Kwansei Gakuin University
Report of Research Outcome

2025/03/14

To President

Department : Science and Technology
Position : Postdoctoral fellow
Name : Hermanus Nawaly

I report the outcome of the research as follows.

Name of the Fund/Program	□Sabbatical leave with grant □Sabbatical leave with no grant □KGU Joint Research □Individual Special Research ☒Postdoctoral fellow ※Please report by designated form as for "International Research Collaboration".
Research Theme	1. Functional characterization of putative chloroplast envelope located transporters belonging to the SLC-4 family by CRISPR/Cas9 mediated genome editing in the marine diatoms, *P. tricornutum*. 2. Functional characterization of putative theta type carbonic anhydrase, Tp-θCA2, by genome editing.
Research Site/Venue	Matsuda lab, Building IV, Department of Bioscience, Kwansei Gakuin University, Kobe-Sanda Campus
Research period	2024/04/01 ～ 2025/03/31 （12 month）

◆ **Summary of the research outcome** （approx. 2,500 words）

Please write down the outcomes in detail regarding the research theme above.

Marine diatoms, a category of microalgae, are responsible for 20% of the Earth's primary production.In the context of seawater, dissolved inorganic carbon (DIC) predominantly exists as HCO_3^-. Conversely, CO_2 concentrations remain below 25 μM, a level insufficient to support the K_m [CO_2] of RubisCO. Our previous studies demonstrated that plasma membrane solute carriers (SLCs), namely PtSLC4-1, PtSLC4-2, and PtSLC4-4, facilitate the movement of bicarbonate from the environment into the cytoplasm of the marine diatom, *Phaeodactylum tricornutum*.However, the mechanisms underlying bicarbonate transport from the cytoplasm to the chloroplast remain to be fully elucidated. Our earlier findings, utilizing a C-terminal tagging approach with EGFP, have successfully localized the bicarbonate transporters, PtSLC4-6 and PtSLC4-7, within the chloroplast membrane. Furthermore, transformants co-expressing PtSLC4-2:EGFP and PtSLC4-7:EGFP, and PtSLC4-2/PtSLC4-7 transformants, exhibited effects on photosynthetic parameters and DIC uptake rate under high CO2 conditions (Nakajima, unpublished data).To understand the function of PtSLC4-6 and PtSLC4-7, a knockout approach with Cas9 was used. Specifically, the knockout of PtSLC4-7 was followed by the knockout of PtSLC4-6. In the initial round of knockout, the production of monoclonal mutants with -13 bp and -19 bp mutations for each allele was observed.

A series of measurements were conducted to assess the photosynthetic parameters of PtSLC4-7 KO cells cultivated in two distinct carbon dioxide (CO_2) environments: high CO_2 (HC, containing 1% CO_2) and low CO_2 (LC, containing 0.04% CO2). These measurements were then compared with those of the wild-type (WT) cells. The results obtained revealed that there was no significant difference in photosynthetic parameters between the WT and the KO mutant under HC conditions (Figure 1, left panel, Tabel 1). However, under LC conditions, there was a tendency for a decrease in photosynthetic capacity, although this decrease was not statistically significant when compared with the WT (Figure 1, right panel, Tabel 1). This outcome may be attributed to the complementary function of PtSLC4-6, which is located in the same position as PtSLC4-7, based on unpublished data (Nakajima, unpublished data). Subsequent experiments were conducted with PtSLC4-6 knockout in PtSLC4-7 mutants by transfecting vectors containing different selection markers with Cas9 and sgRNA targeting PtSLC4-6. This resulted in the isolation of a PtSLC4-6 mutant that exhibited a 10 bp deletion. Subsequently, the photosynthetic parameters of the double KO PtSLC4-6 and PtSLC4-7 (ΔPtSLC4-7/PtSLC4-6) were measured under both HC and LC conditions. Measurement of photosynthetic parameters showed a decrease of 2-fold in P_{max} and an increase of 1.7-fold in $K_{0.5}$ value under LC in ΔPtSLC4-7/PtSLC4-6 compared to WT (Figure 1, table 1). A decrease in P_{max} in the ΔPtSLC4-7/PtSLC4-6 was also seen in HC conditions by 1-fold compared to WT. However, there was no significant difference in $K_{0.5}$ between the WT and ΔPtSLC4-7/PtSLC4-6 (Figure 1, table 1). The growth rate measurements in ΔPtSLC4-7/PtSLC4-6 in LC and HC conditions showed a decrease in doubling rate of about 16-30% and 32-45% compared to WT in LC and HC conditions, respectively (Fig.2).

