>C3-C4</td>
<td>53</td>
<td>8</td>
<td>recombinant protein</td>
<td>(Engelmann <em>et al.</em>, 2003)</td>
</tr>
<tr>
<td>C4- like</td>
<td><em>Flaveria brownii</em></td>
<td>C4- like</td>
<td>108</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td><em>Flaveria trinervia</em></td>
<td>C4</td>
<td>269</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td><em>Alternanthera sessilis</em></td>
<td>C3</td>
<td>36</td>
<td></td>
<td>recombinant protein</td>
<td>(Gowik <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>C3-C4</td>
<td><em>Alternanthera tenella</em></td>
<td>C3-C4</td>
<td>42</td>
<td>8</td>
<td>recombinant protein</td>
<td></td>
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<tr>
<td>C4</td>
<td><em>Alternanthera pungens</em></td>
<td>C4</td>
<td>157</td>
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<td></td>
<td></td>
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<tr>
<td>Green algae</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>PEPC1</td>
<td>240</td>
<td>8.4</td>
<td>native protein</td>
<td>(Rivoal <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEPC2</td>
<td>100</td>
<td>8.1</td>
<td></td>
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<td></td>
<td><em>Selenastrum minutum</em></td>
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<td>2230</td>
<td>8</td>
<td>native protein</td>
<td>(Schuller <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEPC2</td>
<td>570</td>
<td>8</td>
<td></td>
<td></td>
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<td>Diatom</td>
<td><em>Phaeodactylum tricornutum</em></td>
<td>mitochondrial PEPC</td>
<td>890</td>
<td>8</td>
<td>recombinant protein</td>
<td>(Chang <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td>Diatom</td>
<td><em>Phaeodactylum tricornutum</em></td>
<td>plastidic PEPC1</td>
<td>1000</td>
<td>8</td>
<td>recombinant protein</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mitochondrial PEPC2</td>
<td>1600</td>
<td>8.5</td>
<td></td>
<td></td>
</tr>
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</table>
Table 3.6. Comparison of $K_m$ (HCO$_3^-$) in land plants and a diatom.

<table>
<thead>
<tr>
<th>Photosynthesis</th>
<th>Species</th>
<th>C3 or C4 PEPC</th>
<th>$K_m$ (HCO$_3^-$) (µM)</th>
<th>Assay pH</th>
<th>PEPC protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kranz C4</td>
<td>Zea mays</td>
<td>C4</td>
<td>27</td>
<td></td>
<td></td>
<td>(Gutierrez et al., 1974)</td>
</tr>
<tr>
<td></td>
<td>Gonphrena globosa</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Kranz C4</td>
<td>Eleusine indica</td>
<td>C4</td>
<td>24</td>
<td>7.5</td>
<td>native protein from plant</td>
<td></td>
</tr>
<tr>
<td>Kranz C4</td>
<td>Eleusine coracana</td>
<td>C4</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kranz C4</td>
<td>Panicum maximum</td>
<td>C4</td>
<td>27</td>
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<tr>
<td>Kranz C4</td>
<td>Zea mays</td>
<td>C4</td>
<td>100</td>
<td>8</td>
<td>recombinant protein</td>
<td>(Dong et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C3</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>Cucumis sativus L.</td>
<td>C3</td>
<td>240</td>
<td>8</td>
<td>native protein from plant</td>
<td>(Nisi &amp; Zocchi, 2000)</td>
</tr>
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<td>Diatom</td>
<td>Phaeodactylum tricornutum</td>
<td>mitochondrial PEPC</td>
<td>168</td>
<td>8</td>
<td>recombinant protein</td>
<td>(Chang et al., 2014)</td>
</tr>
<tr>
<td>Diatom</td>
<td>Phaeodactylum tricornutum</td>
<td>plastidic PEPC1</td>
<td>850</td>
<td>8</td>
<td>recombinant protein</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mitochondrial PEPC2</td>
<td>110</td>
<td>8.5</td>
<td></td>
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</table>
Chapter 4

PEP Carboxylase contributes to carbon fixation in the diatom *Phaeodactylum tricornutum* at low concentrations of inorganic carbon

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4. PEP Carboxylase contributes to carbon fixation in the diatom *Phaeodactylum tricornutum* at low concentrations of inorganic carbon

4.1 Abstract

Some diatoms have been shown to possess carbon concentrating mechanisms (CCMs), which increase the CO\(_2\) concentrations in the proximity of Rubisco to allow more efficient photosynthesis in a carbon-limited environment. Previously published data indicated that a C4-type CCM may exist in *Thalassiosira weissflogii*, but whether this is also true for other diatoms is controversially discussed. Phosphoenolpyruvate Carboxylase (PEPC) is one of the key enzymes involved in C4-type photosynthesis in higher plants. In the diatom *Phaeodactylum tricornutum*, two isoforms are present, one of them is located in the periplastidic space (PEPC1), while the other in the mitochondria (PEPC2).

In wild type *P. tricornutum*, mRNA transcription, protein expression and total PEPC enzyme activity of both PEPCs were not different between low and high CO\(_2\) conditions. We generated transcription activator-like effector nuclease (TALEN)-mediated knockout mutants of PEPC1, PEPC2, and double-knockouts. The total PEPC activity in PEPC1 knockout cell lines was not significantly affected, while PEPC2 knockout cell lines had 40% residual PEPC activity (resulting from the functional PEPC1) compared to wild type cells. No PEPC activity could be detected in a PEPC1/PEPC2 double-knockout mutant.

We further analyzed the growth phenotype of the knockout cell lines. PEPC2 and PEPC double-knockout mutants grew slower than wild type under ambient air but grew as well as wild type under high CO\(_2\) conditions. These mutants also showed lower Fv/Fm values than wild type under ambient air conditions, whereas no significant differences were observed in PEPC1 knockout mutants.

Measuring whole cell O\(_2\) evolution at different dissolved inorganic carbon (DIC) concentrations, PEPC2 and PEPC double-knockout mutants grown under ambient air both exhibited up to 40% less O\(_2\) evolution than wild type when incubated at low DIC concentrations. The photosynthetic activity could be recovered to wild type level by supplying dissolved HCO\(_3^-\). Furthermore, carbon stable isotope experiments revealed that \(^{13}\text{C}/^{12}\text{C}\) ratios (\(\delta^{13}\text{C}\)) were significantly lower in PEPC2 and PEPC double-knockout mutants than wild type under high CO\(_2\). Again, none of these differences were observed in PEPC1 knockout mutants.

The obtained data suggest that PEPC2 plays an important role in a putative biochemical CCM, which may contribute a small proportion to the total inorganic carbon assimilation in *P. tricornutum*. 
This PEPC2 mediated C4-like photosynthesis is apparently not regulated by the DIC concentration in *P. tricornutum*.

**Keywords:**
Carbon concentrating mechanism (CCM), C4 photosynthesis, diatoms, *Phaeodactylum tricornutum*, TALEN, Phosphoenolpyruvate carboxylases (PEPC), reverse genetics
4.2 Introduction

Diatoms are an important part of the marine phytoplankton, contributing approximately up to 40% of the global oceanic organic carbon production (Nelson et al., 1995). Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), the key enzyme of CO₂ fixation, catalyzes the carboxylation reaction of ribulose-1,5-bisphosphate (RuBP), producing two molecules of the C3 compound 3-phosphoglycerate, the primary product of carbon fixation in most photosynthetic organisms. However, in seawater the dissolved inorganic carbon (DIC) corresponds to a CO₂ concentration of 10-15 µM at pH 8.2 (Riebesell et al., 1993), which is much lower than the Michaelis–Menten value (K_m) (20-60 µM) for CO₂ of the diatom Rubisco enzyme (Badger et al., 1998; Whitney et al., 2011). To circumvent CO₂ limitation, diatoms use carbon concentrating mechanisms (CCMs) that actively take up and transport inorganic carbon to increase the CO₂ concentration in the proximity of Rubisco. This improves photosynthesis efficiencies especially in a carbon-limited environment (Burkhardt et al., 2001; Matsuda et al., 2001; Giordano et al., 2005; Reinfelder, 2010; Hopkinson et al., 2011). There are generally two types of CCMs, called biophysical and biochemical CCMs. A biophysical CCM may involve bicarbonate transporters (Rotatore et al., 1995; Burkhardt et al., 2001; Nakajima et al., 2013) and carbonic anhydrases (CAs) (Morel et al., 1994; Colman & Rotatore, 1995; Burkhardt et al., 2001; Morel et al., 2002; Hopkinson et al., 2013; Kikutani et al., 2016). Recent studies have shown great progress in understanding biophysical CCMs in diatoms (Hopkinson et al., 2011; Nakajima et al., 2013; Kikutani et al., 2016; Clement et al., 2017; Matsuda et al., 2017; Shen et al., 2017; Young & Hopkinson, 2017).

In biochemical CCMs, C3 compounds are carboxylated, mostly by using HCO₃⁻, and leading to the formation of C4 acids (hence these mechanisms are also called C4 photosynthesis). The C4 compounds are then transported into the chloroplast and decarboxylated by a decarboxylase to release CO₂ in close proximity to Rubisco (Sage, 2004). In most terrestrial C4 plants, carboxylation and decarboxylation processes are separated spatially and take place in different cells: the fixation of atmospheric CO₂ into C4 acids occurs in mesophyll cells, while CO₂ is released from the C4 acids in the specialized bundle sheath cells (Sage et al., 2012). Single-cell C4 photosynthesis has been described for terrestrial plants, which functions by spatial separation of two cytoplasmic domains which contain dimorphic chloroplasts (Voznesenskaya et al., 2001; Edwards & Voznesenskaya, 2010). Furthermore, single-cell C4 photosynthesis is also found in the aquatic plant Hydrilla verticillata (Salvucci & Bowes, 1981; Ascencio & Bowes, 1983; Magnin et al., 1997), and in some other aquatic species like Egeria densa (Salvucci & Bowes, 1981; Casati et al.,...
The marine, macroscopic green alga *Udotea flabellum* has been reported to perform C4-like photosynthesis (Reiskind *et al.*, 1988; Reiskind & Bowes, 1991). Moreover, observations of principal $^{14}$C-labelled intracellular C4 compounds (malate), $^{14}$C transfer from malate to phosphoglyceric acid (PGA) and sugar in CO$_2$ stressed cells and the highest activity of phosphoenolpyruvate carboxylase (PEPC) at low CO$_2$ concentrations indicate the existence of a biochemical CCM in the marine diatom *Thalassiosira weissflogii* (Reinfelder *et al.*, 2000).

In diatoms, C4 photosynthesis occurs in *T. weissflogii*, however, some biochemical and molecular data also suggest C4-assisted photosynthesis in *T. pseudonana* and *P. tricornutum*. Beardall *et al.* (1976) showed predominantly early labeling of C4 compounds in *P. tricornutum*, assuming a C4 pathway (Beardall *et al.*, 1976). McGinn & Morel (2008) observed three-fold greater PEPC transcript abundances in *T. pseudonana* under low CO$_2$ growth conditions (McGinn & Morel, 2008). Recently, Kustka *et al.* (2014) found a 1.8 fold increase of PEPC transcript abundance under low CO$_2$ conditions in *T. pseudonana*, and thus, based on a theoretical decarboxylating activity of the plastidic pyruvate carboxylase (PYC), postulated a C4 CCM (Kustka *et al.*, 2014). However, other experimental evidence is contradictory: Pyruvate-orthophosphate dikinase (PPDK) silencing experiments via RNAi (Haimovich-Dayan *et al.*, 2013) and isotope labeling experiments (Roberts *et al.*, 2007b) did not support the presence of a C4-like CCM in *P. tricornutum* and *T. pseudonana*.

Lastly, a recent study indicates that C4-like CCM is absent in all of the examined diatom species, including *T. weissflogii*, but bicarbonate transporters and CAs are important components of diatom CCMs (Clement *et al.*, 2017).

If single-cell C4 photosynthesis exists in diatoms, the release of CO$_2$ in the vicinity of Rubisco is a crucial step to support an efficient C4 pathway. Genomic analysis of *T. pseudonana* (Armbrust *et al.*, 2004) and *P. tricornutum* (Bowler *et al.*, 2008) showed that genes required for the C4 pathway exist in both diatom species (Kroth *et al.*, 2008). However, a GFP fusion experiment in *T. pseudonana* confirmed that two carboxylating enzymes, PEPC1 and PEPC2, are localized at the matrix of the periplastidal compartment and the mitochondria respectively (Tanaka *et al.*, 2014). The decarboxylation enzymes, NAD$^+$-dependent malic enzyme (NAD-ME) and phosphoenolpyruvate carboxykinase (PEPCK), are localized in the cytosol and the mitochondria respectively (Tanaka *et al.*, 2014). Meanwhile, a GFP fusion experiment in *P. tricornutum* confirmed PEPC1 to be localized in periplastidic space, PEPC2 in the mitochondria, and the decarboxylases PEPCK and MEs within the mitochondria (Ewe *et al.*, 2018).
Recently, molecular tools like gene knockout technologies including TALENs (Transcription activator-like effector nucleases) and CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) have been established for diatoms (Daboussi et al., 2014; Nymark et al., 2016; Serif et al., 2017; Kroth et al., 2018; Serif et al., 2018). In this work, we used reverse genetics, TALEN-based approach to knock out the two existing PEPCs in *P. tricornutum*, the key enzymes of a putative biochemical CCM. A comprehensive molecular, biochemical and physiological investigation of wild type and PEPC knockout cell lines allowed us to gain new insights regarding the role of a C4-CCM in *P. tricornutum*.

