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Genetic Engineering of Nitrate Assimilation in Chlamydomonas reinhardtii to Accelerate Growth for Algal Wastewater Treatment

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Abstract. Algae-based wastewater treatment offers a sustainable alternative to conventional bacterial methods but is hindered by slow treatment times and difficulties in harvesting microscopic cells. Although photogranules, the clusters formed from the co-cultivation of the green alga Chlamydopodium sp. and the cyanobacterium Leptolyngbya PCC-6306, solve the harvesting issue, their structural stability depends on maintaining a precise 30:1 species ratio. This combination is inherently unstable due to the significantly faster growth rate of the cyanobacterium. The other bottleneck is the slow growth rate of the nutrient-absorbing green algae, which prolongs treatment time far beyond the standard 6-8 hours used in existing infrastructure. This research aims to overcome these two limitations by genetically enhancing nitrogen metabolism in a model green alga, Chlamydomonas reinhardtii (C.reinhardtii). To accelerate nitrogen uptake and conversion into biomass, thereby aligning algal growth rates with practical treatment timelines and stabilizing photogranule combinations, this experiment overexpresses key genes involved in nitrate assimilation: Nitrate Transporter (NRT1), Nitrate Reductase (NR), and Nitrite Reductase (NiR). We detailed the design of transformation plasmids and extensive efforts to isolate the target genes, and then updated the plan due to discoveries of the characteristics of the high-GC-content genome of C. reinhardtii. Successful target gene extraction proved challenging, leading to a recommendation for artificial gene synthesis in future work.

Keywords: Algal Wastewater Treatment, Genetic Engineering, Chlamydomonas reinhardtii, Nitrogen Assimilation

1. Introduction

1.1. Current previous wastewater treatment method

Water is one of the most important resources that people use in everyday life. Wastewater treatment plays an important role in supporting urban life. Current technologies are sufficient to provide fundamental functions, but they often contain drawbacks that need other solutions. One of the crucial processes in wastewater treatment is the uptake of nitrogen and phosphorus. Current wastewater treatment often uses bacteria as a tool to dissolve excessive nitrogen and phosphorus in

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the municipal wastewater, preventing eutrophication after it is released as effluents [1]. However, the current process emits large amounts of carbon dioxide and consumes large amounts of oxygen as bacteria process through cellular respiration. Bacteria also create lots of sludge after treatment. The sludge often gets buried or burned, but it all creates secondary pollution to the air and land [2].

1.2. Current algae treatment technology

As sustainable development has become a goal to reach over the past years, many companies have started to explore the wastewater treatment function of many other organisms, including algae, which are known to have ecosystem services of water treatment in the wetland ecosystem. Through years of research, people have discovered algae's potential in wastewater treatment, such as Chlorella Vulgaris, which could remove more than eighty percent of total nitrogen and one hundred percent of total phosphorus within thirteen days [3,4]. These algae work well in the laboratory, but they face many application problems, such as harvesting issues where algae clusters are too small to condense, and algae are also growing too slowly, which would not reach real-world application levels. People have found solutions for the harvesting problem, in which we cultivate species of Chlamydopodium sp. and Leptolyngbya_PCC-6306 [5]. Two species bind together to form a bigger cluster named photogranules that can be easily condensed and harvested in a short time. Leptolyngbya_PCC-6306 is a linear-shaped cyanobacterium that is mainly used to support the large cluster structure, and Chlamydopodium sp. is a spherical green algae that is responsible for nitrogen and phosphorus uptake. However, this structure is not stable as it only remains when the Chlamydopodium sp. to Leptolyngbya_PCC-6306 ratio is 30 to 1. Leptolyngbya_PCC-6306 grows at a much faster rate than cyanobacteria, which makes the cluster shape unstable.

1.3. Current problem

The current problem for algae wastewater treatment is that the natural growth rate of green algae cannot be accommodated by the current wastewater treatment process. The current wastewater treatment process sets six to eight hours of treatment time in the biological treatment section, where bacteria are used [6,7]. However, algae need more than three days to treat the water to a releasable standard, which is much more than the time needed before. Also, the low growth rate of green algae poses a challenge to the previous study to maintain the 30:1 ratio between green algae and cyanobacteria [5].

1.4. Research aim

Nitrogen systems were believed to be the most crucial nutrient for algae growth [8]. This research aims to increase Chlamydomonas reinhardtii (C. reinhardtii)'s growth rate by overexpressing genes of NRT1, NR, and NiR, which stimulate nitrogen consumption in algae and increase their transformation from NO_3 to a usable nitrogen source [9]. With abundant nitrogen sources in the wastewater body, overexpressing Nitrogen Reductase allows algae to have more usable nutrients, supporting larger nitrogen requirements as they grow faster. Hypothesizing that the overexpression of NRT1, NR, and NiR can increase the nitrogen uptake rate and the growth rate of algae, plans were made to overexpress these genes by constructing a plasmid as a vector to transform into the organism, and a comparison test is conducted comparing the engineered organism to the wild type to examine this hypothesis. Wild type will be set in the exact condition, with the same temperature,

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same nutrients, and the same light as the engineered ones, and their growth rate will be calculated based on the daily concentration measured using a spectrophotometer.