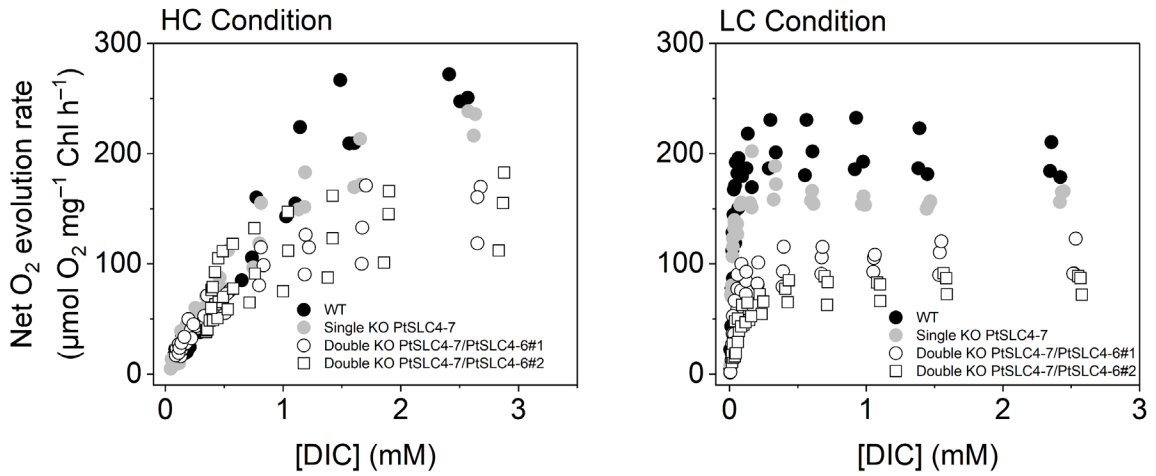

Figure 1 Kinetics for determining photosynthetic parameters in the single ΔPtSLC4-7 and ΔPtSLC4-7/PtSLC4-6 mutant compared to WT under HC and LC conditions. Plots of photosynthetic O_2 evolution rates at different DIC concentrations at pH 8.2 and 20 ^{O}C in WT (Black circle), ΔPtSLC4-7 (Gray circle), ΔPtSLC4-7/PtSLC4-6 (open circle and open square) grown in HC and LC.

Tabel 1. Photosynthetic Parameter of ΔPtSLC4-7 and ΔPtSLC4-7/PtSLC4-6 mutant

Sample	High CO_2		Low CO_2	
	$K_{0.5}$[DIC] (μm)	P_{max} (μmol O_2 mg^{-1} Chl h^{-1})	$K_{0.5}$ (μm)	P_{max} (μmol O_2 mg^{-1} Chl h^{-1})
PtWT	904 ± 223 (n=3)	294 ± 50 (n=3)	23 ± 7 (n=3)	199 ±20 (n=3)
ΔPtSLC4-7	767 ± 202 (n=3)^{NS}	238 ± 14 (n=3)^{NS}	18 ± 3 (n=3)^{NS}	161±8 (n=3)^{NS}
ΔPtSLC4-7/SLC4-6_1	505 ± 114 (n=3)^{NS}	143 ± 33 (n=3)**	38 ± 6 (n=3)*	99 ±12 (n=3)**
ΔPtSLC4-7/SLC4-6_2	488 ± 71 (n=3)^{NS}	143 ± 36 (n=3)**	73 ± 31 (n=3)*	78 ±10 (n=3)**

