Kwansei Gakuin University
Report of Research Outcome

2024/03/16

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	2023/04/01 ～ 2024/03/31 （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 are microalgae responsible for 20% of primary production on Earth. In seawater, dissolved inorganic carbon (DIC) is mostly present as HCO_3^- and CO_2 concentrations are below 25 µM, which is insufficient for the K_m [CO_2] of RubisCO. Our previous studies showed that plasma membrane solute carriers (SLCs), PtSLC4-1, PtSLC4-2, and PtSLC4-4, can transport bicarbonate from the environment into the cytoplasm of the marine diatom, *Phaeodactylum tricornutum*. However, bicarbonate transport from the cytoplasm to the chloroplast is poorly understood. Previous results confirmed the localization of the bicarbonate transporters, PtSLC4-6 and PtSLC4-7, by C-terminal tagging with EGFP in the chloroplast membrane. Furthermore, transformants co-expressing PtSLC4-2:EGFP and PtSLC4-7:EGFP, PtSLC4-2/PtSLC4-7 transformants, showed effects on photosynthetic parameters and DIC uptake rate under high CO2 conditions (Nakajima, unpublished data).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. Sequential knockout of PtSLC4-7 followed by knockout of PtSLC4-6 was performed. For the first run, PtSLC4-7 knockout produced monoclonal mutants with -13 bp and -19 bp mutations for each allele (Figure 1A).

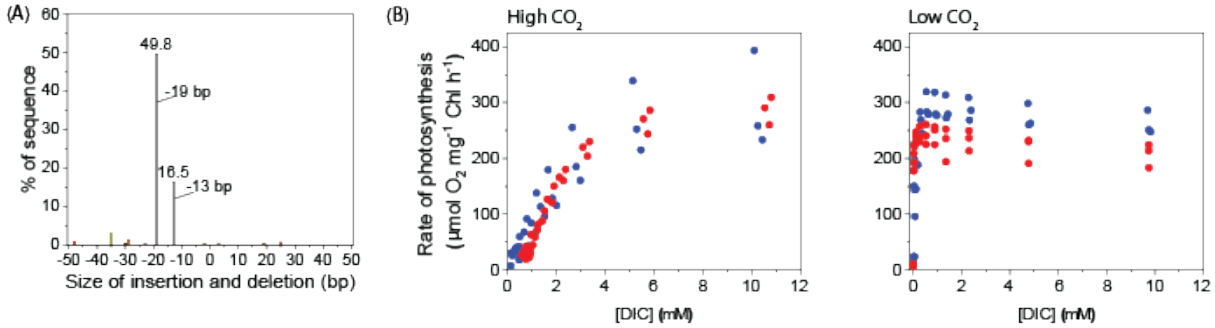

Figure 1. Knockout of PtSLC4-7. (A) Tide analysis (http://shinyapps.datacurators.nl/tide/) of the monoclonal KO mutant PtSLC4-7. (B) Kinetics for determining photosynthetic parameters in the KO PtSLC4-7 mutant compared to WT. Plots of photosynthetic O2 evolution rates at different DIC concentrations at pH 8.2 and 20 OC in WT (blue) and PtSLC4-7 KO (red) grown in HC and LC.

Measurements of photosynthetic parameters were carried out on PtSLC4-7 KO cells grown in high CO_2 (HC, 1% CO_2) and low CO_2 (LC, 0.04% CO_2) and compared with WT. The results showed that there was no significant difference between WT and KO mutant in HC. While at LC, there is a tendency to decrease photosynthetic capacity. However, it is not significantly different from WT (Figure 1B). No significant difference may be due to the complementary function of PtSLC4-6, which shows the same location as PtSLC4-7 based on unpublished data (Nakajima, unpublished data). Further experiments with PtSLC4-6 knockout in PtSLC4-7 mutants were carried out by sending vectors with different selection markers with Cas9 and sgRNA targeting PtSLC4-6. As a result, a PtSLC4-6 mutant with a 10 bp deletion was obtained. The photosynthetic parameters of the double KO PtSLC4-6 and PtSLC4-7 were measured under HC and LC conditions. The results showed that there was a decrease in photosynthetic capacity and an increase in K_m of the PtSLC4-6/PtSLC4-7 mutant compared to WT (Figure 2). These results indicated that PtSLC4-6 and PtSLC4-7 play an important role in the transport of DIC from the cytoplasm to the center of the chloroplast for the CO_2 fixation process. However, further studies still need to be done.

