Applications of Genome Editing in Yeast with an Example of Tup1 Mutants Construction

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Abstract. Genome editing, which allows for the artificial modification of genes in organisms, has received significant attention in recent years. Currently, various genome editing tools have been developed based on different principles. ZFNs (zinc finger nucleases), TALENs (transcription activator-like effector nucleases), and CRISPR-Cas9 are the three genome editing technologies that are predominantly utilized. This article reviews the principles and characteristics of these three genome editing tools and summarizes the advantages and disadvantages of each of them accordingly. Genome editing is also playing an increasingly important role in yeast, opening up unforeseen applications for human beings. Tup1, a crucial transcriptional corepressor factor in yeast, is associated with multiple metabolic pathways and plays a key role in gene expression regulation. Currently, our understanding of how Tup1 regulates gene transcription remains limited. To gain a better understanding of the detailed mechanisms of Tup1, genome editing can be utilized to deepen our knowledge of its function. Therefore, this article proposes a method that utilizes CRISPR-Cas9 to construct mutant variants of Tup1 in yeast. The main process of this method involves introducing plasmids containing artificially designed sgRNA and Cas9 protein sequences into yeast cells, allowing them to express and edit the yeast Tup1 sequence, ultimately generating yeast Tup1 mutants. This method allows for the efficient construction of engineered Tup1 mutants, facilitating further research on Tup1.

1. Introduction

Genome editing refers to the process of making precise and intentional changes to the DNA sequence of an organism's genome. This technique involves using the tools such as nucleases to make targeted modifications to genes in organisms, with the ultimate goal of changing their biological traits. Over the last few decades, there has been significant progress in the field of genome editing, with rapid advancements and a growing interest among researchers.

Yeast, a common single-celled eukaryotic organism with a small and easily analyzable genome, serves as a valuable model organism for studying gene expression regulation. Due to yeast's efficient homologous recombination mechanism, it is exceptionally well-suited for genome editing studies[1]. Currently, a wide range of genome editing tools, including ZFNs, TALENs, and CRISPR systems, have been widely employed in yeast to facilitate gene deletion, integration, and other applications[2]. Tup1 is a classical transcriptional repressor in yeast, which typically forms a complex with Ssn6 as a tetramer to regulate the transcription of yeast genes. Tup1 is involved in the repression of multiple genes in yeast, but the detailed mechanism of its inhibitory action remains unclear[3]. Therefore, the construction of yeast Tup1 mutants is advantageous for understanding of the regulatory mechanisms of gene expression.

In this article, I introduce the classification of genome editing tools and their fundamental principles. Based on this, I specifically summarize the advantages and disadvantages of these various genome editing tools and provide a detailed comparison to elucidate the differences between them. Subsequently, I discuss the application of genome editing in yeast and analyze which genome editing tool is more suitable for yeast. Finally, using the construction of yeast Tup1 mutants as an example, I present a method for creating yeast mutants, highlighting the significance of genome editing in advancing yeast research. This method enables the efficient construction of yeast Tup1 mutants, addressing the limitation of fewer natural mutations. This is likely to promote advancements in research of yeast Tup1 to a large extent.

2. Genome editing

In genome editing, the first step is to cut specific DNA segments using appropriate genome editing tools. The three genome editing tools frequently used include ZFNs, TALENs and the CRISPR-Cas9 system. When they function, they cause DNA double-strand breaks (DSBs), which trigger the cell's repair mechanisms.

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2.1. Classification of genome editing

2.1.1 ZFNs

Zinc Finger Nucleases (ZFNs) are relatively early discovered type of genome editing tools. They are a type of protein artificially synthesized, initially formed by combining restriction endonucleases and zinc finger proteins[4]. ZFNs primarily consist of two components: the DNA binding domain, and the DNA cleavage domain. The DNA binding domain is assembled by sequentially linking several zinc finger protein domains. The DNA cleavage domain is composed of the restriction endonuclease FokI, which has a cutting effect on DNA molecules. A single FokI has no cutting effect on DNA. Only when two ZFNs simultaneously bind to DNA, their FokI domains will approach and activate each other, enabling them to cut DNA. This also means that ZFNs must bind to DNA in the form of dimers to function[5]. In summary, the specificity of ZFNs comes from the recognition capabilities of their zinc finger protein domains. Under the direction of these zinc finger proteins, the FokI within ZFNs can cleave specific DNA sites, achieving genome editing.