### 4.3 Materials and methods

#### 4.3.1 Strains and culture conditions

The marine diatom *P. tricornutum* strain UTEX 646, obtained from Culture Collection of Algae, University of Texas (https://utex.org/) was grown in f/2 medium with artificial half-concentrated sea salts (16.6 g L⁻¹, Tropic Marin Dr. Biener, GmbH, Wartenberg, Germany) and other supplements (Guillard & Ryther, 1962; Guillard, 1975). Solid f/2 media contained 12 g L⁻¹ agar (Becton, Dickinson & Co., Le Pont de Claix, France). Cells were grown at 20 °C, illuminated with a light intensity of 65-70 μmol photons m⁻² s⁻¹ (16 h light: 8 h dark cycle). The cultures inoculated from the exponential phase cultures were grown under either high CO₂ (0.6 vol%), pseudo ambient CO₂ (0.04 vol%) or low CO₂ (0.01 vol%) in two-tier culture vessels (Yu et al., 2017) or under ambient air in Erlenmeyer flasks on a horizontal shaker. The cultures used for DIC dependent O₂ evolution experiments were grown under continuous illumination (50-75 μmol photons m⁻² s⁻¹) at 20 °C under constant aeration with 1 vol% CO₂ or ambient air. The cultures used for light intensity dependent O₂ evolution experiments were grown under ambient air on a horizontal shaker.

#### 4.3.2 Construction of PEPC TALEN knockout vectors, the nuclear transformation of *P. tricornutum* and screening of transformants

Potential TALEN target sites for the *PEPC1* (JGI Protein ID 56026) and *PEPC2* (JGI Protein ID 20853) genes were generated using the TAL Effector Nucleotide Targeter 2.0 software (Doyle et al., 2012). *PEPC* TALEN knockout vectors were generated as previously described (Serif et al., 2017). The *PEPC* TALEN plasmids were assembled and the resulting plasmids were digested with the restriction enzyme AfeI and then sequenced (GATC, Konstanz, Germany) to verify correct integration and orientation.
PEPC TALEN plasmids were transformed into *P. tricornutum* by the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) fitted with 1100 or 1350 psi rupture disks as previously described (Zaslavskaia *et al.*, 2000). Before transformation, about $1 \times 10^8$ exponential phase *P. tricornutum* cells were spread on each plate and the plates were left under continuous light for 24 hours. 5 µg of each up- and down-stream TALEN plasmid DNA were precipitated onto tungsten particles and biolistically transferred in order to generate PEPC1 or PEPC2 knockout mutants. Accordingly, 5 µg of each TALEN plasmids were used to generate PEPC double-knockout mutants by simultaneous transformation. After transformation, cells were allowed to recover for 24 hours at 35 μmol photons m$^{-2}$ s$^{-1}$ at 18°C. The next day, the recovered cells were plated onto the f/2 media plates containing 75 μg mL$^{-1}$ zeocin (Invitrogen, Carlsbad, CA, USA) and 150 μg mL$^{-1}$ Nourseothricin (ClonNat, Werner Bioagents, Jena, Germany) for resistance selection. The cells were grown under constant illumination (50-75 μmol photons m$^{-2}$ s$^{-1}$) at 20 °C. After ca. 40 days the transformed cell lines were further screened.

4.3.3 DNA extraction, allele-specific PCR and sequencing

Genomic DNA was extracted from wild type and transformed cell lines using the nexttec™ 1step DNA isolation from tissues & cells kit according to the manufacturer's instructions (Biozym, Hessisch Oldendorf, Germany). 14 mL 3-4 $\times 10^6$ cells mL$^{-1}$ of *P. tricornutum* culture at exponential phase were centrifuged and the pellets were incubated with extraction buffer at 56 °C for 1.5 hours. After incubation, the samples were centrifuged at 6000 g for 1 min and the supernatants were transferred to the pre-equilibrated column. The genomic DNA was then eluted from the column by centrifugation at 700 g for 1 min. The concentration of the genomic DNA was determined by a Nanodrop 2000 UV/VIS Spectrometer (Thermo Fisher, Schwerte, Germany). PCRs were performed with Taq B polymerase (Biozym, Hessisch Oldenburg, Germany) under the following PCR condition: 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 68 °C for 2 min. Allele-specific PCR was performed as described (Serif *et al.*, 2017), primers with an allele-specific difference on the 3’terminal base are listed in Table S4.1. PCR products were separated by agarose gel electrophoresis and cleaned using the Gene clean Turbo Kit (MP Biomedicals, Eschwege, Germany) according to the manufacturer's instructions. The purified DNA was ligated into a pGEM-T vector (Promega) and sequenced by GATC (Konstanz, Germany) or Source Bioscience (Berlin, Germany) using primers shown in Table S4.1.
4.3.4 Southern blot

For Southern blotting, 1.5 µg genomic DNA was digested with BamHI, HindIII, BsrGI, and XhoI (Thermo Fisher, Schwerte, Germany) overnight at 37 °C according to the manufacturer's instructions. Digested samples were separated in 0.8% agarose gels. The agarose gels were incubated in denaturation buffer (0.5 M NaOH, 1 M NaCl) for 20 min, washed with deionized water three times, and subsequently incubated with neutralization buffer (0.5M Tris-HCl, 3M NaCl, pH 7.5) for 20 min. Afterward, the gels were washed three times with deionized water. The genomic DNA was then transferred to the positively charged nylon membrane (Roche, Mannheim, Germany; 11471240001) overnight. On the next day, DNA loaded membranes were exposed to UV light for 60 s for fixation. Digoxigenin (DIG) labeled probes were synthesized using the PCR DIG Probe Synthesis Kit (Roche, 11636090910) according to the manufacturer's recommendations with primers shown in Table S4.1. The following steps of the Southern blot protocol were performed as described (Serif et al., 2017).

4.3.5 Western blot

For western blotting, 50 mL 3-4 ×10⁶ cells mL⁻¹ of P. tricornutum culture at exponential phase were harvested by centrifugation, the pellet was fast frozen in liquid nitrogen and stored at -80 °C. The pellets were thawed in lysis buffer (1mM Tris-HCl pH 8.0, 50mM EDTA, 10 g L⁻¹ SDS) and supplemented with protease inhibitor (Complete™ EDTA-free, Roche, Basel, Switzerland) according to the manufacturer's instructions. Mixtures of glass beads with different diameters (0.1-1 mm) were added and cells were homogenized in a bead mill (Fast Prep FP 120 Bio 101 Savant, Qbiogene Inc., Carlsbad, CA, USA) at maximum speed for 20 s six times, 1 min on ice in between. The disrupted cells were centrifuged at maximum speed at 4 °C for 40 min. The supernatant was transferred to a new Eppendorf tube for western blotting. The protein concentration was determined with a photometer (Ultrospec™ 8000, GE Healthcare Life Sciences, Cambridge, UK) using the Pierce™ 660 nm Protein Assay Kit (Thermo Fisher) according to the manufacturer's instructions. Western blots were performed as described (Serif et al., 2017). Membranes were cut into two pieces at 70 KD size, the upper part membrane was probed using antiserum specific against PEPC (Agrisera AS09 458, Vännas, Sweden), and the lower part membrane was probed with the large subunit of Rubisco-specific antiserum (Agrisera AS03 037, Vännas, Sweden) as a loading control.
4.3.6 Real-time PCR

Wild type *P. tricornutum* cells grown under low CO$_2$ (0.01 vol%) and high CO$_2$ (0.6 vol%) conditions were filtrated on Isopore Polycarbonate filter 1.2 μm (Millipore, Billerica, MA, USA) and immediately frozen in liquid nitrogen. RNA was extracted using RNASPpure and Total RNA Kit (peqGOLD, VWR, Leuven, Belgium). Residual genomic DNA was digested using DNase Digest Kit (peqGOLD, VWR, Leuven, Belgium) during RNA extraction. An equal amount of RNA (1 μg) was used for all samples for reverse transcription using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). The synthesized cDNA was diluted 2.5-fold in nuclease-free water. 1 μL cDNA sample was used in a 20 μL quantitative PCR assay containing the primers and GoTaq qPCR Mastermix (Promega). While ribosomal ribonucleic acid 18S (Ensembl Gen ID: EPrPhatr3G00000013183) was used as the reference gene in *P. tricornutum* under different light intensities (Lepetit *et al.*, 2013), we tested three housekeeping genes including 18S, histone H4 (JGI ID: 26896||34971) and ribosomal protein S1 RPS (JGI ID: 44451) under low and high CO$_2$ conditions. All three genes are stably transcribed under such conditions. We chose 18S as the reference gene. The primers used here are listed in Table S4.1. The real-time PCR was performed on an Applied Biosystems™ 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) under following PCR conditions: denaturation at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 3 s, annealing, and elongation at 57 °C for 30 s. At the end of each run, melting curve analyses (95 °C for 15 s, 57 °C for 1 min, 95 °C 15 s) were performed to confirm the specific amplification of the template and to exclude false positive fluorescence due to side reactions according to Lepetit *et al.* (2013). Cycle threshold values and relative transcript levels were analyzed by using online software Real-time PCR Miner 3.0 (Zhao & Fernald, 2005; Lepetit *et al.*, 2013).

4.3.7 Measurement of PEPC activity

A total number of 2×10$^9$ cells of *P. tricornutum* in the exponential phase was centrifuged (Sorvall RC 6 Plus centrifuge, Thermo Electron Corporation, Osterode, Germany) at 4 °C and 4000 g for 20 min. The pellet was washed with 10 mL of protein lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1mM EDTA, 15 mM MgCl$_2$, 10 vol % glycerol) and again centrifuged at 4 °C and 4000 g for 5 min. The supernatant was discarded, while the pellet was resuspended in 2.5 mL protein lysis buffer and 140 μl 1× EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The sample was then passed three times through a French press (FA-079, SLM Aminco) at 1000 psi setting (ca. 6.89 MPa) in a cooled mini-cell. To separate broken membrane parts and organelles from the soluble protein
solution, the suspension was centrifuged at 50,000 g and 4 °C for 40 min (Optima-max, Beckman Coulter, USA). The supernatant was subjected to another centrifugation step for another 20 min. 2 mL of the resulting supernatant were cleaned up by using a PD-10 Desalting Column (GE Healthcare, Germany) according to the protocol. The protein concentration of the desalted protein extract was determined by Bradford reagent (Bradford, 1976). For PEPC activity measurement, enzymatic buffer (50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, 15 mM MgCl₂, 10 vol% glycerol) was first added in a cuvette (QS, 10.00mm, Neolab, Heidelberg, Germany), followed by adding 20 mM bicarbonate, 25 units of malate dehydrogenase (MDH) (Roth), 0.1mM NADH (Roth) and 100 µg desalted protein extract. The PEPC enzyme activity reaction was initiated by the addition of 15 mM PEP (Applichem), the measurement volume is 1mL in total. The activity was continuously followed by recording a decrease of absorbance of NADH at 340 nm in a photometer (Ultrospec™ 8000, GE Healthcare Life Sciences, Cambridge, UK) according to the manufacturer's instructions.

4.3.8 Determination of photosynthetic parameters

Chlorophyll a determination

200 µL DIC-free f/2 medium washed cells were centrifuged at 13,000 rpm. The supernatant was discarded, 100 µL DMSO was added to the pellet and the cells were vortexed at maximum speed. Afterward, 1 mL methanol was added and mixed again at maximum speed. The sample was centrifuged at 13,000 rpm for 2 min and the supernatant was used for Chl a measurement with a spectrophotometer (UH5300, Hitachi High-Tech Science Co., Tokyo, Japan). Chl a concentrations were calculated as described (Jeffrey & Humphrey, 1975).

Measurement of maximum photosynthetic efficiency of photosystem II (Fv/Fm), non-photochemical quenching (NPQ) and light intensity dependent O₂ evolution

*P. tricornutum* culture in the mid-logarithmic phase with 1 µg mL⁻¹ Chl a was used for Fv/Fm and NPQ measurements using an AquaPen-C AP 100 (Photon Systems Instruments, Brno, Czech Republic). The maximum photosynthetic efficiency of photosystem II (Fv/Fm) was calculated using the formula Fv/Fm = (Fm - Fo)/Fm; non-photochemical quenching (NPQ) was calculated from the formula NPQ= (Fm – Fm’)/Fm’. Fm and Fo represent the maximum and minimum fluorescence at the dark-adapted period, F’ is the maximum fluorescence at light-adapted state. For light intensity dependent O₂ evolution, *P. tricornutum* culture with 5 µg mL⁻¹ Chl a was used for measurement using a Clark-type oxygen electrode (Hansatech Instruments Ltd, Norfolk, UK). Cells were adapted to dark for 5mins first and then followed with increasing light intensity.
DIC concentration-dependent \( \text{O}_2 \) evolution

\( P. \ tricornutum \) wild type and mutant cultures (60 mL) from mid-logarithmic phase (OD\(_{730} \) = 0.2-0.4) were harvested by centrifugation at 3,000 rpm for 3 min at room temperature (KN-70, KUBOTA Co., Tokyo, Japan). Cell pellets from the low and high CO\(_2\) growth conditions were washed with DIC-free f/2 medium buffered with 10 mM Tris-HCl (pH 8.2) for 3 and 5 times, respectively. Cells were then suspended in the DIC-free f/2 medium at a Chl \( a \) concentration of 10 \( \mu \)g mL\(^{-1}\). The rate of photosynthetic \( \text{O}_2 \) evolution at various DIC concentrations was measured with a Clark-type oxygen electrode (Hansatech Instruments Ltd, Norfolk, UK) (Matsuda \textit{et al.}, 2001). Cells were exposed to 150 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) until the CO\(_2\) compensation point (the DIC concentration at which no net consumption of \( \text{O}_2 \) was measured) was reached. Then, the DIC concentration at the CO\(_2\) compensation point was measured with a gas chromatograph (GC) (GC-8A, Shimadzu Co., Kyoto, Japan) equipped with a methanizer and flame ionization detector (GC-FID) (Birmingham & Colman, 1979). Afterward, the light intensity was increased to 300 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) and various concentrations of DIC were added to measure \( P_{\text{max}} \) and \( K_{0.5} \) [DIC]. \( K_{0.5} \) [DIC] values were determined by rectangular-hyperbola data fitting with non-linear least squares regression.

