Molecular Biotechnology and physiology of *Chlamydomonas reinhardtii*

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Abstract

Plants convert carbon dioxide and water into macromolecular organics such as oxygen, carbohydrates, or lipids by photosynthesis, which can be used as low-pollution biomass energy, which can avoid increasing the greenhouse effect. Algae are suitable for the fixation of greenhouse gasses such as CO2, as well as the production of biodiesel and compounds of high economic value because of their small size, rapid growth rate, high lipid content, and ease of cultivation. This experiment aims to learn how to cultivate *Chlamydomonas reinhardtii* as a model organism, using both broth and solid nutrient medium, and observe *C. reinhardtii* wild-type and mutants of RBO1 221831 (R1-2) and RBO2 048908 (R2-1) for differences in appearance, cell counts, growth density, the production efficiency and accumulation of chlorophyll, starch, carbohydrate, and lipid under physiological nitrogen deficiency conditions.

Referring to an article published by **Alexander Anderson (2014, February) from Hughes Hall University of Cambridge, UK**, we are planning to transform pDBle_RBOL plasmids into *C. reinhardtii* to research the cellular physiological mechanism, in order to verify the association of RBOL1 and RBOL2 genes with NADPH Oxidases (NOX). We have established systems of gene cloning tools, techniques, and strategies, including cell culture, DNA/RNA extraction, PCR/qPCR, plasmid transformation, screening and verification of transformants. Plasmid pUC19 and DH5α competent cells were used to preliminarily verify the feasibility and efficiency of transformation, dilution ratio of colony formation, and the use of antibiotics, ampicillin and carbenicillin included.

Algae PCR for the cassette in mutants

- Extract DNA from RBO2 048908 (R2-2) with TE buffer, and then boil the solution at 100°C for 10 minutes, cool at 4°C for 1 minute, centrifuging after vortex samples to get DNA template.
- PCR verify the presence of transforming cassette
  use primer UBC for colony PCR
  N354 . N355 for cassette from CIB1 in R2-2
  N364 for RBO2 048908 5'end
  N365 for RBO2 048908 3'end

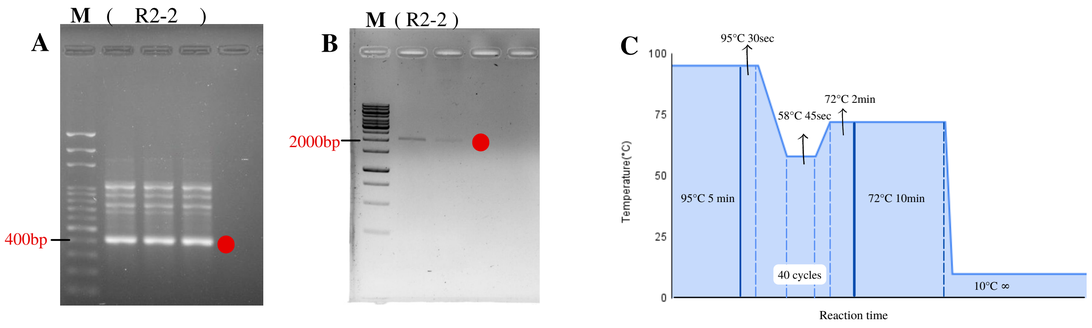

Figure 1. Results of PCR products
(A) R2-2 sample (402 bp) used UBC primer.
(B) R2-2 sample (2100 bp) used N355.N365 primer.
(C) PCR run conditions used N355.N365 primer for the R2-2 sample.

Gene cloning of CrRBOL genes in *C. reinhardtii*

- Use plasmid pUC19 and DH5α competent cells to preliminarily verify the feasibility and efficiency of transformation, dilution ratio of colony formation, and the use of appropriate antibiotics, ampicillin and carbenicillin included.
- Transformation of the plasmid pUC19 and pDBle_RBOL1_GFP from **Alexander Anderson (2014)** into E.coli DH5α competent cell by heat shock in a water bath for 30 seconds at 45 °C. The culture was spread on LB plates plus ampicillin (100 mg/l), then incubated inverted at 37 °C overnight.
- Pick up colonies of transformant by plasmid extraction to check if it has the correct DNA plasmid, then used EcoRI and run electrophoresis to determine its size.
- Assumed the plasmid clones are expressed, bacterial freezing conservation, and isolation and purification of plasmid DNA(pDBle_RBOL1_GFP) will be the next step.