2. Material and methods

2.1. Algae strain

In the cyanobacteria and green algae combination, green algae have a lower growth rate, which makes the cluster unstable. Since Chlamydopodium sp. was less utilized in previous research and has limited availability from the market, C. reinhardtii demonstrated in Figure 1, which is a strain highly similar to Chlamydopodium sp. and is largely studied for biofuel production, was used.

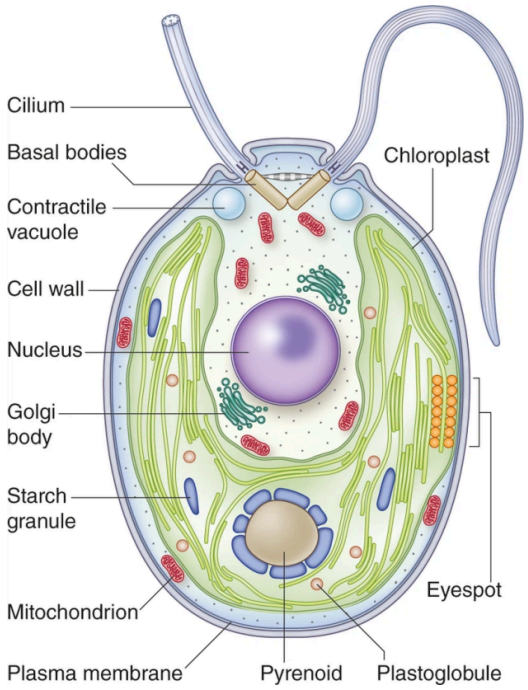

Figure 1. Cell diagram of C. reinhardtii (figure taken from Ref [10])

2.2. Gene extraction

C. reinhardtii has a cell wall, so grinding inside liquid nitrogen is needed to break the cell wall before extracting the genome using a DNA extraction kit.

The genome for C. reinhardtii has lots of exons and introns, which makes the whole genome long. To ensure the PCR process, cDNA is used as the template for PCR. RNA is extracted from C. reinhardtii, and the concentration is measured using a spectrophotometer to ensure the quality of the RNA. The ratio of A260 to A280 should be around 2.0. RNA is then reverse transcribed to cDNA, where only introns exist.

The genome of C. reinhardtii has a high GC content. So, a 5-minute 62 °C water bath is added after the template and primer are added to the tube. The temperature in reverse transcription is adjusted from a 42 °C water bath for 30 minutes to a 50 °C water bath for 60 minutes.

DOI: 10.54254/2755-2721/2026.KA28543

2.3. Plasmid design

C. reinhardtii normally uses Hsp70 and RbcS2 as a promoter and Zeocin to select. pChlamy/Cre and pPTGE34 was used to construct a plasmid with Hsp70 and Rbc S2 promoter and Zeocin

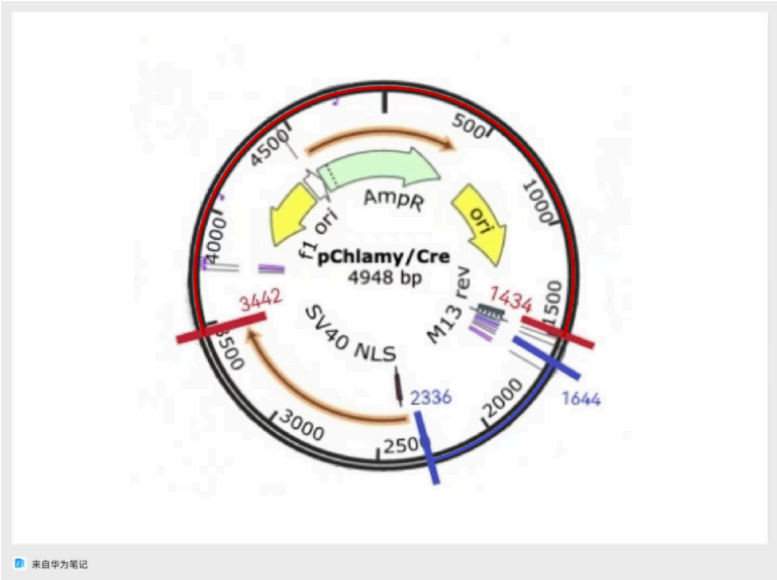

Figure 2. This figure is referenced from the Addgene plasmid map of pChlamy/Cre. The label on the figure has demonstrated the parts that are used later in the new plasmid construction