4.3.9 Stable isotope analyses

Wild type and PEPC knockout mutants grown under 0.01 \%, 0.04 \%, and 0.6 \% \( \text{CO}_2 \) according to Yu \textit{et al.} (2017) were harvested by centrifugation (Allegra 25R Centrifuge, Beckman Coulter) at 4000 \( g \) at room temperature. The pellets were immediately frozen in liquid nitrogen and afterward were freeze-dried overnight (Christ Alpha 1-4, B. Braun Biotech International, Allentown, PA, USA). The dried samples were weighed into 0.3 mm \( \times \) 0.5 mm tin capsules using a micro-analytical balance (aiming to ca. 2.3 mg per sample). The salts used for the preparation of f/2 medium and the KHCO\(_3\)/K\(_2\)CO\(_3\) powders were also weighed into capsules for determination of background \(^{13}\)C/\(^{12}\)C ratios (\( \delta^{13}\)C). Samples were measured and \( \delta^{13}\)C values were calculated according to the method (Dehnhard \textit{et al.}, 2016).

4.3.10 Statistical analysis

Data are given as arithmetic means with standard deviations. The Student’s t-test was applied using Sigma Plot 12.5 software to evaluate the difference between mean values.
4.4 Results

4.4.1 CO₂ dependent regulation of PEPCs in *P. tricornutum* on mRNA, protein and enzymatic activity level

*P. tricornutum* possesses one PEP carboxylase located in the periplastidic space (PEPC1) and one in the mitochondria/ion (PEPC2). In order to determine a putative influence of CO₂ concentrations on the regulation of these two isoenzymes in *P. tricornutum*, we incubated *P. tricornutum* wild type (WT) cells at low (0.01 vol%) and high (0.6 vol%) CO₂. Interestingly, the relative amount of both PEPC1 and PEPC2 transcripts did not change significantly between low and high CO₂ conditions (Figure 4.1a). To investigate whether this result is reflected by the amount of PEPC protein in *P. tricornutum*, we quantified the PEPC proteins under both low and high CO₂ conditions. Unfortunately, the only available functional PEPC antiserum cannot differentiate between PEPC1 and PEPC2, as both isoforms have a similar size in SDS gels (see chapter 3). Thus, we could only compare the total amounts of PEPCs. The respective western blots showed that the total PEPC protein amounts did not change remarkably between low and high CO₂ conditions in WT cells (Figure 4.1b). Finally, we also compared the enzymatic activity of the PEPCs in *P. tricornutum* cell extracts. As shown in Figure 4.1c, we could not detect a significant difference between the PEPC enzyme activities under low or high CO₂ conditions.
Figure 4.1. Relative gene expression of *PEPC1* and *PEPC2*, protein expression, as well as enzyme activity analyses of PEPC in wild type *P. tricornutum* cells cultured under 0.01 vol% CO$_2$ (LC) and 0.6 vol% CO$_2$ (HC) in a day/night cycle (16h light: 8h dark). (a) Quantitative RT-PCR analysis of changes in *PEPC* transcript levels in response to cultivation between low and high CO$_2$ concentrations. Relative *PEPC*
transcript levels with reference to 18S gene expression were normalized to the expression at LC. The error bars indicate SDs of three independent replicate experiments. (b) Western Blot analysis of PEPC protein expression under low and high CO₂. Total soluble protein was extracted from wild type cells, separated via SDS-PAGE, blotted and incubated with antiserum against PEPC. The anti-PEPC antibody recognizes both PEPC1 and PEPC2. Antiserum-based detection of rbcL protein was used to verify identical loading. Three independent wild type replicates were used for western blotting under each CO₂ condition. (c) The specific enzymatic activity of PEPC in cellular extracts under low and high CO₂ condition. The error bars indicate SDs of at least three independent biological replicates.

4.4.2 Bicarbonate transporters were more highly expressed under low than high CO₂ condition

In order to characterize the general response of P. tricornutum to different CO₂ concentrations during culturing, we determined the relative transcript amounts of the bicarbonate transporters SLC4-1, SLC4-2, and SLC4-4. As shown in Figure 4.2, SLC4-1, SLC4-2, and SLC4-4 were much higher expressed under low CO₂ than under high CO₂ conditions.

Figure 4.2. Relative gene expression of bicarbonate transporters SLC4-1, SLC4-2, and SLC4-4 of wild type P. tricornutum between low and high CO₂ conditions. Relative SCL4 transcription levels with reference to 18S gene expression were normalized to the expression under LC. Cells were acclimated to 0.01 vol% CO₂
(LC) and 0.6 vol% CO₂ (HC) in a day/night cycle (16h light: 8h dark). The errors bars indicate SDs of three independent biological replicates. * Student's t-test P values < 0.05.

4.4.3 Generation and screening of PEPC knockout transformants in *P. tricornutum*

To further investigate the function of the PEPCs, we knocked out the *PEPC1* and *PEPC2* genes individually, as well as both genes simultaneously via TALEN nucleases. In total, 38 *PEPC1* knockout transformants, 23 *PEPC2* knockout transformants, and 23 *PEPC1/PEPC2* double-knockout transformants were collected after biolistic transformation and subsequent antibiotic selection for further analyses. We first screened PEPC knockout transformants by PCR, amplifying fragments of approximately 1000 base pair (bp) length by using primers (Table S4.1) located in the regions targeted by the TALENs. Following gel electrophoreses of the PCR products, transformants that showed modified bands or no bands at all compared to wild type were selected for further analysis. To be able to differentiate between the two alleles in the diploid diatom, we tried to detect allele-specific sequence differences between the two alleles of *PEPC1* and *PEPC2*, respectively. We could only detect differences for the *PEPC1* gene (three different base pairs) (Figure S4.1), but there were no allele-specific differences in the *PEPC2* gene. Therefore, we could apply allele-specific PCR only to the potential PEPC1 knockout strains. The PCR products were cloned into a pGEM-T vector and sequenced. In the PEPC1-23 knockout line, a 540 bp deletion in the region of the TALEN target sites, into which a 143 bp sequence from chromosome 17 was inserted, was identified in allele 1 (Figure 4.3a and Figure S4.2). However, no amplicons could be amplified for allele 2 in this cell line, which indicates that a larger deletion or insertion in allele 2 impedes successful amplification. For the PEPC1-26 knockout line, the PCR results showed bands corresponding to shorter amplicons for allele 1 and allele 2. Sequencing results showed that a 199 bp deletion disrupted allele 1 and that allele 2 was disrupted by an 819 bp deletion (Figure 4.3b, Figure S4.4, and Figure S4.5). PCR products also contained an additional band for allele 2, which showed that most likely a fragment of this allele inserted to another location of the genome with a 4bp deletion between the two target sites (Figure 4.3b and Figure S4.6). Two bands of smaller size were amplified in the PEPC1-37 knockout line, and sequencing results showed that there was a 485bp deletion in allele 1 and a 667bp deletion in allele 2 (Figure 4.3c and Figure S4.3).
Figure 4.3. Scheme of modifications of two alleles of *PEPC1* by TALENs in *PEPC1* knockout mutants. (a) Modification in *PEPC1*-23 knockout, 540 bp were deleted near target sites. In the deleted region, a 143 bp fragment from another chromosome was then inserted. (b) Modification in *PEPC1*-26 knockout, 199 bp were deleted in allele 1, 819 bp and 4 bp were deleted in allele 2. (c) Modification in *PEPC1*-37 knockout, 485 bp were deleted in allele 1, 667 bp were deleted in allele 2. The grey arrows indicate the *PEPC1* alleles, brown regions in the *PEPC1* gene indicate TALEN target sites, blue arrows show DNA insertion, black braces show DNA deletions.
In order to further characterize the mutants, we performed Southern blot analyses of genomic DNA extracted from wild type (WT) or PEPC knockout mutants. The restriction enzymes HindIII, BsrGI, BamHI and XhoI was used to digest the genomic DNA. At least one of these restriction enzymes were used to digest genomic DNA for each PEPC knockout mutant. The WT strain always showed a single band in the size range of 3-7kb in all blots (shown in Figure 4.4). Two shifted bands were observed in the PEPC1-23 knockout mutant, where the lower band corresponds to allele1 and the higher band to allele 2, indicating the insertion of a big fragment (Figure 4.4a-2). In strain PEPC1-26, the large deletions in both alleles were confirmed by the location of the two bands below the WT band (Figure 4.4a-1). Only one shifted band appeared in the PEPC1-37 knockout mutant because the deleted two alleles are of similar size and cannot easily be separated from each other in southern blots (Figure 4.4a-3). In the PEPC2-6 knockout mutant, DNA digested with BsrGI showed two bands shifted to higher sizes, indicating insertions of larger DNA fragments into the *PEPC2* gene of this mutant (Figure 4.4b-1). In the PEPC2-13 knockout mutant, DNA digested with BsrGI showed two shifted smaller bands, indicating modification of *PEPC2* gene by deletion (Figure 4.4b-1); DNA digested with HindIII showed one shifted band for each PEPC2 knockout (Figure 4.4b-2). Two shifted bands were detected in the PEPC1/2-7 double-knockout mutant using the *PEPC1* probe, indicating that both *PEPC1* alleles were modified by insertions (Figure 4.4c-1,2). One band shifted towards larger product size was observed in this double knockout mutant using the *PEPC2* probe, indicating an insertion into the *PEPC2* gene (Figure 4.4c-3,4). In general, southern blot revealed no band of exact WT size in the examined PEPC knockout mutants, confirming that indeed the respective *PEPC* genes were knocked out.
Figure 4.4. Southern blots of wild type (WT) and PEPC knockout mutants. *P. tricornutum* genomic DNA was digested with HindIII, BsrGI, BamH I and Xhol, separated by agarose gel electrophoresis, blotted and hybridized with DIG-labelled probes to *PEPC1* or *PEPC2*. (a) Southern blot of PEPC1 knockout lines and WT using *PEPC1* probes. (a-1) The genomic DNA of WT and PEPC1-26 were digested with HindIII and probed with *PEPC1*-probe1. (a-2) The genomic DNA of WT and PEPC1-23 were digested with BamHI and Xhol, and probed with *PEPC1*-probe1. (a-3) The genomic DNA of WT and PEPC1-37 were digested with BamHI and Xhol, and probed with *PEPC1*-probe3. (b) Southern blot of PEPC2 knockout lines and WT using *PEPC2* probe. The genomic DNA of WT and PEPC2-6 and PEPC2-13 were digested with BsrGI and HindIII, and probed with *PEPC2*-probe2. (c) Southern blot of PEPC double-knockout line and WT using *PEPC1* and *PEPC2* probes respectively. (a-1,2) The genomic DNA of WT and PEPC1/2-7 were digested with BamHI and Xhol and probed with *PEPC1*-probe3. (a-3,4) The genomic DNA of WT and PEPC1/2-7 were digested with HindIII and BsrGI, and probed with *PEPC2*-probe3. PEPC1-23, PEPC1-26, and PEPC1-
37 are PEPC1 knockout mutants; PEPC2-6 and PEPC2-13 are PEPC2 knockout mutants; PEPC1/2-7 is the PEPC1/PEPC2 double-knockout mutant.

4.4.4 Protein quantification and enzymatic activity of PEPC in PEPC knockout mutants

To further characterize the PEPC knockout mutants, PEPC proteins of cells in exponential growth phase were probed by western blot. There was a complete lack of PEPC protein in the PEPC1/PEPC2 double-knockout strain PEPC1/2-7 (Figure 4.5a). Partial reduction of the total amount of PEPC protein was observed in the PEPC1 knockout lines 23, 26, and 37 (resulting from PEPC2 expression). Similarly, we also detected PEPC1 protein in the PEPC2 knockout lines 6 and 13 as shown in Figure 4.5a. In all lines, no major differences in the rbcL loading control were observed.

PEPC enzyme activities were also measured within protein extracts of WT and PEPC knockout mutants. PEPC1 knockout mutants showed similar activity as WT cells, but PEPC2 knockout mutants showed ca. 60% reduced PEPC activity compared to WT. However, the PEPC1/PEPC2 double-knockout mutant showed no PEPC activity compared to WT and the other PEPC knockout mutants (Figure 4.5b).
Figure 4.5. Western blot analyses of PEPC protein and PEPC enzymatic activities of wild type (WT) and PEPC knockout mutants. (a) Western blot of PEPC knockout lines and WT. Total soluble protein was extracted from selected lines, separated by SDS-PAGE, blotted and incubated with antisera to *P. tricornutum* PEPC. RbcL protein was detected as a loading control. (b) The PEPC enzymatic activity of WT and PEPC knockout mutants. The error bars indicate SDs of at least three independent biological replicates. Student's t-tests were performed to calculate the differences between WT and PEPC knockout mutants. ** Student's t-test P values < 0.01.
Since diatoms are diploid organisms, it is difficult to obtain biallelic knockout mutants. However, in this study, the genetic characterization of PEPC knockout mutants by allele-specific PCR, sequencing, southern blot, western blot and PEPC enzymatic activity measurement strongly indicated that PEPC1-23, PEPC1-26, and PEP1-37 are biallelic PEPC1 knockout mutants; that PEPC2-6 and PEPC2-13 are biallelic PEPC2 knockout mutants; and that PEPC1/2-7 is a biallelic PEPC1/PEPC2 double-knockout mutant (Table 4.1, Figure 4.3, Figure 4.4 and Figure 4.5).