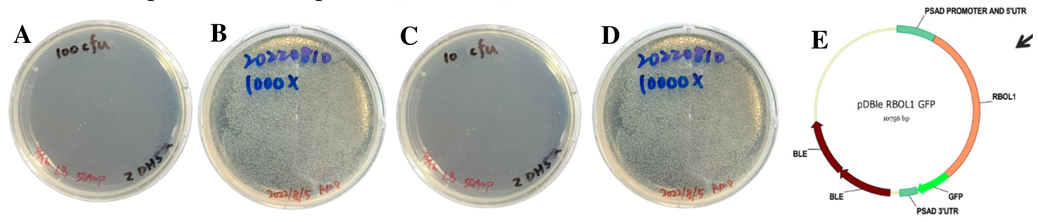

Figure 2. Transformation of pUC19 and DH5α
(A) 10μl DH5α without any ampicillin
(B) 10μl DH5α plus ampicillin (50 mg/l) get 5000 cfu
(C) 2μl DH5α without any ampicillin
(D) 2μl DH5α plus ampicillin (50 mg/l) get 1000 cfu
(E) pDBle_RBOL1_GFP plasmid including BLE, zeocin resistance gene under HSP70A_RBCS2 promoter from **Alexander Anderson (2014)**

Physiological mechanism of nitrogen deficiency in *Chlamydomonas reinhardtii*

Cultivation of *Chlamydomonas reinhardtii*

- Subculture : Measure the optical density (OD750nm) to know the subculture volume for Tris-Acetate-Phosphate medium (TAP), and determine the expected growth days according to the growth curve.
- Conserve microalgae : Streak-culture on TAP agar plates is used for microalgae .

Analyze of nitrogen deficiency experiment

- **Bright-field microscopy** : Observate the appearance size of wild-type and mutants preliminary.
- **Hemocytometer** : Use ''Analyze particles'' of ***ImageJ software (NIH, USA)*** to count cell number and density.

  Instructions of ImageJ : To create a monochrome photograph, first, convert the image with a color deviation into an 8-bit grayscale image, then remove the background and select how many cells to count. Upon filling the holes in the nucleus and removing the overlapped parts, the cell numbers can be automatically calculated.

- **Iodine-starch test** : Use Lugol's Liquor with 0.4% of Iodine for dyeing cells in the dark for 30 min.
- **Enzyme activity test** : Measure protein concentration and Enzyme activity on the cell membrane, and four concentrations of bovine serum albumin were assayed as the standard curve compared with samples.
- **RNA extraction - DNase treatment - cDNA synthesis - qPCR- sequencing to understand the sequence expressed as protein.**