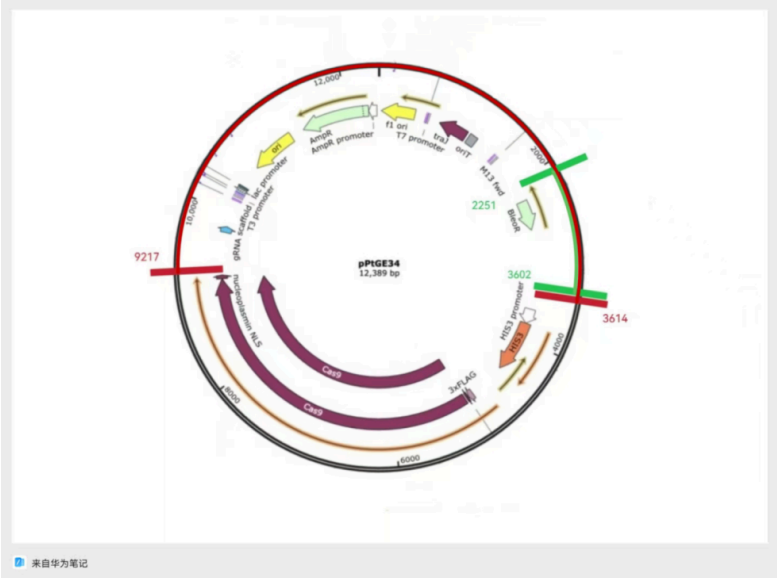

Figure 3. This figure is referenced from the Addgene plasmid map of pPtGE34. The label on the figure has demonstrated the parts that are used later in the new plasmid constructions

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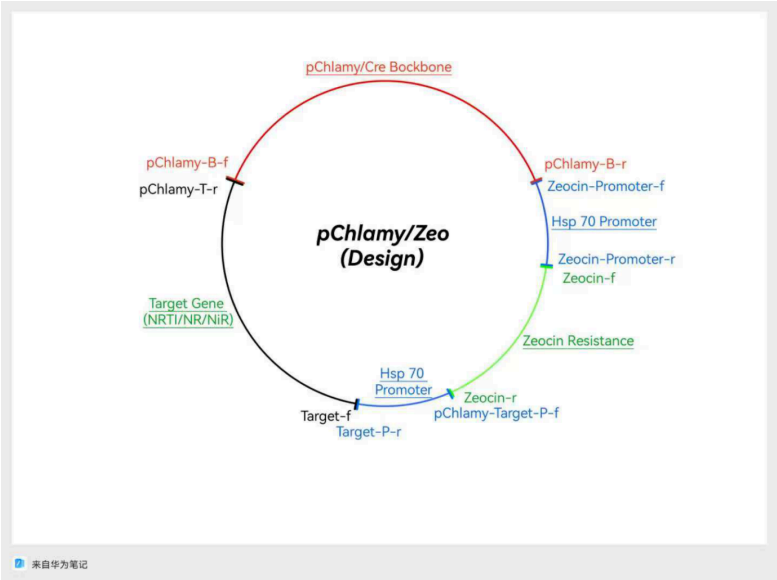

Figure 4: A design for plasmid based on backbone from pChlamy/Cre, and Zeocin Resistance from pPtGE34

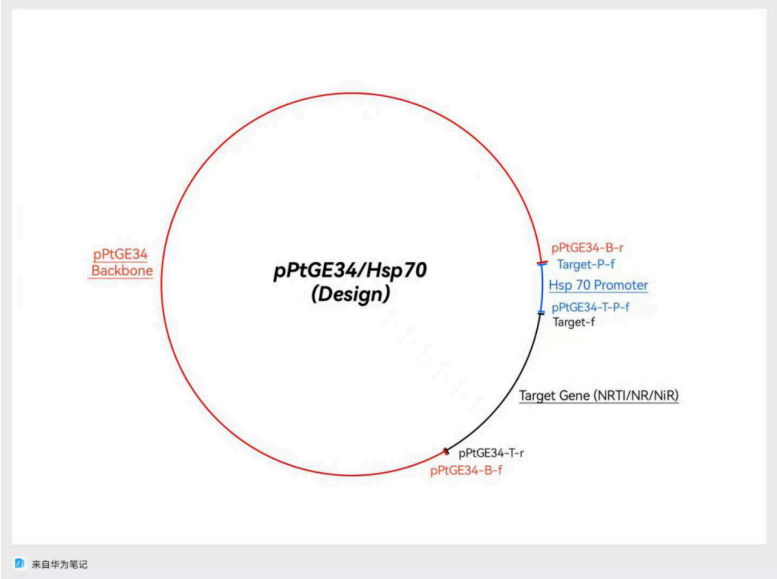

Figure 5. A design for plasmid based on backbone from pPTGE34, and Hsp 70 Promoter from pChlamy/Cre