Table 4.1. Summary of genetic and enzymatic characterization of PEPC knockout mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PEPC _allele 1 (sequencing)</th>
<th>PEPC _allele 2 (sequencing)</th>
<th>Southern blot</th>
<th>Western blot</th>
<th>Enzymatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPC1-23</td>
<td>540bp deletion,143bp insertion</td>
<td>No PCR products</td>
<td>Shifted bands</td>
<td>Less protein</td>
<td>Similar as WT</td>
</tr>
<tr>
<td>PEPC1-26</td>
<td>199bp deletion</td>
<td>819bp and 4bp deletion</td>
<td>Shifted bands</td>
<td>Less protein</td>
<td>Similar as WT</td>
</tr>
<tr>
<td>PEPC1-37</td>
<td>485bp deletion</td>
<td>677bp deletion</td>
<td>Shifted bands</td>
<td>Less protein</td>
<td>Similar as WT</td>
</tr>
<tr>
<td>PEPC2-6</td>
<td>No PCR products</td>
<td>Shifted bands</td>
<td>Less protein</td>
<td>40% of WT</td>
<td></td>
</tr>
<tr>
<td>PEPC2-13</td>
<td>No PCR products</td>
<td>Shifted bands</td>
<td>Less protein</td>
<td>40% of WT</td>
<td></td>
</tr>
<tr>
<td>PEPC1/2-7</td>
<td>No PCR products</td>
<td>Shifted bands</td>
<td>No protein</td>
<td>No activity</td>
<td></td>
</tr>
</tbody>
</table>

4.4.5 Reduced growth and Fv/Fm of PEPC2 and PEPC1/PEPC2 double knockout mutants under ambient air

The growth characteristics of the PEPC1, PEPC2, and PEPC1/2 knockout lines were determined under two different CO\textsubscript{2} conditions. The cells were cultured under ambient air (low CO\textsubscript{2}) and 0.6 vol\% CO\textsubscript{2} (high CO\textsubscript{2}) by using the two-tier flask system (Yu et al., 2017) The pH of the culture in high CO\textsubscript{2} conditions was between 7.17-7.65 and ambient air conditions between 7.87-9.96. The analysis of doubling times of all cell lines indicated that cells generally grow faster under high CO\textsubscript{2} (shorter doubling time). Under ambient air, the PEPC2 and PEPC1/2 double knockout mutants grew slower than WT, showing significant higher doubling time, while the PEPC1 knockout mutants were not affected (Figure 4.6). However, both PEPC2 and PEPC1/2 knockout mutants grew as fast
as WT cells under high CO₂ conditions, while they grew significantly slower than WT under ambient air. This indicates that the high CO₂ condition may replenish the slower growth of the knockout mutants. We further examined the maximum PSII quantum yield (Fv/Fm) and the capacity for rapid photoprotection based on non-photochemical quenching (NPQ) in WT and PEPC knockout mutants under ambient air conditions, in order to investigate whether photosynthesis is somehow impaired by the knockout of the PEPCs. Indeed, while all PEPC1 knockout mutants did not show any differences in Fv/Fm and NPQ compared to WT, the PEPC2 and the double-knockout mutants showed significantly lower Fv/Fm values compared to WT but no differences in NPQ (Figure 4.7).

**Figure 4.6.** Growth curves of wild type and PEPC knockout mutants under different CO₂ conditions. Cells were cultured under ambient air (low CO₂) and 0.6 vol% CO₂ (high CO₂). Mean ± SD of at least three independent biological replicates is shown. In all plots, closed black circles represent wild type cells, closed gray, blue and red circles represent mutants. Doubling time during the exponential phase of PEPC knockout mutants and wild type was calculated and compared. ** indicates the significant differences of doubling time between wild type and PEPC knockout mutants. ** Student's t-test P values < 0.01.
Figure 4.7. Maximum PSII quantum yield and non-photochemical quenching (NPQ) analyses in wild type and PEPC knockout mutants grown under ambient air. The PSII quantum yield was calculated as $(Fm - Fo)/Fm = Fv/Fm$; NPQ was calculated as $(Fm - Fm')/Fm'$. Error bars represent standard error of the mean from at least three independent biological replicates. Student's t-test was performed to calculate the differences between wild type and PEPC knockout mutants. * Student's t-test P values < 0.05.
4.4.6 Knocking out PEPC2 in *P. tricornutum* leads to reduced photosynthetic O₂ evolution under low DIC

The photosynthetic O₂ evolution of WT and PEPC knockout *P. tricornutum* cells grown under low (ambient air) and high (1 vol%) CO₂ conditions was determined in a medium of pH 8.2 (Figure 4.8a and Table 4.2). The DIC concentration that results in half maximum rate of photosynthesis (\(K_{0.5} \text{[DIC]}\)) in the cells pre-cultivated at high CO₂ concentrations was more than 5 times higher than in the cells grown at low CO₂ (Table 4.2). Grown under low CO₂ condition, the maximum rate of net photosynthesis (\(P_{\text{max}}\)) of all PEPC knockout cell lines was not affected compared to WT (Figure 4.8a-1 and Table 4.2). We also observed unaffected \(P_{\text{max}}\) values of all PEPC knockout mutants at saturating light intensity grown under ambient CO₂ concentration (Figure S4.7). However, grown under low CO₂ condition, significantly increased \(K_{0.5} \text{[DIC]}\) was observed in PEPC2-13 and PEPC1/2-7 mutants (Table 4.2). Strains PEPC2-6, PEPC2-13 and PEPC1/2-7 showed significantly reduced photosynthetic O₂ evolution compared to WT when supplied with very low DIC concentrations from 29 to 381 µM (Figure 4.8b-1 and Table 4.3). When the DIC concentration in the chamber was 29 µM, the corresponding photosynthetic O₂ evolution in PEPC2-6 was reduced by 34 %, by 37 % in PEPC2-13, and by 44 % in PEPC1/2-7 (Table 4.3). But when supplied with saturating DIC concentrations (662-2502 µM), the photosynthetic O₂ evolution in PEPC2-6, PEPC2-13 and PEPC1/2-7 recovered as much as in the WT cells (Figure 4.8a-1 and Table 4.3). In contrast to these findings, no significant differences of these photosynthetic parameters were observed in PEPC1 knockout mutants grown under low CO₂ condition (Figure 4.8 and Table 4.2). Under high (1 vol %) CO₂ growth condition, \(P_{\text{max}}\) of the PEPC2-13 and PEPC1/2-7 knockout cell lines was reduced compared to WT (Figure 4.8 a-2, Table 4.2), while \(K_{0.5} \text{[DIC]}\) (Table 4.2) and the O₂ evolutions of these mutants which were supplied with low DIC (Figure 4.8b-2) was not affected.
Figure 4.8. Kinetic plots of photosynthetic rates in wild type and PEPC knockout mutants. (a) Plots of cultures supplying with low and high DIC concentrations during O\textsubscript{2} evolution measurements. (a-1) Cultures used for O\textsubscript{2} evolution measurements were pre-cultivated under low CO\textsubscript{2} (ambient air); (a-2) Cultures used for O\textsubscript{2} evolution measurements were pre-cultivated under high CO\textsubscript{2} (1 vol%). (b) Plots of cultures supplying with only low DIC concentrations. (b-1) Cultures used for O\textsubscript{2} evolution measurements were pre-cultivated under low CO\textsubscript{2} (ambient air). (b-2) Cultures used for O\textsubscript{2} evolution measurements were pre-cultivated under high CO\textsubscript{2} (1 vol%). Data represent mean ± SD of at least four independent biological replicates. In all blots, closed black triangles represent the wild type, closed colorful circles represent PEPC knockout mutants. X axis is the DIC concentration, Y axis is the rate of photosynthesis.
Table 4.2. Photosynthetic characteristics in wild type and PEPC knock-out cell lines grown at low (ambient air) or high (1 vol%) CO₂ concentrations.

<table>
<thead>
<tr>
<th>CO₂</th>
<th>Strain</th>
<th>pH in assay</th>
<th>P&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;1&lt;/sup&gt;</th>
<th>K&lt;sub&gt;0.5[DIC]&lt;/sub&gt;&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>μmol O₂ mg&lt;sup&gt;-1&lt;/sup&gt; Chl h&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>(μM)</td>
</tr>
<tr>
<td>Ambient air&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>8.2</td>
<td>254 ± 45</td>
<td>29 ± 6</td>
<td></td>
</tr>
<tr>
<td>PEPC1-23</td>
<td>8.2</td>
<td>241 ± 33</td>
<td>26 ± 10</td>
<td></td>
</tr>
<tr>
<td>PEPC1-37</td>
<td>8.2</td>
<td>225 ± 8</td>
<td>31 ± 7</td>
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</tr>
<tr>
<td>PEPC2-6</td>
<td>8.2</td>
<td>243 ± 36</td>
<td>70 ± 7</td>
<td></td>
</tr>
<tr>
<td>PEPC2-13</td>
<td>8.2</td>
<td>222 ± 17</td>
<td>50 ± 20*</td>
<td></td>
</tr>
<tr>
<td>PEPC1/2-7</td>
<td>8.2</td>
<td>271 ± 21</td>
<td>87 ± 44*</td>
<td></td>
</tr>
<tr>
<td>1% CO₂&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>8.2</td>
<td>197 ± 21</td>
<td>520 ± 78</td>
<td></td>
</tr>
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<td>PEPC1-23</td>
<td>8.2</td>
<td>249 ± 13</td>
<td>595 ± 34</td>
<td></td>
</tr>
<tr>
<td>PEPC1-37</td>
<td>8.2</td>
<td>223 ± 37</td>
<td>581 ± 106</td>
<td></td>
</tr>
<tr>
<td>PEPC2-6</td>
<td>8.2</td>
<td>202 ± 16</td>
<td>449 ± 66</td>
<td></td>
</tr>
<tr>
<td>PEPC2-13</td>
<td>8.2</td>
<td>140 ± 5*</td>
<td>399 ± 53</td>
<td></td>
</tr>
<tr>
<td>PEPC1/2-7</td>
<td>8.2</td>
<td>165 ± 16*</td>
<td>546 ± 55</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Maximum photosynthetic rate.
<sup>2</sup> DIC concentration give half P<sub>max</sub>
<sup>3</sup> Low CO₂
<sup>4</sup> High CO₂

All values are mean ± SD of at least four independent biological replicates

* Student t test P values < 0.05
Table 4.3. Representing of Figure 4.8a-1 with values. Relative photosynthetic O$_2$ evolution in PEPC knockout mutants compared to wild type (WT) (in %). Cultures supplying with low and saturating DIC concentrations during O$_2$ evolution measurements were pre-cultivated under low CO$_2$ (ambient air). The last two lines indicate no significant differences of photosynthetic O$_2$ evolution between WT and PEPC knockout strains under saturating DIC conditions.

<table>
<thead>
<tr>
<th>DIC (µM)</th>
<th>WT</th>
<th>PEPC1-23</th>
<th>PEPC1-37</th>
<th>PEPC2-6</th>
<th>PEPC2-13</th>
<th>PEPC1/2-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>100</td>
<td>89</td>
<td>82</td>
<td>66*</td>
<td>63**</td>
<td>56**</td>
</tr>
<tr>
<td>51</td>
<td>100</td>
<td>82</td>
<td>78</td>
<td>64*</td>
<td>60**</td>
<td>60*</td>
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<tr>
<td>79</td>
<td>100</td>
<td>89</td>
<td>82</td>
<td>72</td>
<td>65**</td>
<td>67*</td>
</tr>
<tr>
<td>114</td>
<td>100</td>
<td>90</td>
<td>85</td>
<td>77</td>
<td>70**</td>
<td>73*</td>
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<tr>
<td>198</td>
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<td>91</td>
<td>87</td>
<td>85</td>
<td>77*</td>
<td>84</td>
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<td>381</td>
<td>100</td>
<td>94</td>
<td>87</td>
<td>89</td>
<td>82*</td>
<td>92</td>
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<td>662</td>
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<td>93</td>
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<td>2506</td>
<td>100</td>
<td>95</td>
<td>88</td>
<td>96</td>
<td>87</td>
<td>103</td>
</tr>
</tbody>
</table>

* Student t test P values < 0.05; ** Student t test P values < 0.01; Blue colour: low DIC concentration; grey colour: saturating DIC concentrations. Red color indicates the PEPC2 and PEPC double knockout cell lines are significantly different from WT at certain low DIC concentrations.

4.4.7 Lower δ$^{13}$C values in PEPC2 and PEPC1/PEPC2 double knockout mutants

We measured δ$^{13}$C values of WT and all PEPC knockout mutants grown under low and high CO$_2$ conditions using an Isotope Ratio Mass Spectrometer (IRMS). The δ$^{13}$C values are dependent on the δ$^{13}$C value of the DIC in the medium and the CO$_2$ fixation mechanism of *P. tricornutum* cells. Cells using a biochemical C4 CCM usually have higher amounts of δ$^{13}$C than cells relying on a C3, as Rubisco discriminates $^{13}$C (O’Leary, 1981). We observed a different δ$^{13}$C pattern for WT and PEPC knockout strains grown under three different CO$_2$ conditions (Figure 4.9). The δ$^{13}$C values of KHCO$_3$ and K$_2$CO$_3$ used in this study are -32.22 ‰ and -4.59 ‰ respectively. So to examine the effects of PEPC knockouts on δ$^{13}$C values, only δ$^{13}$C values between the different strains, but grown in the same CO$_2$ condition can be compared. We observed that PEPC1 knockout mutants did not show any significant differences to WT neither under low CO$_2$ nor high CO$_2$ conditions (Figure
However, PEPC2-6, PEPC2-13, and PEPC1/2-7 mutants showed a significantly lower value of $\delta^{13}C$ under high CO$_2$ (0.6 vol% CO$_2$); PEPC2-13 also showed a lower value of $\delta^{13}C$ under low CO$_2$ (0.01 and 0.04 vol % CO$_2$) (Figure 4.9). These results indicate that the PEPC2 and double-knockout mutants fixed less $^{13}$CO$_2$ compared to WT under the same growth condition.