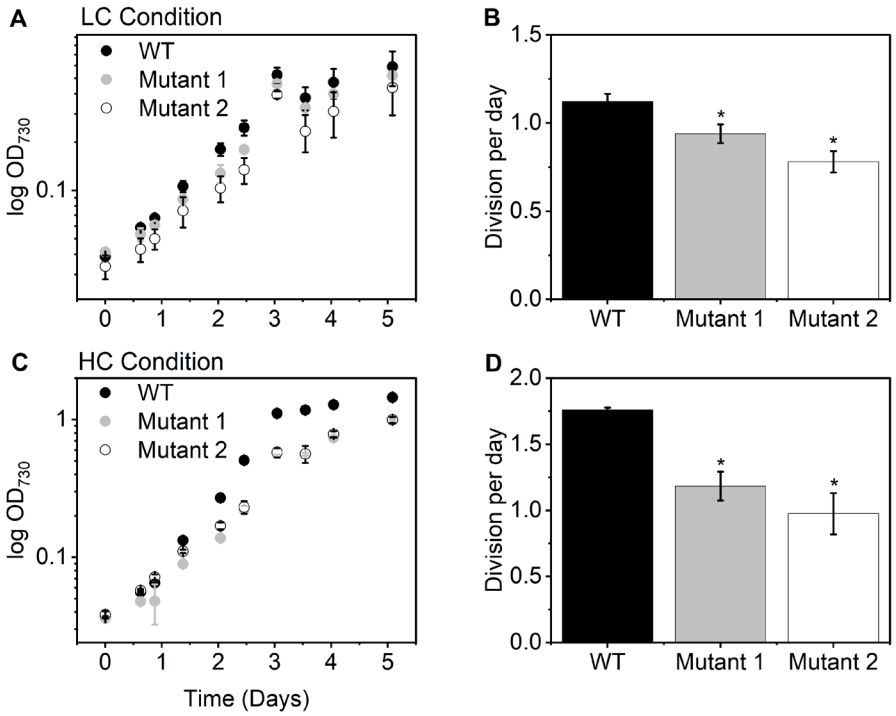

Figure 2 Growth analysis of WT and ΔPtSLC4-7/PtSLC4-6 mutant under HC and LC conditions. Error bars indicated three biological replications. Significant differences compared to the wild-type were determined using Student's *t*-test: *P<0.05

Carbonic anhydrase (CA) is an essential factor in the CO_2 concentrating mechanism of many aquatic photoautotrophs, which play a crucial role in world primary production. CA catalyzes the interconversion of carbon dioxide (CO_2) and bicarbonate ions (HCO_3^-) in both directions. Four potential gene sequences in the genome of the centric marine diatom, *Thalassiosira pseudonana*, are responsible for encoding θ-type carbonic anhydrase (CA), a recently discovered type of CA seen in marine diatoms and green algae. My previous study identified the precise subcellular positions of four θ-CAs, namely Tp-θCA1, Tp-θCA2, Tp-θCA3, and Tp-θCA4, by expressing GFP-fused proteins of these Tp-θCAs in T. pseudonana. Subsequent analysis of the C-terminal GFP fusion proteins of Tp-θCA1, Tp-θCA2, and Tp-θCA3 revealed their presence in the chloroplast. Specifically, Tp-θCA2 was found in the chloroplast's central part, while the other two Tp-θCAs were distributed throughout the chloroplast. Transmission electron microscopy immunogold labeling was conducted on the transformants expressing Tp-θCA1:GFP and Tp-θCA2:GFP using anti-GFP monoclonal antibodies. The Tp-θCA-1:GFP protein was shown to be concentrated in the stroma, specifically in the peripheral pyrenoid area.Tp-θCA2: GFP is distinctly positioned in a linear pattern within the center region of the pyrenoid structure. This linear arrangement is hypothesized to result from the penetration of thylakoids into the pyrenoid (PPT). Furthermore, this study postulated that Tp-θCA2 plays a crucial role in CCM. However, attempts to eliminate this protein using Cas9 nickase (D10A) were unsuccessful (Nawaly et al., 2023). To address this challenge, a conventional Cas9 was employed in conjunction with dual sgRNA to achieve the knockout of *Tp-θCA2*. The outcome of this approach was the successful execution of genome editing at the *Tp-θCA2* locus, culminating in a 41-base pair deletion. Subsequent bioinformatic analysis revealed that this deletion led to the emergence of an early stop codon, thereby indicating the inactivation of Tp-θCA2. Subsequently, the photosynthetic parameters of the Tp-θCA2 knockout under LC and HC conditions were measured and compared to those of the WT. The results demonstrated a decrease in photosynthetic capacity and an increase in Km in the Tp-θCA2 mutant compared to the WT under HC conditions. Under LC conditions, the photosynthetic parameters of the Tp-θCA2 mutant were found to be inferior to those under HC conditions (Figure 3). These results indicate that Tp-θCA2 is essential for *T. pseudonana* under both HC and LC conditions, suggesting a critical role for Tp-θCA2 in the CCM of *T. pseudonana*.