In case of unexpected challenges, there were two designs that were based on pChlamy/Cre and pPtGE34 backbones, respectively. For the first design shown in Figure 4, the origin and Hsp 70 promoter come from the pChlamy backbone, and the range included in this fragment is demonstrated with red marks and blue marks in Figure 2, respectively,. The zeocin resistance gene is from the pPtGE34 plasmid fragment shown in green in Figure 3. The second design is based on the origin provided by the pPtGE34 backbone fragment shown with a red mark in Figure 3, and uses the same Hsp 70 promoter as the first design. The designs are shown in the following figures (Fig.4 & Fig.5)

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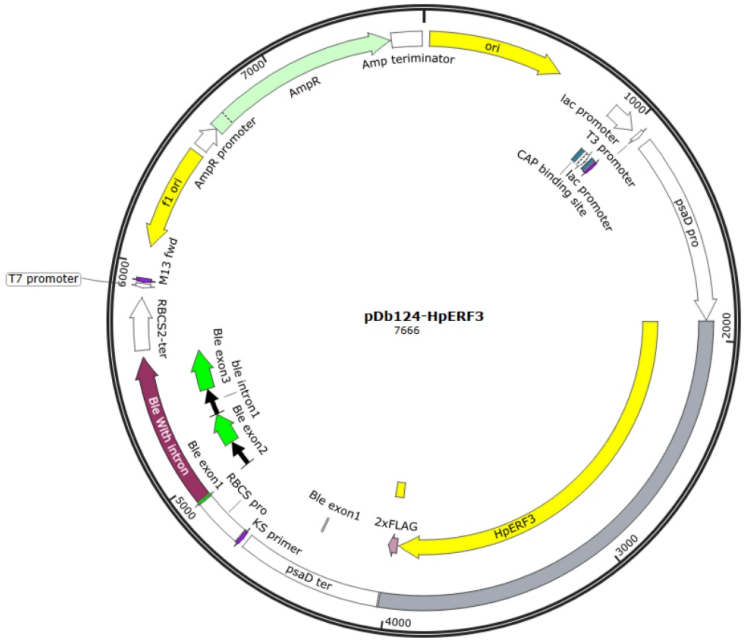

Figure 6. This figure demonstrates the map of plasmid pDb124-HpERF3 [11]

In Figure 6, the plasmid is designed with NdeI and Spe I restriction enzyme cutting site around the target gene intersection region. In this experimental design, target gene is extracted from the genome using primer with overhang containing restriction enzyme site.

2.4. Primer design

To get fragments as designed, we need to design a primer, and with so many fragments to connect, Gibson assembly will be the best choice for this purpose. So, primers were designed with an overhang that ensures Gibson assembly.

The template used is cDNA, which means it only exists in exons. The primers were designed within 20 base pairs after the start codon, and 20 base pairs before the stop codon.

Table 1. Primers with overhang designed for Gibson assembly

Name	Sequence (5' to 3')	Length	%GC	Tm
pChlamy-B-f-NRT1	CTGCCTGCGCCCTTCAGGTAGCTCTGCAGCGGCCGCCATAT	41	65.8	76.9°C
pChlamy-B-f-NR	CAAGGACCGCCAGATCCAGTTCTAGCTCTGCAGCGGCCGCCATAT	45	60.0	75°C
pChlamy-B-f-NiR	GCCACCGCCACCGCCTAACTCTGCAGCGGCCGCCATAT	38	68.4	77.3°C
pChlamy-B-r	GAAGATCCACTAGTTCTAGAGCGGCGTCGGGAAACCTGTCGTGCC	45	57.8	72.7°C
Zeocin-Promoter-f	GGCACGACAGGTTTCCCGACGCCGCTCTAGAACTAGTGGATCTTC	45	57.8	72.7°C
Zeocin-Promoter-r	GCACTGGTCAACTTGGCCATGGGTCCTCGAATCTCCTGCAAATG	45	53.3	71.6°C
Zeocin-f	CATTTGCAGGAGATTCGAGGTACCCATGGCCAAGTTGACCAGTGC	45	53.3	71.6°C

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Table 1. (continued)