Figure 4.9. Carbon stable isotope signatures of wild type and PEPC knockout cell lines. All cultures were grown under very low CO$_2$ (0.01 vol% CO$_2$), pseudo ambient air (0.04 vol% CO$_2$) and high CO$_2$ (0.6 vol% CO$_2$) in two tier-vessels according to (Yu et al., 2017). The $\delta^{13}C$ value of the examined *P. tricornutum* cells are at approximately -20‰, -26‰ and -46‰, in the 0.01 %, 0.04 % and 0.6 vol% CO$_2$ conditions respectively, reflecting differences in the composition of the carbon sources (HCO$_3$-/CO$_3$²⁻ buffers mixed at different ratios). $\delta^{13}$C values were calculated using Pee Dee Belemnite (PDB) as an internal standard. Error bars represent standard errors of means from at least three independent biological replicates. * Student t test P values < 0.05; ** Student t test P values < 0.01. The red line indicates the average value of WT.
4.5 Discussion

4.5.1 The potential presence of biochemical carbon pre-fixation in diatoms

It has been debated for a long time whether there is a C4-like photosynthetic pathway in diatoms. While the genes for enzymes required for such a pathway have been identified now for a couple of years in the two model diatoms *P. tricornutum* and *T. pseudonana* (Kroth et al., 2008), physiological and biochemical proof or disproof of their role in carbon fixation is scarce and in many cases ambiguous. The intracellular localization of putative C4 enzymes indicates that there might be no C4-like pathway in these two diatoms, because no plastidic decarboxylase could be identified (Tanaka et al., 2014; Ewe et al., 2018). However, $^{14}$C labelling experiments, PEPC enzyme activity, PEPC mRNA sequencing and PEPC inhibition experiments showed evidence that a C4-like pathway may exist in diatoms (Beardall et al., 1976; Reinfelder et al., 2000; Reinfelder et al., 2004; McGinn & Morel, 2008; Kustka et al., 2014). In this study, we found new evidence for biochemical pre-fixation of inorganic carbon in *P. tricornutum*, which contributes to the overall carbon fixation when DIC concentrations are low.

4.5.2 The PEPCs of *P. tricornutum* are not regulated by CO$_2$

We investigated the *PEPC* mRNA levels, protein amounts and enzymatic activities in wild type (WT) *P. tricornutum* cells under different CO$_2$ concentrations. Consistent with McGinn & Morel (2008), we did not detect any changes of *PEPC* transcript amount in response to low or high CO$_2$ (Figure 4.1a). Accordingly, different CO$_2$ concentrations during cultivation also had no significant effect on the total amount of PEPC protein and total PEPC enzymatic activity (Figure 4.1b,c), all indicating a constitutive expression and activity of PEPCs under different CO$_2$ conditions in *P. tricornutum*. Similar results were observed in *T. pseudonana* by Robert et al. (2007b) regarding protein expression of PEPC. However, Clement et al. (2017) observed lower PEPC activity of *P. tricornutum* as well as *T. pseudonana* under low CO$_2$ conditions (Clement et al., 2016; Clement et al., 2017), in contrast to an elevated *PEPC* transcript amount in *T. pseudonana* (McGinn & Morel, 2008; Kustka et al., 2014). Thus, there may be differences in the regulation of PEPC in both diatoms species, but the data availability is contradictory. Besides employing different experimental setups to induce CO$_2$ limitation, one possible explanation for the stable total PEPC activity of *P. tricornutum* in our experiments could be the usage of different *P. tricornutum* strains (UTEX 646 Pt4 strain in our study, RCC Pt1_8.6 (equivalent to CCMP2561) in Clement et al. (2017)).
Chapter 4

4.5.3 Reduced PEPC activity in PEPC2 and PEPC double-knockout mutants

In the PEPC double-knockout mutant PEPC1/2-7 we could not detect any PEPC activity, which confirms that our knockouts are complete and that there is no unidentified additional enzyme that might catalyse the same reaction. In PEPC2 knockout mutants, we still could detect about 40% of the total activity observed in WT strains (Figure 4.5b). This activity thus can be attributed to PEPC1. Interestingly, in PEPC1 knockout mutants, total activity of the mutant strain is similar as in WT cells. As we have confirmed the complete inactivation of PEPC1, we conclude that the missing PEPC1 activity might be compensated by increased PEPC2 activity. The remaining PEPC1 activity in PEPC2 knockout mutants plus PEPC2 activity remaining in PEPC1 knockout mutants is more than the total PEPC activity in WT cells, which indicates that the residual PEPC activity in PEPC1 and PEPC2 knockout mutants may increase.

4.5.4 PEPC2 is involved in CO2 accumulation in P. tricornutum

PEPC is one of the key enzymes that functions in the CO2 assimilation pathways of known C4 species (Sage, 2004). Accordingly, inactivation of PEPC could be a good indicator for the presence of a C4–type CCM if a lower affinity of mutant cells to CO2 under low CO2 conditions could be observed. In this study, we measured DIC dependent O2 evolution of PEPC knockout mutants to investigate their photosynthetic DIC affinity as well as their maximum photosynthetic activity ($P_{\text{max}}$) under low DIC concentrations. In all the PEPC knockout mutants and the wild type strain, $K_{0.5}$ [DIC] values of those strains grown under high CO2 (1 vol% CO2) are more than five times higher than that grown under low CO2 (ambient air) (Table 4.2). This indicates that a CCM is active in low CO2 conditions. Similar results were also observed in P. tricornutum as well as in other diatom species (Burkhardt et al., 2001; Trimborn et al., 2009; Clement et al., 2016; Clement et al., 2017). Our results showed that $K_{0.5}$ [DIC] values of PEPC1 knockout mutants grown under low CO2 did not show any differences compared to WT, indicating that PEPC1 may not be involved in this CCM. However, the $K_{0.5}$ [DIC] values of the PEPC2-13 and PEPC double-knockout mutants are significantly higher than that of WT grown under low CO2 (Table 4.2), indicating a reduced affinity for DIC in the PEPC2-13 and PEPC double knockout mutants compared to WT. Grown under low CO2 conditions, the photosynthetic O2 evolution is significantly reduced in PEPC2-6, PEPC2-13 and PEPC1/2-7 knockout mutants which were supplied with low DIC concentrations during O2 evolution measurements (Figure 4.8b-1 and Table 4.3). However, we further observed restored photosynthetic O2 evolution of these PEPC knockout mutants after addition of saturating DIC concentrations (Table 4.2). These reduced and recovered photosynthetic O2 evolution observations
are in agreement with the study by McGinn & Morel (2008) in other diatom species. The net CO₂ fixation was blocked by the PEPC specific inhibitor DCPC (3, 3-dichloro-2-(dihydroxyphosphinoyl-methyl)-propenoate) (Jenkins, 1987) at 400µM DIC in T. pseudonana and T. weissflogii, but was restored to 50% and 80%, respectively, after adding KHCO₃ (McGinn & Morel, 2008). Reinfelder et al. (2004) also observed that T. weissflogii photosynthesis, after DCDP treatment, can be restored by elevated CO₂ concentration (Reinfelder et al., 2004). Complete inhibition of the PEPC activity in P. tricornutum by knocking out PEPCs reduces photosynthetic O₂ evolution under low DIC only by ca. 40% (Table 4.3). This small reduction in P. tricornutum may indicate that PEPC2-mediated biochemical carbon pre-fixation in P. tricornutum only contributes to a small proportion to the total carbon fixation. The reduced O₂ evolution of PEPC2 and double-knockout mutants supplied with low DIC concentrations in combination with unaffected Pₘₐₓ values of those mutants supplied with saturating DIC concentrations indicate that in P. tricornutum knocking out PEPC does not affect CO₂ fixation by Rubisco but may affect CO₂ assimilation during photosynthesis.

Photosynthetic organisms use both ¹²CO₂ and ¹³CO₂ during CO₂ fixation. PEPC does less discrimination against ¹³CO₂ than Rubisco, therefore, C3 and C4 plants can be distinguished by their carbon stable isotope ratios (O'Leary, 1981). Accordingly, observation of less ¹³C in PEPC knockout mutants compared to WT would indicate that PEPC mediated carbon pre-fixation exists in P. tricornutum. δ¹³C measurements of the PEPC knockout mutants and WT cells by Isotope Ratio Mass Spectrometer (IRMS) showed that less ¹³CO₂ was fixed in PEPC2 and double-knockout mutants when grown under high CO₂, and also significantly less ¹³CO₂ was fixed in PEPC2-13 under low CO₂, which was not observed in PEPC1 knockout mutants (Figure 4.9). These results indicate that PEPC2 may be involved in carbon fixation. Because PEPC2 activity must be absent in PEPC2 and PEPC double-knockout mutants, less pre-fixation of ¹³CO₂ via PEPC2 may occur in these mutants. Interestingly, also under high CO₂, we still observed less ¹³CO₂ fixation in PEPC2 and double knock out mutants compared to WT, indicating that the PEPC2 mediated carboxylation is also active at high CO₂ conditions, which is consistent with our finding that PEPC mRNA, protein and enzyme activities are not affected by CO₂ concentrations. Such a constitutive expression of C₄ CCM component is similar with the biophysical CCM component in P. tricornutum, the recently identified thylakoid luminal 0-CA is also not transcriptionally regulated by DIC (Kikutani et al., 2016).

In summary, the physiological characterization of PEPC knockout mutants showed that no significant effects regarding CO₂ assimilation were observed in the PEPC1 knockout cell lines,
while significantly lower PEPC enzyme activity, growth, Fv/Fm, photosynthetic O\(_2\) evolution, and \(\delta^{13}\)C values were observed in PEPC2 knockout cell lines as well as in the PEPC double-knockout cell line (Table 4.4). This indicates that PEPC2 seems to be involved in CO\(_2\) assimilation during photosynthesis.

Table 4.4. Summary of physiological characterization of PEPC knockout mutants.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PEPC1 Knockouts</th>
<th>PEPC2 Knockouts</th>
<th>PEPC1/2 double-Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPC enzymatic activity level</td>
<td>Similar to WT</td>
<td>40% activity of WT</td>
<td>No enzymatic activity</td>
</tr>
<tr>
<td>Growth under low CO(_2)</td>
<td>Similar to WT</td>
<td>Slower than WT</td>
<td>Slower than WT</td>
</tr>
<tr>
<td>Growth under high CO(_2)</td>
<td>Similar to WT</td>
<td>Similar to WT</td>
<td>Similar to WT</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>Similar to WT</td>
<td>Lower than WT</td>
<td>Lower than WT</td>
</tr>
<tr>
<td>NPQ</td>
<td>Similar to WT</td>
<td>Similar to WT</td>
<td>Similar to WT</td>
</tr>
<tr>
<td>Photosynthetic O(_2) evolution under low DIC</td>
<td>Similar to WT</td>
<td>60% of WT</td>
<td>60% of WT</td>
</tr>
<tr>
<td>(P_{\text{max}}) under saturated DIC</td>
<td>Similar to WT</td>
<td>Similar to WT</td>
<td>Similar to WT</td>
</tr>
<tr>
<td>(\delta^{13})C</td>
<td>Similar to WT</td>
<td>Less than WT</td>
<td>Less than WT</td>
</tr>
</tbody>
</table>

4.5.5 The biophysical CCM seems to be the main mechanism in \(P.\) tricornutum at low CO\(_2\) concentrations

The biophysical CCM is based on the activity of HCO\(_3\)\(^-\) transporters and carbonic anhydrases (Colman & Rotatore, 1995; Rotatore et al., 1995; Burkhardt et al., 2001; Hopkinson et al., 2011; Nakajima et al., 2013; Hopkinson, 2014; Kikutani et al., 2016). Confirming data by Nakajima et al. (2013), in our \(P.\) tricornutum strain three of the HCO\(_3\)\(^-\) transporters SCL4 (Figure 4.2) are much higher expressed under low than under high CO\(_2\) concentrations. Carbonic anhydrases facilitate the fast conversion of CO\(_2\) and HCO\(_3\)\(^-\) in both directions, preventing the diffusion of CO\(_2\) out of the cell, and allowing the release of CO\(_2\) from the accumulated HCO\(_3\)\(^-\) in the chloroplast stroma (Hopkinson et al., 2016; Kikutani et al., 2016). Numerous carbonic anhydrases have been detected in different compartments of \(P.\) tricornutum (Hopkinson et al., 2016): two \(\gamma\)-CAs are located in the
mitochondria, five α-type CAs are located in the periplastidic space (PPS), and two β-CAs localized in the pyrenoid (Tachibana et al., 2011; Kikutani et al., 2012; Hopkinson et al., 2016). A newly identified θ-type CA was recently found in the thylakoid lumen. This CA was not transcriptionally regulated by CO₂, but was proven to play a crucial role in CO₂ assimilation and growth (Kikutani et al., 2016). In this study, the maximum reduction of photosynthetic O₂ evolution in PEPC2 and double-knockout mutants was only about 40 %, which means that under low CO₂ concentrations the PEPC mediated C4 photosynthesis only contributes to a small portion of the CCM. Apparently, the biophysical CCMs via HCO₃⁻ transporters and CAs are most important in P. tricornutum (Burkhardt et al., 2001; Matsuda et al., 2001; Hopkinson et al., 2011; Hopkinson et al., 2013; Nakajima et al., 2013; Kikutani et al., 2016). Reinfelder et al. (2004) though found that in T. weissflogii PEPC inhibition results in more than 90% inhibition of photosynthesis. Marine diatoms are diversely distributed in different water regions in the ocean: intertidal regions, coasts, open-ocean surface, upwelling regions, and deep oceans. The nutrient uptake, growth and photoacclimation characteristics among diatoms can be quite different (Strzepek & Harrison, 2004; Marchetti et al., 2006; Lavaud et al., 2007), which might also be the case for DIC acquisition (Reinfelder, 2010). Possibly, differential expression and regulation of PEPCs in diatoms may result in the utilization of C4 pathways in diatoms to a different extent. As mentioned above, the regulation of PEPCs could be different among the different diatom species since C4 CCM is also diverse in different species.