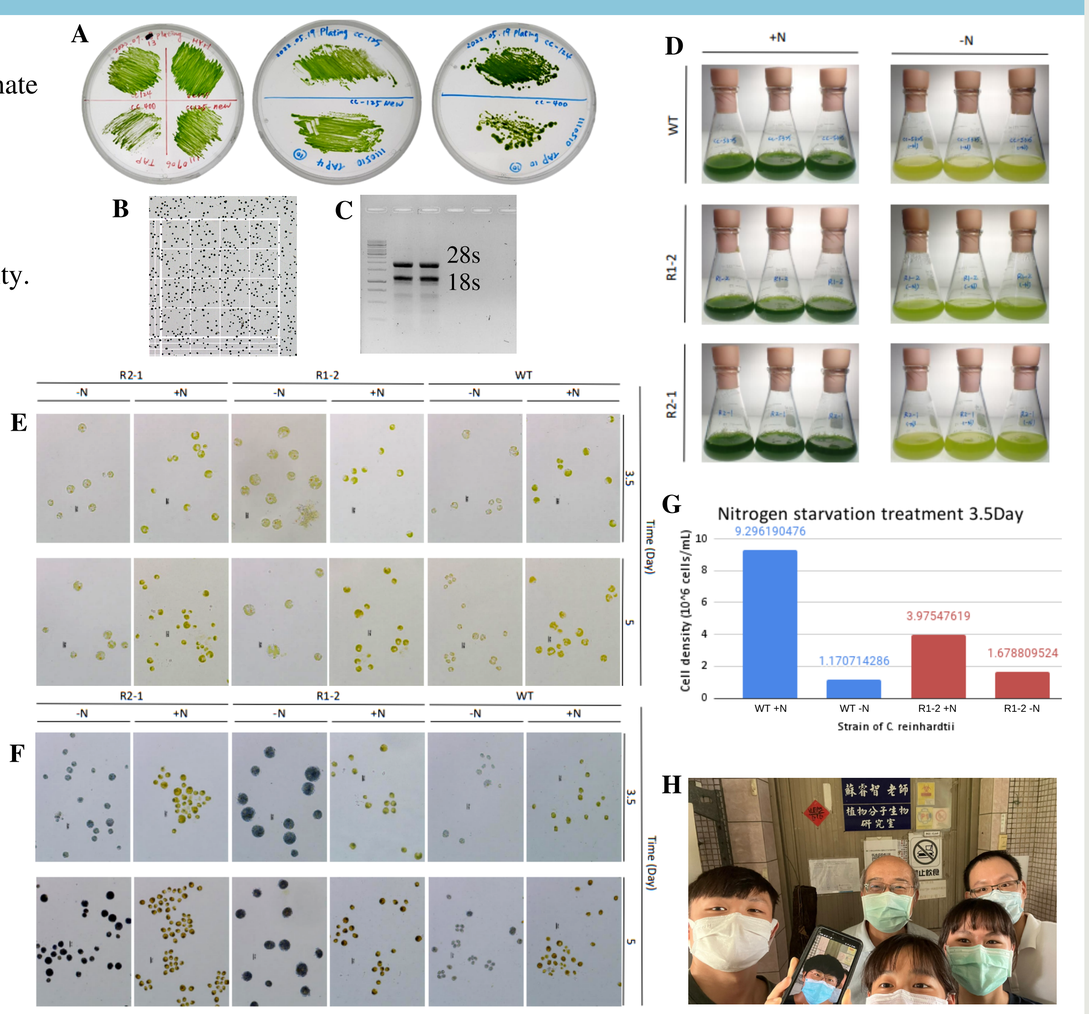

Figure 3. Results of nitrogen deficiency experiment
(A) Streak culturing of strain cc124, cc125, cc125-new, cc400 on TAP agar plates without any antibiotics.
(B) C. reinhardtii R1-2 cells of hemocytometer under a microscope.
(C) Strain R1-2 RNA extraction, 28S:18S = 1:1, RNA concentration : 1749.2μg/μl , A260/230=1.95 , A260/280=2.21
(D) Appearance comparison between WT, R1-2, and R2-1 in nitrogen deficiency test.
Appearance, cell counts, and growth density comparison between WT, R1-2, and R2-1 in nitrogen deficiency test of Bright-field(E) and Iodine–starch test(F). (Fig.D.E.F by Yi-Lin Chien(2022))
(G) Cell density (10^6 cell/mL) comparison between WT and R1-2 in nitrogen deficiency test 3.5 day.
(H) Learning gene cloning in professor Ruey-Chih Su's lab at Fu Jen Catholic University.

Conclusion

- By testing the process of Heat shock transformation, the 1/8 success rate of broth culture, and the spread plate method used with serial dilution, we can confirm the functions of the competent cells and ampicillin. Using the colonies' growth conditions of pUC19 and DH5α transformant, it is expected that if we want to get 100 CFU, we need to dilute the successfully transformed cells 50 times before coating the LB plate with ampicillin (100 mg/l).
- Under nitrogen deficiency conditions(-N), cell density decreased in all samples. WT cells of Chlamydomonas reinhardtii shrunk, but mutants of R1-2 and R2-1 both swelled, and the volume of R1-2 was 3 times that of nitrogenous conditions(+N) on the same day. According to speculation, it is related to the Reactive Oxygen Species (ROS) that promotes rapid cell division produced by NADPH oxidases.
- The mutant strain can accumulate starch in the absence of nitrogen. A potential explanation is that its NADPH oxidase is inhibited, so the TOR stops working and autophagy is activated. Because of this, the chloroplast will not decompose temporarily, resulting in a high carbon fixation capacity, allowing the cells to produce more carbohydrates and accumulate starch.