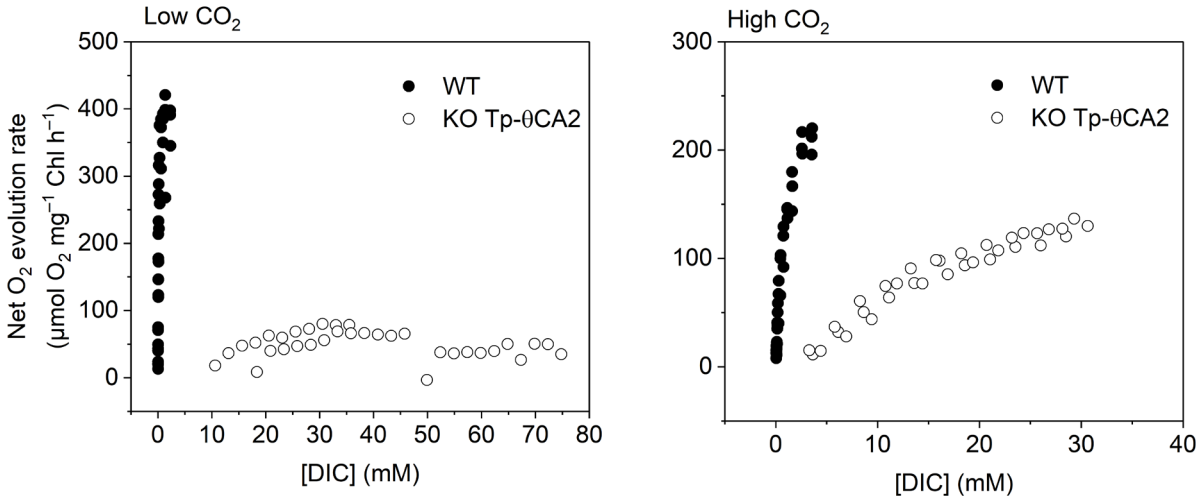

Figure 1 Kinetics for determining photosynthetic parameters in the KO Tp-θCA2 mutant compared to WT. Plots of photosynthetic O_2 evolution rates at different DIC concentrations at pH 8.2 and 20 ^{O}C in WT (Black circle) and double KO Tp-θCA2 (white circle) grown in HC and LC.

These results indicate that Tp-θCA2 is essential for *T. pseudonana* under both HC and LC conditions, suggesting a critical role for Tp-θCA2 in the CCM of *T. pseudonana*. Growth measurements also confirm the importance of Tp-θCA2 on HC and LC conditions, which can be seen in the slow growth rate of mutants that lack Tp-θCA2 compared to WT (Figure 4).