4.5.6 Proposed C4 pathway in P. tricornutum

Our data indicate that PEPC2 in P. tricornutum might play a role in C4 photosynthesis, albeit contributing only a small portion to the CCM. This finding was surprising considering that PEPC2 is located in the mitochondria, whereas the carboxylation by PEPC in C4 plants happens in the cytosol. Based on our results, we propose the following pathways to be involved in a PEPC2 mediated C4 photosynthesis in P. tricornutum (Figure 4.10): HCO₃⁻ is prefixed by PEPC2 in the mitochondrion to form oxaloacetate (OAA), which can be converted into malate. Flori et al. (2017) observed physical contacts between the mitochondria and chloroplasts in P. tricornutum by using focused ion beam scanning electron microscopy (FIB-SEM). The physical interaction between plastids and mitochondria (Flori et al., 2017) as well as the existence of genes encoding malate shuttle transporters (Prihoda et al., 2012) seem to allow the energetic exchange between these two organelles (Bailleul et al., 2015). Thus, we propose that malate produced in the mitochondria could be transported into the plastid by the malate shuttle transporters or through the physical interaction
between the two organelles. In the plastid, the malate could be decarboxylated by a plastidic decarboxylating enzyme. The only annotated decarboxylating enzymes in *P. tricornutum*, malate (ME) and phosphoenolpyruvate carboxykinase (PEPCK), were predicted and demonstrated to be located in mitochondria (Kroth *et al.*, 2008; Ewe *et al.*, 2018), although we cannot exclude the possibility that there might be unknown enzymes in the plastids to perform a decarboxylation of malate, generating CO₂ in proximity to Rubisco. Rechecking the protein sequences of decarboxylating enzymes of *P. tricornutum*, we found that one of ME isoforms (ME2) (JGI protein ID 27477) can be targeted to either mitochondria or plastid membrane-associated compartment by using software signal P 3.0 NN (Emanuelsson *et al.*, 2007; Gruber & Kroth, 2017). The ME2-GFP fusion protein located in mitochondria indicated by Ewe *et al.* (2018) was not completely convincing, because the image showed the ME2-GFP signal is probably located in periplastid space rather than in mitochondria. ME2 could function as a decarboxylating enzyme, generating CO₂ close to Rubisco during the putative C4-type CCM if it targets to the periplastid space. Kustka *et al.* (2014) also suggested that the plastidic PYC in a reverse reaction to its regular function could decarboxylate C4 acids, requiring carbonic anhydrases which are present in the plastid of *T. pseudonana* and *P. tricornutum* (Kustka *et al.*, 2014). The pyruvate carboxylase (PYC) mediated decarboxylation in *T. pseudonana* proposed by Kustka *et al.* (2014) was based on the decarboxylating activity of PYC isolated from chicken liver (Attwood & Cleland, 1986), however, there is no experimental verification of this reaction in *T. pseudonana*. Pyruvate generated by decarboxylating of malate in plastids during the proposed C4 cycle could be converted into PEP by the pyruvate-orthophosphate dikinase (PPDK) and could be transported back to the mitochondria as a substrate for PEPC2 via the malate shuttle transporters and the physical interaction between mitochondria and chloroplasts as suggested above. The regeneration of PEP from pyruvate by the PPDK would be required to keep this potential C4 cycle going. However, a PPDK silencing mutant of *P. tricornutum* by Haimovich-Dayan *et al.* (2013) indicates that PPDK inhibition has little effect on photosynthesis. Possibly silencing PPDK may not be sufficient to affect a C4 pathway, which in *P. tricornutum* is not strongly involved anyway, knocking out the PPDK gene may yield clearer results here. Furthermore, the mitochondrial PYC1 could also supply OAA for this C4 pathway, as it converts pyruvate to OAA (Figure 4.10). This could be a reason why a knockout of PEPC2 may only have a small effect on carbon fixation in *P. tricornutum*, because PYC1 has the same function as PEPC2 to supply C4 compounds. Therefore, we assume that knocking out both PEPC2 and PYC1 could have even more pronounced impacts.
Figure 4.10. Model of the biophysical CCM and the proposed PEPC2 mediated C4 CCM in *P. tricornutum*. The model of biophysical CCM pathway is indicated by green arrows (Young & Hopkinson, 2017). The proposed C4 pathway is marked by light blue arrows. The dashed blue arrows indicate pathways which have not yet been verified by experiments. Green lines represent the four plastid membranes, orange lines represent mitochondria with two membranes, black line indicates cytoplasmic membrane. Red arrows indicate the Entner–Doudoroff pathway. Abbreviations: CA: carbonic anhydrase; PEPC: phosphoenolpyruvate carboxylase; MDH: malate dehydrogenase; ME: malic enzyme; PPDK: pyruvate-phosphate dikinase; PYC: pyruvate carboxylase; PEPCK: phosphoenolpyruvate carboxykinase; PK: pyruvate kinase; PDC: pyruvate dehydrogenase complex; PEP: phosphoenolpyruvate; OAA: oxaloacetate; GAP: glyceraldehyde-3-phosphate; 6PG: 6-phospho-gluconate; 2K3DPG: 2-keto-3-deoxyphosphogluconate; PPS: periplastidic space.
In land plants, C4 photosynthesis has evolved many times with great diversity among different species (Sage, 2004). C4 photosynthesis including Kranz anatomy or temporal separation of reactions in the CAM mechanism are the most common types of biochemical CCMs in plants. A few land plants and some aquatic species evolved single-cell C4 pathways, in which the C4 photosynthesis can be accomplished within a single chlorenchyma cell (Voznesenskaya et al., 2001; Edwards et al., 2004). The land plants Borszczowia aralocaspica and Bienertia cycloptera which belong to the Chenopodiaceae, both perform C4 photosynthesis within a single cell (Voznesenskaya et al., 2001; Sage, 2002; Voznesenskaya et al., 2002). The single-cell C4 photosynthesis functions by the intercellular distribution of enzymes and organelles in two different cytoplasmic compartments, and involves the existence of dimorphic chloroplasts (Edwards et al., 2004). In the single-cell C4 photosynthesis, the first step is prefixation of $\text{HCO}_3^-$ in the cytosol by a cytosolic PEPC to produce C4 compounds. Then the C4 compounds can be transported to the mitochondria and be decarboxylated by a mitochondrial NADP-ME to release CO$_2$. The released CO$_2$ from the mitochondria can be captured by Rubisco, which is localized in a specialized type of chloroplasts surrounding the mitochondria. The released pyruvate can then be transported back to the other type of chloroplasts and converted to PEP by PPDK as a substrate for PEPC (Edwards et al., 2004). Apart from the terrestrial plants, single-cell C4 has also been found in aquatic plants, for instance in Hydrilla verticillata and Egeria densa (Ascencio & Bowes, 1983; Bowes, 2010). These aquatic plants neither contain Kranz anatomy nor dimorphic chloroplasts, they show different C4 mechanism compared to the Kranz and single-cell C4 land plants. PEPC uses the $\text{HCO}_3^-$ catalyzed from CO$_2$ by a cytosolic CA to produce OAA in the cytosol. OAA and Asp transported into the chloroplast are converted into malate by plastidic MDH. The converted malate can be decarboxylated by a chloroplastic ME to release CO$_2$ in close proximity of Rubisco, and inhibits its oxygenase activity. The generated pyruvate is then converted to PEP which can return back to the cytosol as the substrate for PEPC (Bowes, 2010). Similarly, the single-cell C4 pathway could also be present in diatoms and function in two different compartments of a single cell.
4.5.7 Alternative role of PEPC2 in mitochondria and PEPC1 in periplastidic space

As shown in Figure 4.10, OAA and malate produced in mitochondria are also required for anaplerotic reactions in the tricarboxylic acid (TCA) cycle, supplying carbon skeletons. And the metabolic intermediates used for biosynthetic reactions are drawn away from the TCA cycle. Therefore, usage of OAA produced by PEPC2 in the mitochondria for anaplerotic/anabolic pathways could act as a parallel input of DIC into organic biomass. Obviously continuous pyruvate and ATP are produced in mitochondria via glycolysis, providing cells with energy and metabolic intermediates for the TCA cycle, fatty acid synthesis, and amino acid synthesis. *P. tricornutum* possesses unusual metabolic pathways including Entner–Doudoroff pathway which is typical for prokaryotes (Fabris et al., 2012), and the second half of glycolysis in the mitochondria (Kroth et al., 2008) (Figure 4.10). However, the origin and functions of these two pathways in mitochondria of *P. tricornutum* remain unknown. Fabris et al. (2012) suggest that the Entner–Doudoroff pathway may be a strategy for diatoms to adjust rapidly to changing environments. Mitochondrial glycolysis is only present in diatoms and non-photosynthetic oomycetes (Ginger et al., 2010). The mitochondrial glycolysis may play an important role in generating pyruvate and energy for the intermediates used in biosynthesis. PEPC, PEPC, PYC, MDH, and MD which involve in the interconversion of C3 and C4 intermediates are indicted as components of pyruvate hub in mitochondria of diatoms (Smith et al., 2012). The mitochondrial of diatoms functions as carbon flux regulator, which plays an important in the distribution of intracellular organic carbon (Smith et al., 2012).

Interestingly, we could not find evidence that the periplastidic PEPC1 is involved in photosynthesis, which raises the question of why this enzyme is targeted to this compartment. One possibility could be the generation of carbon skeletons for amino acid synthesis. In *Arabidopsis thaliana*, PEPCs localized in the cytosol play a role in amino acid synthesis. OAA produced by PEPC enters the TCA cycle, the α-ketoglutarate generated from the TCA cycle can be used as a precursor of glutamate synthesis in the chloroplast (Taylor et al., 2010). The periplastidic PEPC1 in *P. tricornutum* could fulfill a similar function as the cytosol PEPC in *A. thaliana*. OAA produced by PEPC1 might be converted into α-ketoglutarate by the aspartate aminotransferase 4 (AAT4, JGI Protein ID 22909) which is predicted to be localized in the chloroplast (Ewe, 2015). The α-ketoglutarate would then be a substrate for the glutamate synthase (Taylor et al., 2010).
4.6 Conclusion

Our results show that part of the primary carbon fixation in *P. tricornutum* is achieved by the mitochondrial PEPC2 enzyme. The contribution of this C4-type carbon fixation to the biomass formation is higher at low DIC concentrations compared to normal (in the range of air saturated growth medium) DIC concentrations. We hypothesize that PEPC acts as a component of a biochemical CCM pathway in *P. tricornutum*, however, a plastidic decarboxylase would be needed for such a pathway. Reverse genetic tools like the gene knockouts used in this study will be helpful to further characterize the putative C4 like carbon fixation in *P. tricornutum*, or other non-model diatoms.

4.7 Acknowledgments

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Chapter 5

General discussion
5. General discussion

5.1 Putative single-cell C4 photosynthesis in diatoms

In land plants, the C4 photosynthesis has evolved many times with great diversity among different species (Sage, 2004). Among those, the Chenopodiaceae Borszczowia aralocaspica and Bienertia cycloptera are exceptional because they possess single-cell C4 pathway that can be accomplished within a single chlorenchyma cell in two different cytoplasmic compartments (Voznesenskaya et al., 2001; Edwards et al., 2004). Also, the monocot Hydrilla verticillata is using single-cell C4 photosynthesis (Edwards & Voznesenskaya, 2010).

Single-cell C4 pathway can also be present in diatoms and may function in two different compartments. Beardall et al. (1976) observed predominantly labeled C4 compounds after short-term (10s or less) incubation of diatoms to $^{14}$CO$_2$. However, Holdsworth & Colbeck (1976) and Mortain-Bertrand et al. (1987) in short term labeling experiments found a predominance of C3 compounds among the initial products of photosynthetic CO$_2$ fixation (Holdsworth & Colbeck, 1976; Mortain-Bertrand et al., 1987). Later, in a reinvestigation, Reinfelder et al. (2000) found strong evidence for single-cell C4 photosynthesis in the marine diatom *T. weissflogii*. In their study, short-term $^{14}$C (10 to 15s) assimilation in CO$_2$ stressed cells showed that the principal $^{14}$C-labelled intracellular compound was a C4 molecule (malate), they further observed $^{14}$C transfer from malate to phosphoglyceric acid (PGA) (Reinfelder et al., 2000). Moreover, cells acclimated to different concentrations of CO$_2$ showed the highest activity of PEPC at low CO$_2$ concentrations, indicating that PEPC might be involved in inorganic carbon uptake in this species (Reinfelder et al., 2000). These findings later have been substantiated by Morel et al. (2002) and Reinfelder et al. (2004).
Table 5.1. Putative photosynthetic carbon assimilation pathway in three diatom species.