Zeocin-r	GAAGATCCACTAGTTCTAGAGCGGCCGCCTCGTATCTTTTAATGATGG	48	47.9	68.9℃
pChlamy-Target-P-f	CCATCATTAAAAGATACGAGGCGGCCGCTCTAGAACTAGTGGATCTTC	48	47.9	68.9℃
Target-P-r-NRT1	CCGTCTCGCCGTCTTGACATGGGTCCTCGAATCTCCTGCAAATG	45	55.6	72.1℃
Target-P-r-NR	GGCTGCTCGGCAACGGTCATGGGTCCTCGAATCTCCTGCAAATG	45	57.8	73.6℃
Target-P-r-NiR	AAGCACTGGCGCGACTGCATGGGTCCTCGAATCTCCTGCAAATG	45	55.6	73.6℃
Target-f-NRT1	CATTTGCAGGAGATTCGAGGTACCCATGTCAAGACGGCGAGACGG	45	55.6	72.1℃
Target-f-NR	CATTTGCAGGAGATTCGAGGTACCCATGACCGTTGCCGAGCAGCC	45	57.8	73.6℃
Target-f-NiR	CATTTGCAGGAGATTCGAGGTACCCATGCAGTCGCGCCAGTGCTT	45	55.6	73.6℃
pChlamy-T-r-NRT	ATATGGCGGCCGCTGCAGAGCTACCTGAAGGGCGCAGGCAG	41	65.8	76.9℃
pChlamy-T-r-NR	ATATGGCGGCCGCTGCAGAGCTAGAACTGGATCTGGCGGTCCTTG	45	60.0	75℃
pChlamy-T-r-NiR	ATATGGCGGCCGCTGCAGAGTTAGGCGGTGGCGGTGGC	38	68.4	77.3℃
pPtGE34-B-f-NRT1	CTGCCTGCGCCCTTCAGGTAGCGACTTTGGCTGGGACACTTTCA	44	59.1	74.7℃
pPtGE34-B-f-NR	CAAGGACCGCCAGATCCAGTTCTAGCGACTTTGGCTGGGACACTTTCA	48	54.2	73℃
pPtGE34-B-f-NiR	GCCACCGCCACCGCCTAACGACTTTGGCTGGGACACTTTCA	41	61.0	74.9℃
pPtGE34-B-r	GAAGATCCACTAGTTCTAGAGCGGCGTAACTTACACGCGCCTCGTATCTT	50	50.0	71℃
pPtGE34-T-P-f	AAGATACGAGGCGCGTGTAAGTTACGCCGCTCTAGAACTAGTGGATCTTC	50	50.0	71℃
pPtGE34-T-r-NRT1	TGAAAGTGTCCCAGCCAAAGTCGCTACCTGAAGGGCGCAGGCAG	44	59.1	74.7℃
pPtGE34-T-r-NR	TGAAAGTGTCCCAGCCAAAGTCGCTAGAACTGGATCTGGCGGTCCTTG	48	54.2	73℃
pPtGE34-T-r-NiR	TGAAAGTGTCCCAGCCAAAGTCGTTAGGCGGTGGCGGTGGC	41	61.0	74.9℃

In Table 1, the report has shown a high GC content and high annealing temperature for the primers. High annealing temperature can make gene targeting hard, especially when some of it is higher than the extension temperature in phase three of PCR, which is 72 degrees Celsius. To ensure accurate gene targeting from the C. reinhardtii genome, two alternative plans were designed. Alternative plan one is to have the higher quality primers in Table 2 that is further from the start codon to get the target gene roughly. Then, use the previous set of primers with overhang to enable Gibson assembly to connect the fragments. Alternative plan two is using plasmid three, which previously had restriction enzymes Nde I and Spe I cutting sites around the target gene intersection area. Table 3 shows the new primers designed with Nde I and Spe I cutting sites.

DOI: 10.54254/2755-2721/2026.KA28543

Table 2. High quality and low annealing temperature primers designed for target gene

Name	Sequence (5' to 3')	Length	%GC	Tm
NRT1-f-1	CAAGAATGTCAAGACGGCGAG	21	52.4	56.5℃
NRT1-r-1	GCTGCCGCCTCTTGCTTCCTC	21	66.7	63℃
NRT1-f-2	AGAATGTCAAGACGGCGAGACG	22	54.5	59.6℃
NRT1-r-2	CTGCCGCCTCTTGCTTCC	18	66.7	58.5℃
NR-f	CCAGTATGACCGTTGCCG	18	61.1	55.6℃
NR-r	CTGTGCCAGCCCTTATCC	18	61.1	54.8℃
NiR-f	AAATAACCCGAGGCTTGTG	19	47.4	52.7℃
NiR-r	TCGCTCCAAACACCAACG	18	55.6	55.7℃

Table 3. Primers designed with restriction enzyme site

Name	Sequence(5’ to 3’)	Length	%GC	Tm
NRT1-f-res	CATATGTCAAGACGGCGAGACGG	23	56.5	60.1℃
NRT1-r-res	ACTAGTCTGCCGCCTCTTGCTTCCTC	26	57.7	64.1℃
NiR-f-res	CATATGCAGTCGCGCCAGTGCTT	23	56.5	62.8℃
NiR-r-res	ACTAGTTCGCTCCAAACACCAA CGCT	26	50.0	63℃
NR-f-res	CATATGACCGTTGCCGAGCAGCC	23	60.9	62.9℃
NiR-r-res	ACTAGTCTGTGCCAGCCCTTATCCGG	26	57.7	63.7℃

2.5. Link method

Gibson assembly is used in the first two plasmid constructions. The first two plasmid designs with pxy/Cre and pPtGE34 as backbones involved the synthesis of many fragments, which made the common application of restriction enzymes challenging due to a design challenge that required checking if the enzyme uses only one targeted site. The short binding site of the restriction enzyme can make it unspecific, which increases the chance of a binding error. Gibson assembly is used in the design of the first two plasmids, since Gibson assembly ensures a binding of more than 20 base pairs between the overhangs of primers.

