Gene Editing of Saccharomyces Cerevisiae Using CRISPR

Chenyu Yang[1,*] and Yilin Li[2]
[1]*Queen Mary College, Nanchang University, the Medical College, Block C, No. 999, Xuefu Avenue, Honggutan New District, Nanchang, China*
[2]*Biomedical Science Department, Southern University of Science and Technology, No. 1088, Xueyuan Avenue, Nanshan District, Shenzhen, China*

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Abstract: This experiment aims to integrate the mCherry gene into saccharomyces cerevisiae (S.cerevisiae) genome and examine its expression in S.cerevisiae. This experiment is to detect whether the integration and mCherry gene expression are successful. By applying the CRISPR/Cas9 and PCR techniques, we have shown that the integration into S. cerevisiae is successful, and the mCherry gene is expressed perfectly. This experiment shows the feasibility of the CRISPR/Cas9 technique, which can be applied in clinical treatments or mass production of certain medical products.

1 INTRODUCTION

CRISPR/Cas9 is a mechanism of gene editing, applied to form guide RNA (gRNA) plasmids and integrate target gene into cells, in order to express the target gene (L.S.Q, 2013).

CRISPR/Cas9 is categorized into two types: Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR). NHEJ is a pathway that repairs double-strand break mutations by insertions or deletions, before knocking out the gene. HDR repairs the template to complete gene editing.

The technique has the potential to affect scientific research. It enables more precise gene editing, a higher probability of successful transformation and a purer transformation product. This technique supports experts to express certain DNA and obtain the target substances (Yan, 2022). CRISPR/Cas9 is a perfect tool to transform cells, making them produce certain substances, which can also be applied in medical treatments, mass production of certain medical products, and so on.

CRISPR/Cas9 employs the Cas9 Nickases from BJ5464-p414-Cas9 as a catalyst. The gRNA binds with and cuts the target sequence, which is the pre-designed target gene (Wu, 2014). In this experiment, the binding happens with the plasmids and transforms the S.cerevisiae. The advantage of CRISPR/Cas9 is its high efficiency, which increases its ability to modify the gene accurately and efficiently. Nucleic acid probe-based qPCR identifies the mutation site rapidly. It can be used for genotyping to the subsequent formation of a colony (Vasu, 2021). CRISPR/Cas9 is more accurate than the double enzyme digestion technique, and it provides blunt ends, which means the sequence cut by CRISPR/Cas9 will be less possible to adhere to other sequences. The sequence cut by the technique is more likely to be pure target fragments. The difficulty in purifying and detecting the aim proteins (the expression of the target gene) will decrease. However, as a pre-mature technique, CRISPR/Cas9 still needs to be optimized.

The experiment aims to use S.cerevisiae to express the fluorescent protein gene by the CRISPR/Cas9 technology. The exogenous expression box (YPet fluorescent protein) has already been structured. In this experiment, we have activated the S. cerevisiae BJ5464/ p414-TEF1p-Cas9-CYC1t culture and made the competent cells to gain S. cerevisiae host bacteria.

2 MATERIALS AND METHODS

2.1 Materials

S. cerevisiae BJ5464/ p414- TEF1p- Cas9- CYC1t, E.coli.

PCR centrifuge tubes, 1.5ml centrifuge tubes.

PCR Machine, electrophoresis apparatus.

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2.2 Methods

2.2.1 Design of gRNA Plasmids

Design the gRNA sequence to participate in the reaction with the plasmids in the experiment. Anneal the primer, bind the gRNA with the enzyme carrier (p426- pSNR-gRNA- tSUP4) of E. coli, let the transformed plasmids proliferate in the E. coli, and then extract the plasmids.

1) Guide sequences

LEU- sgRNA target sequence: TTTGTTGCCATCTGCGTCCT;

LEU- sgRNA- F: 5' - GATCTTTGTTGCCATCTGCGTCCTG- 3';

LEU- sgRNA- R: 5' - AAAACAGGACGCAGATGGCAACAAA- 3'.