<table>
<thead>
<tr>
<th>Species</th>
<th>C3 or C4 Photosynthesis</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalassiosira weissflogii</td>
<td>C4</td>
<td>$^{14}$C labeling</td>
<td>(Reinfelder et al., 2000; Morel et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>$^{14}$C labeling,</td>
<td>(Reinfelder et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>PEPC and PEPCK inhibitor</td>
<td>(Reinfelder et al., 2004; McGinn &amp; Morel, 2008)</td>
</tr>
<tr>
<td></td>
<td>C3-C4 intermediate</td>
<td>$^{14}$C labeling</td>
<td>(Roberts et al., 2007b)</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>C4</td>
<td>PEPC and PEPCK inhibitor</td>
<td>(McGinn &amp; Morel, 2008)</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>Transcriptomics</td>
<td>(Kustka et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>C3 or C4</td>
<td>Proteomics</td>
<td>(Nunn et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>$^{14}$C labeling</td>
<td>(Roberts et al., 2007b)</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>C4 enzymatic activity</td>
<td>(Trimborn et al., 2009; Clement et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>C4 enzyme localization</td>
<td>(Tanaka et al., 2014)</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>C4</td>
<td>$^{14}$C labeling</td>
<td>(Beardall et al., 1976)</td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>PEPC and PEPCK inhibitor</td>
<td>(McGinn &amp; Morel, 2008)</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>C4 enzymatic activity</td>
<td>(Cassar &amp; Laws, 2007; Clement et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>C4 gene $PPDK$ silencing</td>
<td>(Haimovich-Dayan et al., 2013)</td>
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<tr>
<td></td>
<td>C3</td>
<td>C4 protein localization</td>
<td>(Ewe et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>C3 and C4</td>
<td>C4 gene $PEPC$ knockout</td>
<td>This study</td>
</tr>
</tbody>
</table>
In the subsequent work, Roberts et al. (2007b) demonstrated that both C3 and C4 compounds were predominately labeled in *T. weissflogii* after 2s and 5s incubation with $^{14}$CO$_2$, however, with a lower ratio of C4 to C3 compounds than determined in the study by Reinfelder et al. (2000). Overall, Robert et al. (2007b) explained the labeling pattern in *T. weissflogii* to represent C3-C4 intermediate photosynthesis rather than pure C4 photosynthesis (Roberts et al., 2007b). However, the $^{14}$C labeling pattern in *T. pseudonana* with the same method used in *T. weissflogii* was different: it showed an exclusive C3 pattern in *T. pseudonana* (Roberts et al., 2007b). It was therefore suggested that the C3 labeling pattern in *T. pseudonana* could override the C4 metabolism after short-term (2s) labeling, because the transfer from C4 to C3 could happen even within 2s (Raven, 2010), and that the C4 compounds could be other organic C4 compounds rather than malate or aspartate (Bowler et al., 2010). Despite the different $^{14}$C labeling pattern in the two *Thalassiosira* species (Roberts et al., 2007b), similar effects were observed for both species after treatment with specific inhibitors of PEPC and PEPCK, which is indicative for C4 photosynthesis (McGinn & Morel, 2008). Although the strong evidence for single-cell C4 photosynthesis in *T. weissflogii* (Reinfelder et al., 2000; Reinfelder et al., 2004; McGinn & Morel, 2008), it is still controversial whether a C4 CCM is generally present in diatoms (Table 5.1). *Phaeodactylum tricornutum* with completely sequenced genome has also been investigated regarding the occurrence of biochemical CCM. $^{14}$C labeling experiment (Beardall et al., 1976) and the similar effect to C4 inhibitors with *T. pseudonana* and *T. weissflogii* (McGinn & Morel, 2008) indicate *P. tricornutum* could do C4 photosynthesis. However, the C4 enzyme activity, C4 enzyme Pyruvate-orthophosphate dikinase (PPDK) silencing mutant and the localization of C4 enzymes indicate that C4 photosynthesis does not exist in *P. tricornutum* (Cassar & Laws, 2007; Haimovich-Dayan et al., 2013; Ewe et al., 2018). The conclusions and methods regarding the occurrence of C4 photosynthesis in *P. tricornutum* compared to *T. pseudonana* and *T. weissflogii* were listed in Table 5.1.
In our study, we showed that the marine diatom *P. tricornutum* may perform a C4 pathway that relies on carboxylation by a mitochondrial PEPC and a putative plastid decarboxylase enzyme (chapter 4, a model is shown in Figure 5.1). The mitochondria and chloroplasts of *P. tricornutum* are closely connected, which may allow molecule exchange between these two compartments (Bailleul et al., 2015; Flori et al., 2017). These two physically contacted compartments in *P. tricornutum* may thus function similarly with the two cytosolic compartments of single-cell C4 photosynthetic land plants *Borszczowia aralocaspica* and *Bienertia cycloptera*, where the carboxylation and decarboxylation of C4 compounds take place. As shown in Figure 5.1, the substrate PEP is produced in mitochondria by the second part of the mitochondrial glycolysis and carboxylated by mitochondrial PEPC2 to produce OAA, which can be converted into malate. We propose that the C4 compound malate can be transported into chloroplast via the physical connection between the two organelles, a putative decarboxylase enzyme then may catalyze the decarboxylation of malate and the releases CO₂ in close proximity to Rubisco. The released pyruvate could be converted to PEP, which would be transported back to mitochondria, and used as a substrate for PEPC. The advantage of a carboxylation reaction in the mitochondria could be that the CO₂ which is generated from photorespiration and respiration can be converted into HCO₃⁻ by a mitochondrial CA, being a substrate for PEPC2 (Figure 5.1). This recapture of CO₂ in mitochondria may reduce the loss of CO₂ during photorespiration/respiration.
The key aspect of the unsolved questions in the proposed C4 CCM in diatoms is the identification of a decarboxylase enzyme which can catalyze C4 compounds and release CO₂ near Rubisco. The localization of decarboxylase enzymes PEPCK and ME in *P. tricornutum* and *T. pseudonana* were identified, and none of them is located in the chloroplast where the Rubisco is located (Tanaka *et al.*, 2014; Ewe *et al.*, 2018). Two MEs were identified in *P. tricornutum* and were shown to be located in mitochondria (Ewe *et al.*, 2018). However, the evidence for the localization of ME2 (JGI protein ID 27477) in mitochondria (Ewe *et al.*, 2018) was not convincing enough. ME2 was predicted to have dual targeting pre-sequence, that either a signal peptide or a mitochondrial transit peptide by using a software signal P 3.0 NN (Emanuelsson *et al.*, 2007; Gruber & Kroth, 2017). Moreover, the ME2-GFP signal according to Ewe *et al.* (2018) showed that the ME2 is probably
localized in the endoplasmic reticulum (ER) or the plastid membrane-associated compartment rather than mitochondria of *P. tricornutum*. Therefore, we speculate that the ME2 of *P. tricornutum* may be involved in the C4 photosynthesis to catalyze the decarboxylation of malate and release CO₂ in this compartment for Rubisco. Since *T. weissflogii* has been strongly proven to perform C4 CCM. If the decarboxylase enzyme in this species can be identified, it probably will give some hints on identifying the C4 decarboxylase enzyme of the other diatom species. Unfortunately, the genome sequence of this species has not been available yet. Reinfelder *et al.* (2000) suggest that in *T. weissflogii*, the C4 compounds could be decarboxylated by PEPCK in plastids and release CO₂ for Rubisco because 75% PEPCK activity was determined in chloroplasts. The molecular and physiological characteristics of a putative C4 CCM are mainly examined among marine diatoms by various methods, and showed diverse results among different species (Beardall *et al.*, 1976; Reinfelder *et al.*, 2000; Reinfelder *et al.*, 2004; Cassar & Laws, 2007; Roberts *et al.*, 2007b; McGinn & Morel, 2008; Haimovich-Dayan *et al.*, 2013; Kustka *et al.*, 2014; Clement *et al.*, 2016). Very few publications are available to show the examination of C4 CCM in the freshwater diatoms (Clement *et al.*, 2017). It could be worthy to do more efforts on the examination of C4 CCM among freshwater diatoms.

5.2 Possible advantages of single-cell C4 photosynthesis in diatoms

The reasons and benefits behind the utilization of single-cell C4 photosynthesis in diatoms are not completely clear, but similar to land plants, one benefit is the accumulation of CO₂ in close proximity to Rubisco in order to increase CO₂ fixation under low CO₂ conditions. Another benefit could be that the carbon prefixed by PEPC can be used for supplementing carbon skeleton for intermediates of the TCA cycle in the mitochondria. In land plants, single-cell C4 system could simply be a variation of the Kranz anatomy-type C4 based on two cell types, being equally complex regarding its control of compartmentation functions, but less complex in requiring the cooperative functions of two cells (Edwards *et al.*, 2004). In addition, single-cell C4 photosynthesis responds more flexible to changing environments (Edwards *et al.*, 2004). For instance, the single-cell C4 species *Hydrilla verticillata* may shift from C3 to C4 and back, depending on the environmental conditions. It performs C3 photosynthesis if high concentrations of inorganic carbon are available and at low temperatures, but shifts to C4 photosynthesis at low inorganic carbon concentrations and warmer conditions (Salvucci & Bowes, 1981; Ascencio & Bowes, 1983; Magnin *et al.*, 1997). Similarly to *Hydrilla verticillata*, a shift from C3 photosynthesis to C4 photosynthesis could be possible in diatoms or these two CCM mechanisms could function in parallel according to the
changing environment. Carbonic anhydrases, which catalyze the inter-conversion of CO$_2$ and HCO$_3^-$ (Badger & Price, 1994), require trace metal zinc (Zn) at the active site for efficient enzyme activity (Coleman, 1998). This essential trace metal is limited in the surface layer of the ocean (Reinfelder, 2010), where has been measured as low as around 2 pM (Bruland, 1989). The utilization of inorganic carbon is limited in the coastal diatom *T. weissflogii*, when cultivated at the concentration of trace metal of surface water from the ocean (Morel *et al*., 1994). Later, Lane & Morel (2000) examined the regulation of the major Zn-requiring isoform of CA (TWCA1) in *T. weissflogii* under different Zn and CO$_2$ concentrations. Results showed TWCA1 protein and total intracellular CA activity levels can be induced by low CO$_2$, but the induction is dependent on the Zn concentration. Low Zn concentration directly leads to low TWCA1 protein and total CA activity levels in *T. weissflogii* no matter under low or high CO$_2$ conditions (Lane & Morel, 2000). Under Zn stressed conditions, Reinfelder *et al*. (2000) observed the C4 pathway contributes a major carbon fixation in *T. weissflogii*. The biophysical CCMs may not function efficiently under Zn limited condition due to the low CA activity. Therefore, using the C4 pathway in parallel with the utilization of incomplete biophysical CCM could be a good strategy for *T. weissflogii* or other diatoms to increase the rate of photosynthesis under Zn limited environment.

In the marine ecosystem, the concentrations of macronutrient like nitrogen can change drastically for a number of reasons including water turbulence or upwelling in the ocean. Nitrogen concentrations are usually high in coastal waters, but limited in the open ocean. As nitrogen is important for protein synthesis, it was shown that the protein level of Rubisco was highly reduced in *P. tricornutum* under nitrogen stress (Levitan *et al*., 2015). Strongly reduced Rubisco amount could reduce the rate of CO$_2$ fixation. Under nitrogen and CO$_2$ limited condition, using C4 type photosynthesis may increase the rate of CO$_2$ releasing (via decarboxylation of C4 acid) in close proximity to less amount of Rubisco, still resulting high rate of CO$_2$ fixation. In this case, the C4 CCM become important for the diatom species which distribute in the open ocean, where the nitrogen is limited. Overall, we speculate that the strategy of using C4 CCM can easily help diatoms to deal with changing DIC and nutrients and may promote their role as major primary producers in the marine ecosystem.

5.3 The diversity of CCMs in plants and diatoms

C4 photosynthesis can be quite diverse among different plant species (Edwards *et al*., 2004; Sage, 2004; Edwards & Voznesenskaya, 2010; Sage *et al*., 2012). The Kranz anatomy type of C4 photosynthesis shows great diversity on their biochemistry as well as chloroplasts (Edwards &
Walker, 1983; Voznesenskaya et al., 1999; Edwards et al., 2004; Edwards & Voznesenskaya, 2010). The C4 biochemical diversity is based on the different types of C4 acid decarboxylase enzymes: NAD-malic enzyme (NAD-ME), NADP-malic enzyme (NADP-ME), and PEP carboxykinase (PEPCK) (Kanai & Edwards, 1999; Edwards et al., 2004). The Kranz C4 plants contain dimorphic chloroplasts in bundle sheath and mesophyll cells (Edwards et al., 2004). The diversity of chloroplasts is reflected in the differences of chloroplast ultrastructures, starch locations and enzymes (Edwards & Walker, 1983; Voznesenskaya et al., 1999; Edwards et al., 2004). Single-cell C4 land plants also contain dimorphic chloroplasts within two separated cytoplasmic compartments (Edwards et al., 2004; Edwards & Voznesenskaya, 2010). While aquatic plants performing single-cell C4 photosynthesis contain monomorphic chloroplasts (Edwards et al., 2004).

We also observed the diversity of biochemical CCMs in the examined diatom species, T. weissflogii, T. pseudonana and P. tricornutum. Firstly, C4 enzymes can be differently regulated in different diatom species. In chapter 4, our experimental data showed that the mRNA, protein and enzyme activity of the C4 enzyme PEPC is stable under low and high CO₂ in P. tricornutum. Instead, in T. weissflogii, the C4 enzyme PEPC activity is highly induced under CO₂ limited conditions (Reinfelder et al., 2000). Similar to T. weissflogii, the mRNA level of C4 gene PEPC was up-regulated in T. pseudonana under low CO₂ concentrations (McGinn & Morel, 2008; Kustka et al., 2014).

Secondly, the C4 CCM seems to contribute differently to the whole CCM among different diatom species, or within the same species but under different environmental conditions. It was shown in this study that P. tricornutum is less dependent on a C4 CCM, but mainly utilize a biophysical CCM under low CO₂. In contrast, T. weissflogii was proven to be more dependent on C4 CCM than T. pseudonana and P. tricornutum under low CO₂ (McGinn & Morel, 2008).

The biophysical and biochemical CCMs of diatoms have been investigated under the criteria of the availability of the genome sequences and genome editing tools of the model organisms P. tricornutum (Bowler et al., 2008) and T. pseudonana (Armbrust et al., 2004). The components of biophysical CCMs (HCO₃⁻ and CAs) have been gradually identified and functionally analyzed. Regarding the diversification of biophysical CCMs among diatom species, intercellular localization of CAs shows high variability, while HCO₃⁻ transporters are rather similar (Shen et al., 2017; Young & Hopkinson, 2017). The C4-like photosynthesis in diatoms shows diversity among different species. Analysis of the PEPC genes indicates that PEPCs are diversely regulated, may further infer that PEPCs have evolved differently within diatom lineages. Diatoms are adapted to different
ecological niches in the marine ecosystem, therefore nutrient uptake, growth, and photoacclimation can be diverse among diatom species (Reinfelder, 2010). It has been shown that micronutrients are selective factors of oceanic and coastal diatoms (Brand et al., 1983; Sunda & Huntsman, 1995). In the marine ecosystem, not only the DIC concentration can affect the carbon uptake by a diatom, but also the nutrients, light as well as other effectors could have effects on CO₂ assimilation in diatoms. We speculate that diatoms use both C3 and C4 CCMs, the ratio of C3 or C4 used in different diatom species are different and may depend on the changing environment.