A restriction enzyme is used in the third plasmid construction. The plasmid backbone of pDb124-HpERF3 already involves Zeocin resistance and promoter for C. reinhardtii. There are existing Nde I and Spe I restriction enzyme cutting sites around the target gene intersection area. Restriction enzyme is applied in this case as it does not require as long an overhang as Gibson assembly did, ensuring primer quality.

2.6. Transformation

Electroporation is one of the most common transformation methods, which only requires common lab equipment. It is suitable for C. reinhardtii due to its ability to break through the thick cell wall.

DOI: 10.54254/2755-2721/2026.KA28543

2.7. Result testing

C. reinhardtii can survive in ampicillin even when the plasmid we designed does not exist in the cell. Zeocin was used to select cells with a plasmid. C. reinhardtii will be cultivated on a petri dish with SE as a nutrient and Zeocin as an antibiotic. Cells that have a plasmid inside will be able to survive on the plate. A single colony from the plate was picked and cultivated for expansion.

A comparison test is then set to test if overexpressing the gene will work. We cultivated the transformed C. reinhardtii with the natural one and in the same absolute environment, and recorded their OD680 to test their concentration change.

3. Data and analysis

3.1. Gene extraction

Table 4. RNA extract purity and concentration.

	A260	A280	A260/A280	Dilution Factor	Concentration (ng/μL)
RNA	0.703	0.383	1.857	15x	421.8

The RNA sample demonstrated in Table 4 is extracted without using liquid nitrogen, using only the RNA extraction kit. The absorption under wavelengths of 260nm and 280nm is measured using a spectrophotometer, and the concentration is calculated using the A260 value multiplied by 40, and multiplied by diluted times.

The cell wall of C. reinhardtii might decrease the efficiency of the RNA extraction, so this experiment was performed again with pre-treatment of grinding using mortar in liquid nitrogen, and the result is shown in Table 5. Comparing results shown in Table 4 and Table 5, the concentration of RNA, the concentration of sample extracted with pre-treatment appear to be higher.

Table 5. RNA extract's purity and concentration with pre-treatment

	A260	A280	A260/A280	Dilution Factor	Concentration (ng/μL)
RNA	0.795	0.428	1.857	15x	477

3.2. Plasmid extraction and cultivation

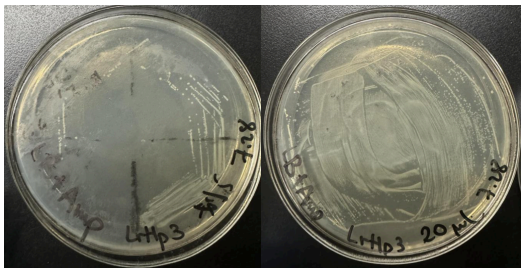

Figure 7. Cultivation of glycerol stock pDb124-HpERF3 on an LB petri-dish with ampicillin. a) demonstrates streak plating using 5ul of glycerol stock, and b) demonstrates spread plating using 20ul of glycerol stock

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In Figure 7, the cultivation on the petri dish showed that to get a clear single colony, this plasmid stored in glycerol stock is best cultivated using streak plating. The results of plasmid extraction are shown in Table 6.

Table 6. pDb124-HpERF3 single colony and multi-colony extracts' purity and concentration

	A260	A280	A260/A280	Dilution Factor	Concentration (ng/μL)
pDb124-HpERF3 (Single)	0.127	0.083	1.53	30x	190.5
pDb124-HpERF3 (5)	0.126	0.082	1.537	30x	189

3.3. PCR

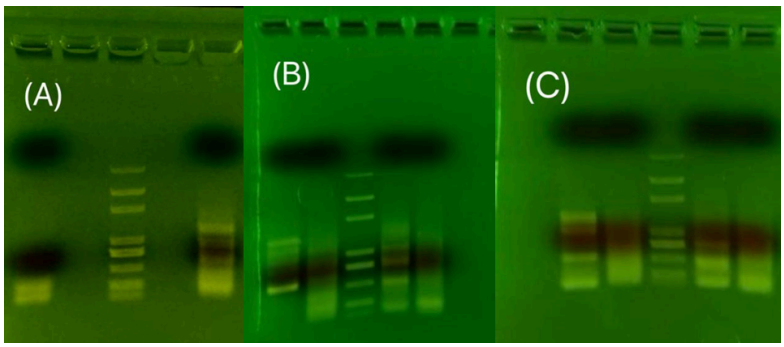

Figure 8. The three pictures show gel-electrophoresis results of the PCR results. (A) Well 1 is Zeocin; Well 3 is Marker; Well 5 is NRT1. This PCR is run with an annealing temperature of 60 ℃ for the first five cycles and 72 ℃ for the remaining 25 cycles. (B) Well 1 is Zeocin; Well 2 is NRT1; Well 3 is Marker; Well 4 is NR; Well 5 is NiR. This PCR sample is run with an annealing temperature of 55 ℃ for the first five cycles and 72 ℃ for the other 25 cycles. (C) Well 2 is Zeocin; Well 3 is NRT1; Well 4 is Marker; Well 5 is NR; Well 6 is NiR. This PCR sample is run with an annealing temperature of 50 ℃ for the first five cycles and 60 ℃ for the remaining 25 cycles