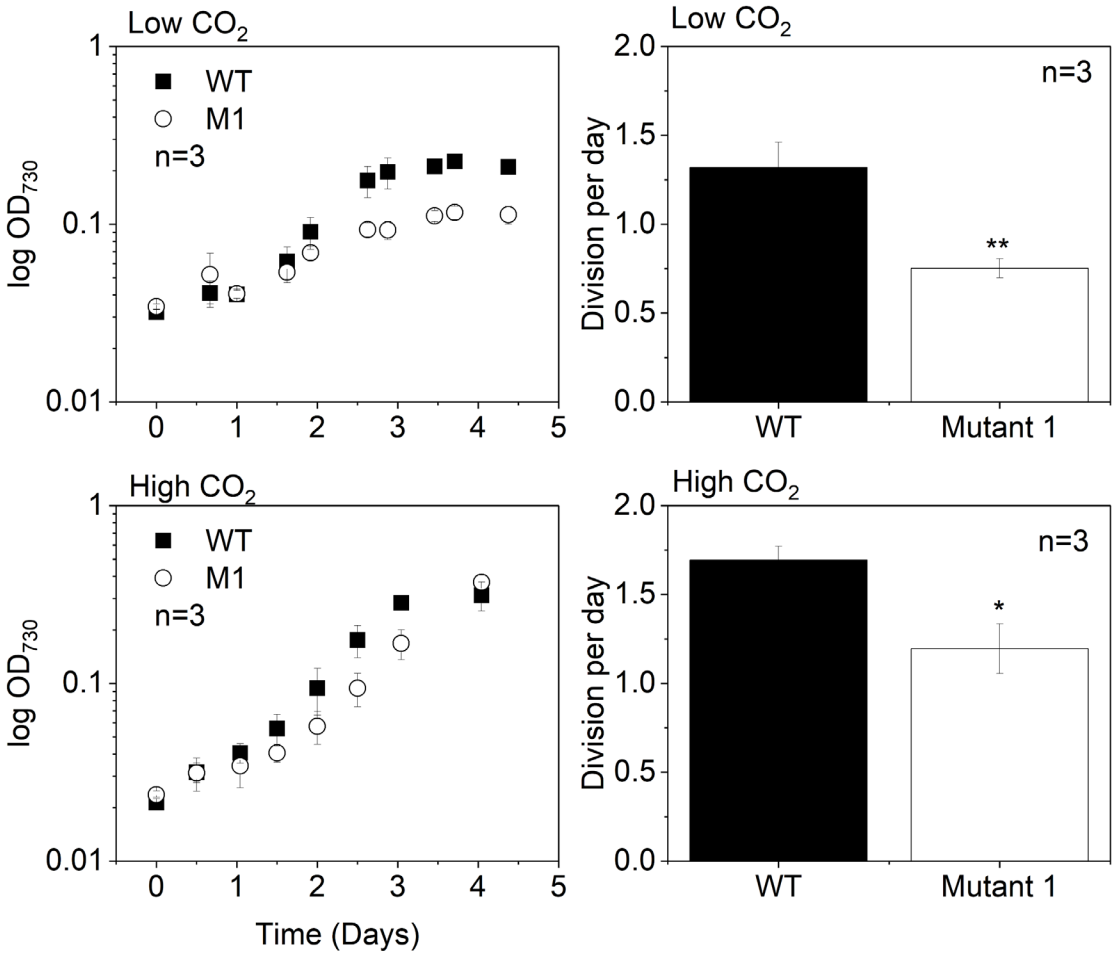

Figure 4 Growth analysis of WT and KO Tp-θCA2 under HC and LC condition. Error bars indicated three biological replications. Significant differences compared to the wild-type were determined using Student's *t*-test: **P*<0.05

Deadline : Within two months after finishing the research period.
Sabbatical leave with grant: Submit this report to President with confirmation by the dean of school you belong to.
※Postdoctoral fellow is required to submit this report with confirmation by the dean of graduate school before the end of employment period.
Where to submit : Organization for Research and Development and Outreach (NUC)
◆We put this report on the web of KGU. If there is any problem about it because of difficulties on your research, please let us know.