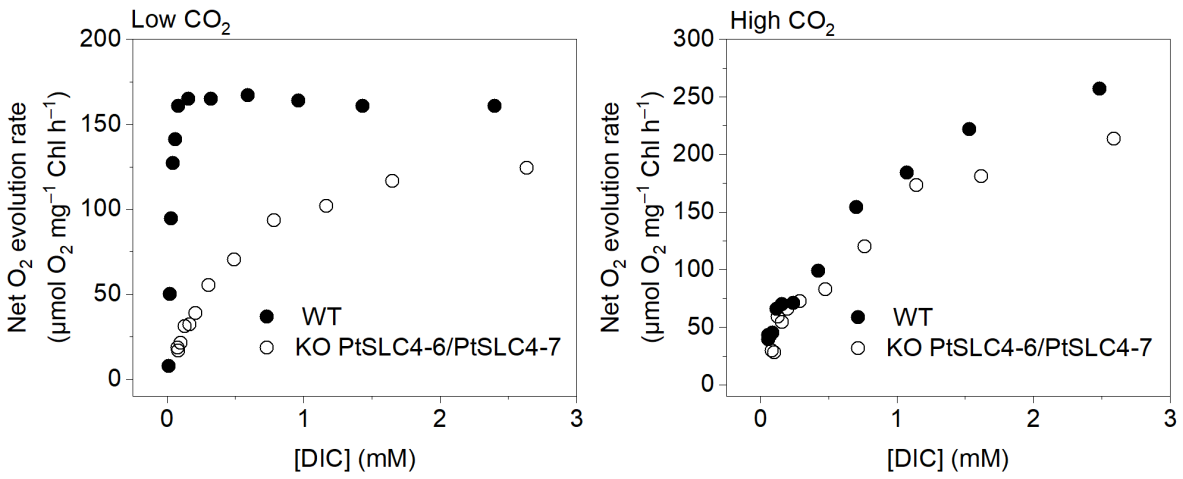

Figure 2 Kinetics for determining photosynthetic parameters in the double KO PtSLC4-6/PtSLC4-7 mutant compared to WT. Plots of photosynthetic O_2 evolution rates at different DIC concentrations at pH 8.2 and 20 ^{O}C in WT (Black circle) and double KO PtSLC4-6/PtSLC4-7 (white circle) grown in HC and LC.

Carbonic anhydrase (CA) is an essential factor in the CO_2 concentrating mechanism of many aquatic photoautotrophs that 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. The 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*. The C-terminal GFP fusion proteins of Tp-θCA1, Tp-θCA2, and Tp-θCA3 were all found in the chloroplast. Tp-θCA2 was 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 TpθCA2: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 pattern is likely caused by thylakoids that are penetrating into the pyrenoid (PPT). This study also postulated that Tp-θCA2 plays a crucial role in CCM, but attempts to eliminate this protein were unsuccessful using Cas9 nickase (D10A) (Nawaly et al., 2023). To overcome this, we used normal Cas9 to try to knock out Tp-θCA2 using dual sgRNA. As a result, we successfully performed genome editing at the *Tp-θCA2* locus, which resulted in a 41 bp deletion. Bioinformatic analysis showed that due to the 41 bp deletion, an early stop codon appears, indicating that Tp-θCA2 is not functional. Next, the photosynthesis parameters of the Tp-θCA2 knockout under LC and HC conditions were measured compared to WT. The results showed that there was a decrease in photosynthetic capacity and an increase in K_m in the Tp-θCA2 mutant compared to WT under HC conditions. Under LC conditions, the Tp-θCA2 mutant photosynthesis parameters were worse than under HC conditions (Figure 3). These results indicate that Tp-θCA2 is essential for *T. pseudonana* under HC and LC conditions. It also suggests that Tp-θCA2 is essential for the CCM of *T. pseudonana*. Further studies are still needed.

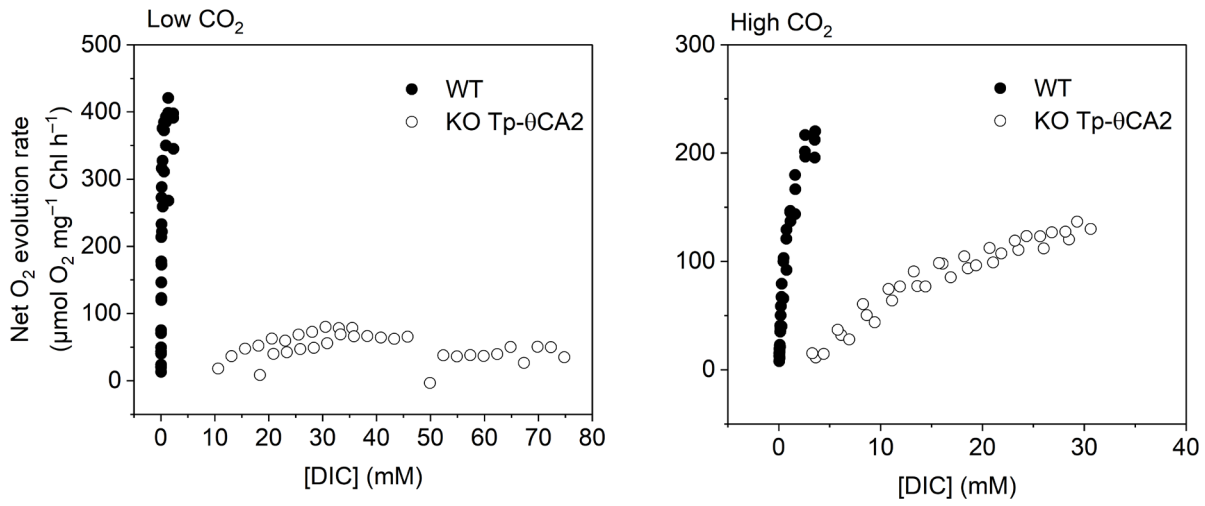

Figure 3 Kinetics for determining photosynthetic parameters in the KO Tp-θCA2 mutant compared to WT. Plots of photosynthetic O_2 evolution rates at different DIC concentrations at pH 8.2 and 20 ^{O}C in WT (Black circle) and double KO PtSLC4-6/PtSLC4-7 (white circle) grown in HC and LC.

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.