2) Primer annealing

Suspend the forward and reverse oligo in 100uM HEPES (pH= 7.9) water.

3) Connection

Dilute the tenfold annealed double strands DNA (0.1μL)

T4 DNA Ligase (0.25μL)

Buffer (0.5 μL)

Carrier (0.5 μL)

Up to 5 μL

Reaction conditions: 16℃, overnight.

4) E. coli transformation

Remove the competent cells (100μL) out from a fridge at -80℃ and place them on ice for about 30 min. Add 10μL connection products and mix them, and place them on ice for about 30 min.

Heat the competent cells with the connection products in a water bath for 90 s, then place them on ice for 2 min immediately. Add the competent cells into 800μL LB medium without screening resistance (preheated to 37℃), cultivate at 37℃, at a 180-rpm table for about 1h.

Centrifuge the cells (at 300 rpm) for 3 min, discard 600μL supernatant, resuspend the rest of the thalli and transfer 200μL liquid smear to the ampicillin-resistance LB solid tablet. Cultivate at 37℃ for about 20-22h.

Collect the transformant and patch on the ampicillin- resistance LB solid tablet and recultivate at 37℃.

5) Colony PCR

Dissolve some thalli in 20μL ddH_2O, at 95℃ for 10 min. Use the supernatant as the template of PCR.

Table 1: Colony PCR.

Reagents	Dosages (μL)	Steps	Conditions
Hief	5	1	94℃, 5 min
F	0.5	2	94℃, 30 s
R	0.5	3	57℃, 30 s
Template	2	4	72℃, 30 s/kb
ddH_2O	2	5	Go to step2 for 33 times more
Up to	10	6	72℃, 10 min
		7	16℃, forever

Detect the PCR product by agarose gel, choose the suspected positive clone strain, inoculate to LB/ Amp+ fluid nutrient medium, at 37℃, and cultivate overnight. Extract the recombinant plasmids to sequence the gene.

2.2.2 Acquisition of the Host Bacteria

Activate the S. cerevisiae BJ5464/ p414-TEF1p-Cas9- CYC1t culture to make competent cells that will be used as host bacteria.

Select a single colony and place it into 5ml SD (without tryptophan) liquid culture medium, at 30℃, and cultivate overnight, until the bacteria solution OD increases to 3.0-5.0.

After the bacteria solution grows to the expected OD600, transfer a proper amount of solution into another 5ml SD (without tryptophan) liquid culture medium. Set the starting OD600 of the solution between 0.2 and 0.4, cultivate at 30℃, 200 rpm for about 4-6 hours. The OD600 should be kept between 0.8-1.0.

Transfer 1 mL solution to a 1.5 mL centrifuge tube, centrifuge for 3 min, at normal temperature, at 7000 rpm, and discard all the supernatant.

Wash away the residual medium, prepare 1 mL wash solution to suspend the bacteria, centrifuge for 3 min, at normal temperature, at 3000 rpm, and discard all the supernatant.

Prepare 200μL lithium cation solution to suspend the bacteria, which is the BJ5464- p414- Cas9 competent cells. Sub-package 25μL in each tube, then either start the transformation of the plasmid or store it in an ultra-low temperature refrigerator at -80℃.

2.2.3 Transformation of the S. cerevisiae

The donor DNA has been pre-designed. After the PCR proliferation, use SDS-PAGE to detect if the plasmids contain the donor sequences. Then purify the plasmids to get the donor DNA. Mix the plasmids transformed by gRNA and the donor DNA to transform the S. cerevisiae. Perform coated-plate culture and colony PCR, then obtain the transformed colony.

Donor Primers:

LEU-Donor- F:

TCATCTCCGATGAAGCCTCCGTTATCCCAGGTTCCTTGGGCATAGCTTCAAAATGTTTC

LEU-Donor-R:

AGTAAGCTACTATGAAAGACTTGTCTGGCAAAGAGGCCAAGGACGCAGATGGCAACAAA.