The variable that changed among the samples used in Figure 8 is the annealing temperature. None of them showed obvious signals, but we can see that the signals for Zeocin are getting better as the temperature decreases. Higher annealing temperature may increase specificity, and lower annealing temperature may increase production. Fig. 8a) and b) show no smear shape, so the annealing temperature is decreased to increase the production of the sample. However, no obvious improvements in DNA band quality was noted.

DOI: 10.54254/2755-2721/2026.KA28543

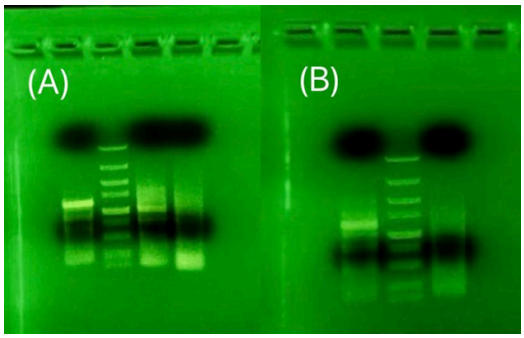

Figure 9. The two pictures show gel-electrophoresis results of the PCR results. The DNA template has increased to 2ul out of a 20ul system. (A) Well 2 is NRT1; Well 3 is Marker; Well 4 is NR; Well 5 is NiR. These PCR samples are run with an annealing temperature of 63℃ for the first five cycles and 72℃ for the remaining 25 cycles. (B) Well 2 is NR; Well 3 is Marker; Well 4 is NiR. These PCR samples are run with an annealing temperature of 67℃ for the first five cycles and 72℃ for the remaining 25 cycles

The variable that changed among the sample used in two gel-electrophoresis shown in Figure 9 is the annealing temperature and the amount of template. The annealing temperature had been increased to the lowest Tm of the primer part without overhang. The temperature was increased to increase specificity. There are obvious signals shown around 1300bp for NRT and NR. The length does not match the target gene length, but this discrepancy may be due to splicing that changes the expressed sequence. This sample was sent to the test sequence, but the result showed no similarity between the tested sample and the target gene.

Previous experiments were run at high annealing temperatures, as the high GC content and the overhang had significantly increased the Tm for primers. New sets of primers were designed with lower annealing temperatures and higher quality.

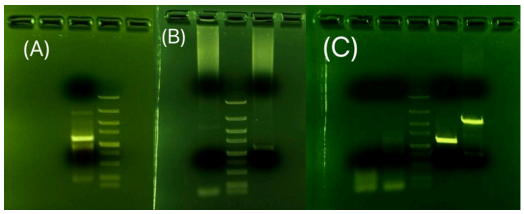

Figure 10. The three pictures show the gel-electrophoresis result of the PCR results. New primers are applied in the three experiments shown above. (A) Well 2 is NRT1; Well 3 is Marker. The template is cDNA. The PCR sample is run under an annealing temperature of 58℃ for all 30 cycles. 58℃ is the lowest Tm for the primers. (B) Well 2 is NRT1 using newly designed primer set 1; Well 3 is Marker; Well 4 is NRT1 using newly designed primer set 2. The template is a genome. The PCR sample is run under an annealing temperature of 55℃ for all 30 cycles. 55℃ is the lowest Tm among the four primers. (C) All wells are a sample of NRT1. Well 2 used a cDNA template transcribed using methods that neglect the high GC content; Well 3 used a cDNA template transcribed using method specially for high GC content sample; Well 4 is Marker; Well 5 use gel extraction product that is 1300 bp from previous experiment; Well 6 used gel extraction product that is 2300bp from the previous experiment. The PCR sample is run under an annealing temperature of 55℃ for all 30 cycles. 55℃ is the lowest Tm among the two primers

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The variables in this set of experiments include the annealing temperature and the template. The previous experiment with cDNA as a template did not give a correct DNA band. The experiment demonstrated in Fig.10(B) using the genome as a template has eliminated possible flaws in the template since it also did not give the band with the targeted length. The signal of two weak bands is amplified in the experiment demonstrated in Fig.10(C). The two strong bands were extracted and sent to the test sequence, but the result comes back with no similarity to the target gene.