5.4 Comparison of origin and localization of PEPC in diatoms, green algae, red algae, and plants

It is not clear yet where eukaryotic PEPCs originated from. In the early time, the PEPCs of land plants were assumed to be obtained from the ancestors of their plastids, the cyanobacteria (Lepiniec et al., 1994). As the ancestors of green algae, red algae are also cyanobacteria, and the ancestor of diatom plastids are red algae (Kroth & Strotmann, 1999; Keeling, 2013), their PEPC might have the same origin of cyanobacteria according to the assumption by Lepiniec et al. (1994). However, later phylogenetic analysis of PEPCs shows that land plants PEPCs are more closely related to gammaproteobacteria than cyanobacteria, indicating the plant PEPCs most probably origin from proteobacteria (Gehrig et al., 2001; Sánchez & Cejudo, 2003). Similar phylogenetic relations were observed in green algae, red algae, and diatoms (Chi et al., 2014), indicating PEPCs of diatom as well as green algae and red algae were also obtained from proteobacteria via horizontal gene transfer (HGT) (Figure 5.2).
Figure 5.2. Horizontal gene transfer from a proteobacterium to a diatom. During evolution, a diatom got PEPC gene from a proteobacterium via horizontal gene transfer. PEPC gene was duplicated into two copies in the diatom during evolution. Nuclear-encoded PEPC1 protein targets to the periplastidic space, while PEPC2 protein targets to mitochondrion. Abbreviation: N: Nuclear; C: Chloroplast; M: Mitochondrion; PPS: periplastidic space.
The number and intracellular localization of PEPCs can be quite diverse among different photosynthetic groups. As shown in Figure 5.3, plants have at least three PEPC genes, for instance, three PEPC genes have been found in Zea mays (Dong et al., 1998), and four PEPC genes in Arabidopsis thaliana (Sánchez & Cejudo, 2003). Only one or two PEPC genes have been found in green algae (Rivoal et al., 2001; Mamedov et al., 2005), red algae (Nozaki et al., 2007), and diatoms (Kroth et al., 2008). For instance, two PEPC gene were found in C. reinhardtii (Mamedov et al., 2005) and P. tricornutum (Bowler et al., 2008) respectively, and one PEPC gene in Cyanidioschyzon merolae 10D (Nozaki et al., 2007). Interestingly, PEPCs are cytosolic enzymes in land plants (both C4 and C3 PEPCs) (Sage, 2002; Sage, 2004), red algae, and green algae (one isoform of PEPCs of C. reinhardtii is predicted to be localized in mitochondria) (Figure 5.3), while PEPCs of the examined diatoms P. tricornutum were identified to be localized in mitochondria and the periplastidic space (Ewe et al., 2018). The proper number and intercellular localization of PEPCs may correlate to the functions that PEPCs are involved in. Both C4 and C3 PEPCs in plants were localized in the cytosol, C4 PEPCs play roles in photosynthesis (Sage, 2004). C3 PEPCs involve other physiological processes (Schuller et al., 1990; Huppe & Turpin, 1994; O’Leary et al., 2011). PEPCs in green algae are involved in the anaplerotic metabolism of carbon and nitrogen (Schuller et al., 1990; Mamedov et al., 2005). In this study, the mitochondrial localized PEPC of the diatom P. tricornutum was shown to be involved in C4 photosynthesis (chapter 4). The periplastidic PEPC1 apparently is not involved in photosynthesis according to the experimental data, but could have an anaplerotic function, supplying intermediates for amino acid synthesis. C4 PEPCs have evolved from none photosynthetic PEPCs in land plants and obtained distinct features: for example, C4 PEPCs show a lower affinity to the substrate PEP, but a much higher affinity to HCO₃⁻ than C3 PEPCs (Gutierrez et al., 1974; Dong et al., 1998; Gowik et al., 2006; Lara et al., 2006). The finding that the mitochondrial PEPC2 from P. tricornutum has similar properties to C4 PEPCs of plants that PEPC2 has a high $K_m$ value for PEP but a low $K_m$ value for HCO₃⁻ (chapter 3), indicating that this isoenzyme may play a role in C4 metabolism. In contrast, the periplastidic PEPC1 has different affinities, which marks it as a non-photosynthetic PEPC.
Figure 5.3. Localization of PEPCs in land plants *Zea mays*, green algae *Chlamydomonas reinhardtii*, red algae *Cyanidioschyzon merolae* 10D, and diatom *Phaeodactylum tricornutum*. Land plants *Z. mays* obtain three copies of *PEPC* gene and the PEPC proteins locate in the cytosol. Green algae *C. reinhardtii* contains two isoforms of PEPC, one isoform is predicted to locate in the cytosol; the other isoform is predicted to locate in mitochondria. Only one PEPC isoform is present in red algae *C. merolae* 10D, which is predicted to locate in the cytosol. Two isoforms of PEPC are found in diatom *P. tricornutum*, one isoform has been identified to be localized in mitochondria and the other isoform in periplastidic space. Abbreviation: N: Nuclear; C: Chloroplast; M: Mitochondria; PPS: periplastidic space.
Author Contributions

Chapter 1. General introduction
Guilan Yu and Peter G. Kroth
GY drafted the chapter, PGK did revisions, GY finalized the chapter.

Chapter 2. Controlled supply of CO\textsubscript{2} to batch cultures of the diatom *Phaeodactylum tricornutum*
Guilan Yu, Peter G. Kroth and Ansgar Gruber
GY and AG designed the study. GY carried out the experiments, analyzed the data and drafted the manuscript. GY, AG and PGK finalized the manuscript.

Chapter 3. Overexpression and enzymatic assay of *Phaeodactylum tricornutum* PEPCs
Guilan Yu and Peter G. Kroth
GY and PGK designed the study. GY carried out the experiments analyzed the data and drafted the manuscript. GY and PGK finalized the chapter.

Chapter 4. PEP Carboxylase contributes to carbon fixation in the diatom *Phaeodactylum tricornutum* at low concentrations of inorganic carbon
Guilan Yu, Kensuke Nakajima, Ansgar Gruber, Carolina Rio Bartulos, Bernard Lepetit, Elizabeth Yohannes, Yusuke Matsuda and Peter G. Kroth
GY, AG, BL, and PGK designed the study. YM gave advice on the design of the O\textsubscript{2} evolution experiments. KN carried out the DIC-dependent O\textsubscript{2} evolution experiments, EY carried out the stable isotope experiments. GY carried out the other experiments, analyzed the data and drafted the chapter. CRB gave supervisions on genetic characterization of PEPC transformed cell lines. GY, AG, BL, PGK finalized the chapter.

Chapter 5. General discussion
Guilan Yu and Peter G. Kroth
GY drafted the chapter, PGK did revisions, GY finalized the chapter.
List of Publications


Guilan Yu, Kensuke Nakajima, Ansgar Gruber, Carolina Rio Bartulos, Bernard Lepetit, Elizabeth Yohannes, Yusuke Matsuda and Peter G. Kroth. PEP Carboxylase contributes to carbon fixation in the diatom *Phaeodactylum tricornutum* at low concentrations of inorganic carbon. (in preparation)
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**Bibliography**


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


**Supplementary Data**

**Supplementary data for Chapter 3**

Table S3.1. The JGI and Genebank accession number of PEPC protein sequences used in alignment.

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### Table S3.2. Primers used in chapter 3.

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### Supplementary Data for Chapter 4

### Table S4.1. Primers used in chapter 4.

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Figure S4.1. Alignment of two alleles of PEPC1 in wild type. Allele-specific PCR was performed with primers PEPC1_ta_all_FW2 and PEPC1_ta_allele1/2_REV2. The PCR products were sequenced with primer PEPC1_ta_all_FW2. Sequences in light green indicate TALEN target sites; sequences in blue and red color show the allele different base pairs which can distinguish two different alleles.
Figure S4.2. Alinement of PEPC1 between PEPC1-23 knockout mutant and wild type. Allele-specific PCR was performed with primers PEPC1Ta_allele1_FW1 and PEPC1Ta_allele1_REV2. The PCR products were sequenced with primer PEPC1Ta_allele1_FW1. Sequences in light green indicate TALEN target sites; the sequence in blue and red color showed the allele different based. The alinement showed 540bp deletion near TALEN target sites and 143bp sequence in yellow from chromosome 17 was inserted in the deletion site.

Supplements
Figure S4.3. Alignment of PEPC1 between PEPC1-37 knockout mutant and wild type. Allele-specific PCR was performed with primers PEPC1_ta_allele1_FW1 and PEPC1_ta_allele1_REV2. The PCR products were sequenced with primer PEPC1_ta_allele1_FW1. The sequence in light green indicates TALEN target sites; the sequence in blue and red color showed the allele different base pairs. Alignment showed 485bp deletion in allele1 and 667bp deletion in allele2 in PEPC1-37 knockout mutant.
Supplements

Figure S4.4. Alignment of PEPC1 in PEPC1-26 knockout mutant (only allele1) and wild type. Allele-specific PCR was performed with primers PEPC1_ta_all_FW2 and PEPC1_ta_allele1_REV2. The PCR products were sequenced with primer PEPC1_ta_all_FW2. The sequence in light green indicates TALEN target sites; the sequence in blue and red color showed the allele different base pairs. Alignment shows 199bp deletion in allele1 of PEPC1-26 knockout mutant.
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<td>CGCCC GCCATGGATGCCTTGGCAGA TTCCG</td>
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Figure S4.5. Alignment of PEPC1 in PEPC1-26 knockout mutant (only allele2) and wild type. Allele-specific PCR was performed with primers PEPC1_ta_allele1_FW1 and PEPC1_ta_allele2_REV2. The PCR products were sequenced with primer PEPC1_ta_allele1_FW1. The sequence in light green indicates TALEN target.
Supplements

sites; the sequence in blue and red color showed the allele different base pairs. Alinement showed 819bp deletion in allele2 in PEPC1-26 knockout mutant.

PEPC1_26_allele2  TCAACAGTGGCCAGTTACCCTGGGAGCTGCTGGCGCCATGACATGGCCAAATCATGTTTCTACCGTTCTCTCCGAATTGGCAATTTCT
WTPT4_allele1    TCAACAGTGGCCAGTTACCCTGGGAGCTGCTGGCGCCATGACATGGCCAAATCATGTTTCTACCGTTCTCTCCGAATTGGCAATTTCT
WTPT4_allele2    TCAACAGTGGCCAGTTACCCTGGGAGCTGCTGGCGCCATGACATGGCCAAATCATGTTTCTACCGTTCTCTCCGAATTGGCAATTTCT

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PEPC1_26_allele2  GTCTCAGCTGGTTGACTTCTGAGCTTTCCAACAAACGTCCCTTGTACCGAATTCGCGAAT
WTPT4_allele1    GTCTCAGCTGGTTGACTTCTGAGCTTTCCAACAAACGTCCCTTGTACCGAATTCGCGAAT
WTPT4_allele2    GTCTCAGCTGGTTGACTTCTGAGCTTTCCAACAAACGTCCCTTGTACCGAATTCGCGAAT

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PEPC1_26_allele2  TAGATGCCATTACGCGTTGGTTGGGAATTGGCTCCTATAGTGAATGGACCGAAG
WTPT4_allele1    TAGATGCCATTACGCGTTGGTTGGGAATTGGCTCCTATAGTGAATGGACCGAAG
WTPT4_allele2    TAGATGCCATTACGCGTTGGTTGGGAATTGGCTCCTATAGTGAATGGACCGAAG

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PEPC1_26_allele2  TTGGTATGTCCCTCGTGCCCTTGGATATTCGCGAGGAGAGTACCAAGCACACGGAAGCGT
WTPT4_allele1    TTGGTATGTCCCTCGTGCCCTTGGATATTCGCGAGGAGAGTACCAAGCACACGGAAGCGT
WTPT4_allele2    TTGGTATGTCCCTCGTGCCCTTGGATATTCGCGAGGAGAGTACCAAGCACACGGAAGCGT

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PEPC1_26_allele2  CGGGCTTCGGTTTGGTGGCCGACGGTTTATTGGTCGATATCATTCGTCGATTGTATGTGT
WTPT4_allele1    CGGGCTTCGGTTTGGTGGCCGACGGTTTATTGGTCGATATCATTCGTCGATTGTATGTGT
WTPT4_allele2    CGGGCTTCGGTTTGGTGGCCGACGGTTTATTGGTCGATATCATTCGTCGATTGTATGTGT

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PEPC1_26_allele2  TTGACGATGCGACTGATTTGATCAAGCCTTTGCGCAT
WTPT4_allele1    TTGACGATGCGACTGATTTGATCAAGCCTTTGCGCAT
WTPT4_allele2    TTGACGATGCGACTGATTTGATCAAGCCTTTGCGCAT

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PEPC1_26_allele2  GTATGGTCAGTCAGACTCTCGTTGAGGAAGCAGTGGATGGTTGGCAAGACGTCGATGCTC
WTPT4_allele1    GTATGGTCAGTCAGACTCTCGTTGAGGAAGCAGTGGATGGTTGGCAAGACGTCGATGCTC
WTPT4_allele2    GTATGGTCAGTCAGACTCTCGTTGAGGAAGCAGTGGATGGTTGG

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Figure S4.6. Alignment of PEPC1 in PEPC1-26 knockout mutant (only allele2) and wild type. Allele-specific PCR was performed with primers PEPC1_ta_allele1_FW1 and PEPC1_ta_allele2_REV2. The PCR products were sequenced with primer PEPC1_ta_allele1_FW1. The sequence in light green indicates TALEN target sites; the sequence in blue and red color showed the allele different base pairs which can distinguish two different alleles. Alignment showed 4bp deletion in allele2 in PEPC1-26 knockout mutant.

![Alignment Figure](image1)

Figure S4.7. The rate of net photosynthesis of WT and PEPC knockout mutants under different light intensity. In all blot, the closed black triangle represents wt, closed colorful circle represent PEPC knockout mutants. X axis is the light intensity, Y axis is the rate of net photosynthesis. Data represent mean ± SD of three separate experiment.

![Rate of Net Photosynthesis](image2)