Table 2. PCR (Amplify the donor fragment)

Reagents	Dosages (μL)	Steps	Conditions
PrimeStar Max Premix(2x)	25	1	94℃, 2 min
F (20 μM)	0.5	2	98℃, 10 s
R (20 μM)	0.5	3	52℃, 30 s
Template	0.2	4	72℃, 2 min
ddH_2O	23.8	5	Go to step2 for 29 times more
Up to	50	6	72℃, 10 min
		7	16℃, forever

2.2.4 Acquisition and Detection of the Products

Cultivate the positive transformant in a tube with YPD culture medium for 12 h, transfer to a 50 mL conical flask to cultivate. Control the starting OD600 to be 0.05. Cultivate in 10 mL YPD, at 30℃, at 200 rpm, for 12 h. Place 200μL solution in a black, transparent 96-pore plate to perform the fluorescence test.

Excitation wavelength: 485nm.

Emission wavelength: 528nm.

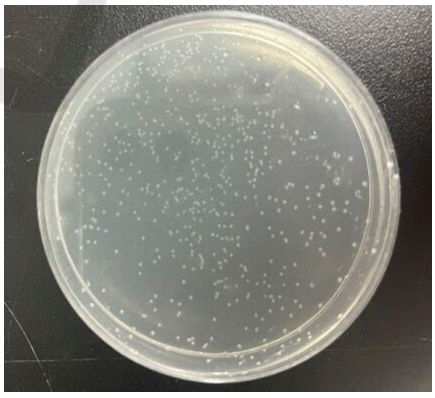

Figure 1: The plate of p426-pSNR52-gRNA (LEU)-tSUP4.

3 RESULTS

Figure 2 represents the p426-pSNR52-gRNA (LEU)-tSUP4 colony PCR results. It shows the suspected positive strain.

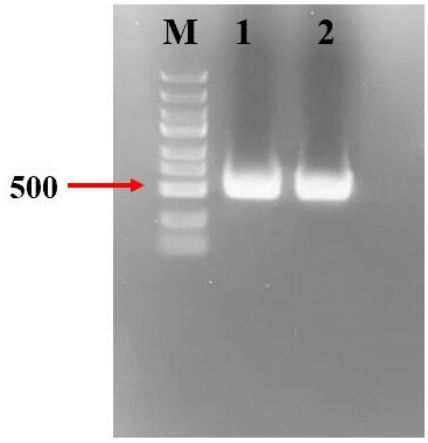

Figure 2: Results of p426-pSNR52-gRNA (LEU)-tSUP4 colony PCR.

Figure 3: Results of concentration detection of p426-pSNR52-gRNA (LEU)-tSUP4 plasmid extraction.

Figure 4: Results of BJ5464-p414-Cas9 streaking of the strain.

This above figure shows the results of the activated S. cerevisiae.

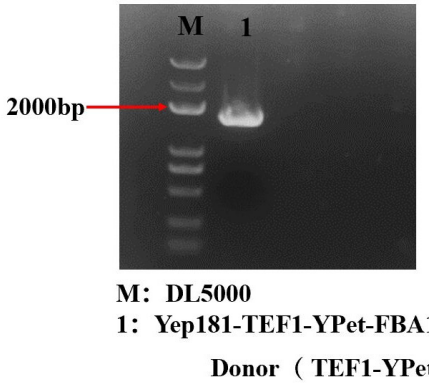

Figure 5: Results of electrophoresis detection of donor fragments on the LEU site.

From Figure 5, we can see that the donor sequence is less than 2000bp.

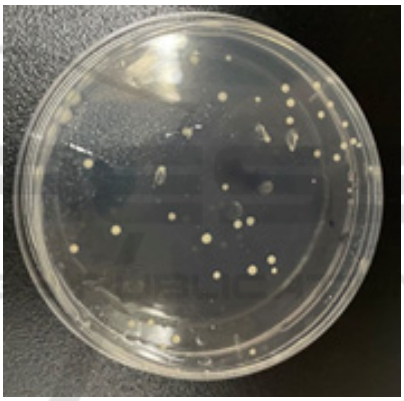

Figure 6: Results of BJ5464::LEU-pTEF1-YPet-tFBA1 transformant coating.