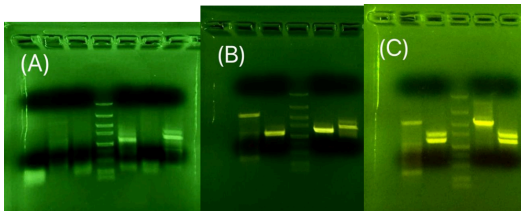

Figure 11. The three pictures show the gel-electrophoresis result of the PCR results. New primers are used for all genes. (A) Templates used are cDNA42, which gets transcribed using methods that neglect the high GC content, and cDNA50, which gets transcribed using methods specially for high GC content samples. Well 1 is NRT1 using cDNA 42; Well 2 is NRT1 using cDNA 50; Well 3 is NR using cDNA42; Well 4 is Marker; Well 5 is NR using cDNA50; Well 6 is NiR using cDNA42; Well 7 is NiR using cDNA50. (B) The templates used here are products from the gel extraction of the previous experiment, demonstrated in Fig.11(A); Well 2 is NRT1; Well 3 is NR; Well 4 is Marker; Well 5 is NiR from recycled band length of 1300bp; Well 6 is NiR from recycled band length of 1700bp. (C) The templates used here are products from the gel extraction of the previous experiment, demonstrated in Fig.11(A) and Fig.11(B). Well 2 is NRT1 from template recycled from Fig.11(A); Well 3 is NiR recycled from Fig.11(A); Well 4 is Marker; Well 5 is NRT1 from template recycled from Fig.11(B); Well 6 is NiR from template recycled from Fig.11(B)

These experiments have shown a strong DNA band with a length close to the target length, but none of them was the target gene after sequence testing. The annealing temperature was kept constant in these three experiments. Experiments shown in Fig.11b and Fig.11c aimed to amplify clear signals. However, the signals for NiR cannot be cleared; it always shows two bands.

The sequencing result shows that the sample is not the target gene, but it is not similar to the previous sequencing result either. Through blasting the primers, many other proteins' sequences have been found to show similarity, which shows the high similarity between these proteins. This led to the failure to obtain the targeted gene from the cDNA.

4. Future directions

4.1. Reflection

As previously planned, the experiments progressed well, with each small experiment feasible and doable for obtaining the whole genome and cDNA. However, the experiment stopped at a crucial step: obtaining the target gene from the cell body. The reason for not obtaining the target gene from cDNA is that it neglected the actual introns eliminated during the splicing process, where the matching code of the primer might not exist in the cDNA. However, even using the genome as a template, the target gene cannot be found. This is due to the high GC content in this organism, which causes high similarity in gene code for many proteins in this organism. Although the primers were designed to match the target gene's ends perfectly, the sequencing results indicate that the primers bind to gene sequences that match approximately eighty percent, suggesting the primer is not

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sufficiently specific to target the gene. The high similarity in the gene sequence made it challenging to obtain from the organism; for the new plans to overcome this problem, artificially synthesizing it should be the best choice. Having long primers to perform Gibson assembly will be problematic, since synthesizing the DNA artificially, using restriction enzyme sites NdeI and Spe I, will be more efficient.

4.2. Gene recombinance

C. reinhardtii is an eukaryotic cell that does not naturally have a plasmid in its cell. They could only temporarily express the gene in the plasmid, and this cannot be inherited. To prolong the ability to consume large amounts of nitrogen and achieve a fast growth rate, the code to overexpress the gene should be added to the genome. Thus, the trait could exist for a long time and be inherited. To achieve this purpose, we need to use the Transposon System, where the added gene is randomly inserted into C. reinhardtii's genome [12]. This method is better than other commonly seen methods, such as homologous recombination and viral infection. This is because homologous recombination has low efficiency in C. reinhardtii, as it needs to target a specific site in the genome. The viral infection efficiency is known due to the existence of a cell wall in C. reinhardtii, which might limit viral uptake.

The Transposon System uses the transposase enzyme to cut the target gene sequence and select the marker out of the plasmid through recognition of the transposon ends; then, it randomly inserts the gene into the cell's genome. To apply the transposon system, the plasmid should include the sequence to produce transposase, the target gene, and zeocin resistance gene flanked with recognizable transposon ends. These are designed in a single plasmid instead of separate plasmids, as it can decrease transform efficiency, which might prohibit two plasmids from existing in one cell at the same time.

4.3. Kill switch

To apply this engineered C. reinhardtii strain in real-life wastewater treatment, a kill switch must be designed to ensure safety [13]. As the purpose of this research, the growth rate of this engineered C. reinhardtii will be higher than it usually is, and it will consume more nitrogen. This is good in terms of wastewater treatment, but if it has been exposed to nature, this engineered species will be invasive. Invasive species may outcompete the native species in the area, threatening other species' survival. The kill switch will prohibit its growth when it is exposed to nature.

A kill switch is designed to limit the living conditions of an organism. In this case, for engineered C. reinhardtii to only live in a wastewater treatment plant, anthropogenic gadolinium might be a viable element to design as a kill switch [14]. Gadolinium is often contained in medical waste that does not exist in natural water bodies. Designed with gadolinium as a kill switch, the engineered organism can only live in the wastewater treatment plant with gadolinium present, preventing it from becoming an invasive species in nature.

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