Figure 6 represents that the colonies have been transformed to S. cerevisiae.

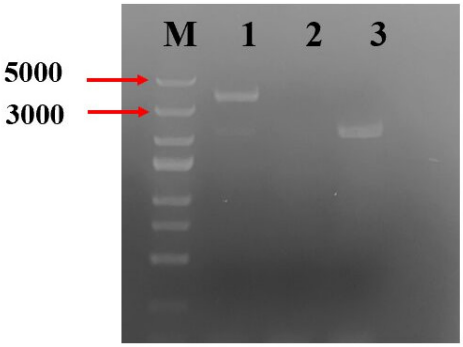

Figure 7: Results of BJ5464::LEU-pTEF1-YPet-tFBA1 colony PCR.

From Figure 7, we know the target sequence is 3000bp, so the result numbered 1 in the figure is a positive stripe.

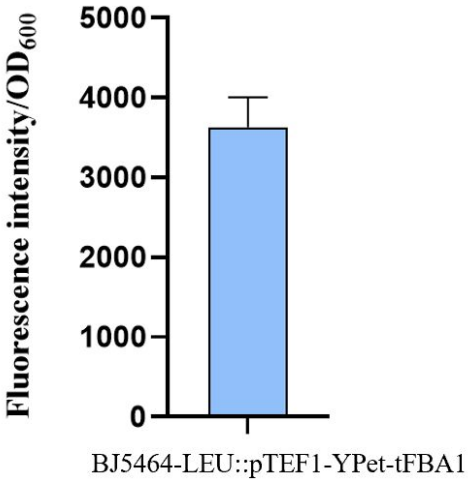

Figure 8: BJ5464::LEU-pTEF1-YPet-tFBA1.

Figure 8 shows the relative fluorescence value of BJ5464::LEU-pTEF1-YPet-tFBA1. The result shows the OD600 is between 3000 and 4000.

4 CONCLUSION

In this experiment, we use the technology of CRISPR/Cas9 to transform the genetic material of S. cerevisiae to produce specific fluorescent protein. The CRISPR/Cas9 system was discovered in the Streptococcus pyogenes CRISPR pathway, and it is widely used in research (Vasu, 2021). CRISPR is also applicable in clinical treatments, with the challenges and prospects to realize the clinical potential widely studied. The transformation method of CRISPR-Cas9 technology is the same as that of most gene technologies, but due to the specificity of Cas enzyme, it can successfully bind to the gene chain at a specific site (Cheng, 2021; Lu, 2015).

CRISPR/Cas9 has the potential in many application prospects, for example, cancer treatment (Khajuria, 2021; Wang, 2021), AIDS (Acquired Immune Deficiency Syndrome) therapies (Xiao, 2019) and cardiac diseases (Schreurs, 2021).

Cancer is one of the major causes of death, and humans have worked hard to treat it for many years. CRISPR/Cas9 optimizes the way people usually treat tumours (Xing, 2020). Experiments have shown that CRISPR/Cas9 technology can be used to methylate DNA to alter genetic performance, which is of great significance for the treatment of many genetic diseases (Katayama, 2021). Another successful trial is that researchers from the University of Sichuan, China, had been able to inject genetically modified lymphocytes for the first time to a patient with lung cancer as a therapeutic approach to promote the immune system's response for eliminating malignant tumor cells (Castillo, 2016).

AIDS is a disease that seriously threatens human health. The duplication of HIV (human immunodeficiency virus) can only be restrained for the time being. Using CRISPR/Cas9 as an effective tool to edit the gene is a possible approach to cure AIDS (Pelletier, 2015).

CRISPR/Cas9 is a technology with great promise. There have also been successes in treating other disease. Sickle cell disease is the name for a group of inherited health conditions that affect the red blood cells. The most serious type is called sickle cell anaemia. Researchers successfully induced hemoglobin for the treatment of sickle cell disease (SCD) (Demirci, 2021; Philippidis, 2021).

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