2.1.2 TALENs

Transcription activator-like effector nucleases (TALENs) are similar to ZFNs, consisting of a DNA binding domain and a DNA cleavage domain. The difference between the two is that the DNA binding domain of TALENs is made up of TALEs, a natural protein from the plant pathogen *Xanthomonas*[6]. The binding of TALEs to DNA is mediated by a repeated sequence of 33-35 amino acids, these repeated sequence domains are similar, except for the two hypervariable amino acids at positions 12 and 13[7]. Studies reported that each repeated sequence domain in TALEs can bind to a specific DNA base[8-9]. The DNA cleavage domain of TALENs is exactly the same as that of ZFNs, which is FokI, so TALENs also need to bind to DNA in the form of dimers to function.

2.1.3 CRISPR-Cas9

CRISPR-Cas9 is the most recent development in genome editing tools and is presently the most commonly employed method for editing genomes. The CRISPR system is an adaptive immune system that serves as a defense against bacteriophage infections in prokaryotes[10]. There are various types of CRISPR systems, with the Type II system, namely the CRISPR-Cas9 system, being the most extensively studied and utilized. This system necessitates only a single protein, Cas9, to bind and cleave the target DNA sequence[11]. In prokaryotes, the expression of CRISPR genes produces two types of non-coding RNAs, namely pre-crRNA and tracrRNA, which form a double-stranded structure through base pairing and assemble with Cas9 protein to form a complex. The double-stranded structure formed by pre-crRNA and tracrRNA serves as a guide RNA (gRNA), which can guide the Cas9 protein to specifically recognize the protospacer adjacent motif (PAM) sequence, thereby cutting the foreign DNA for protection[12]. By utilizing this characteristic of the CRISPR-Cas9 system, the gRNA sequence can be artificially designed to guide the Cas9 protein to cut specific sequences, thereby achieving the purpose of genome editing. When tracrRNA-crRNA is fused into a single-stranded guide RNA (sgRNA), it can also guide Cas9. Therefore, when employing CRISPR-Cas9 as a tool for genome editing, it is frequently designed in the form of sgRNA[13]. The advent of CRISPR-Cas9 has brought genome editing into a new era. Owing to its prominent advantages and huge development potential, it has received widespread attention.

2.2 Comparison of different genome editing tools

Table 1. Comparison of different genome editing tools

	Composition	Principles	Advantages	Disadvantages	References
ZFNs	Zinc finger proteins and FokI	Zinc finger protein binds to the target sequence and FokI cleaves	High specificity ZFNs are small molecules and less likely to cause an immune response Relatively easy to deliver	Hard to design and construct High cost Takes a long time Off-target effects are likely to occur Each zinc finger protein domain can only recognize approximately 3 bases at a time Cannot edit multiple genes simultaneously	Gabriel R, et al., 2011, [23]
TALENs	Transcription activator-like effector and FokI	TALE binds to the target sequence and FokI cleaves	Easy to design and construct TALE repeat domain can recognize a single base Less off-target effects	TALENs are larger molecules, may lead to reduced specificity Hard to deliver Cannot edit multiple genes simultaneously	Guilinger J P, et al., 2014, [19] Yan W, et al., 2013, [24]
CRISPR-Cas9	gRNA and Cas9	gRNA guides the Cas9 protein to the target sequence and the Cas9 protein cleaves	Very easy to design and construct Low cost Efficient cutting Can edit multiple genes simultaneously	High frequency of off-target effects A PAM sequence is required	Riesenberg S, et al., 2019, [25] Zhang Q, et al., 2018, [26]

The range of genome editing tools currently available offers a multitude of options to researchers. Nevertheless, since different tools rely on distinct methods and principles, each comes with its unique strengths and also certain constraints. In practical applications, the selection of the most suitable genome editing tool should be determined based on specific circumstances, see table 1.

The zinc finger protein structural domain of ZFNs can recognize and bind to different DNA sequences through artificial design. This recognition is usually quite precise, with good specificity. In the CRISPR-Cas9 system, the cutting site usually requires a PAM sequence, while ZFNs do not have this restriction and can better control the cutting site artificially[14]. However, ZFNs also have certain drawbacks. For example, the zinc finger protein can only recognize three bases at a time, which puts certain limitations on the corresponding cutting sequence. In addition, the design of the zinc finger protein structural domain of ZFNs often involves complex protein engineering, which requires a long time and high cost, making it unsuitable for clinical use[15-16]. The off-target rate can be minimized when using ZFNs by increasing the number of zinc fingers, improving the specificity of the zinc fingers, and lowering the concentration of ZFNs, thereby generating more efficient outcomes[17].

Compared with ZFNs, the repeated sequences in the DNA binding domain of TALENs can be designed to recognize individual bases, thereby achieving more precise recognition of cutting sequences. Additionally, TALENs are simpler to design, can be produced in high quantities rapidly, and exhibit reduced cytotoxicity[18]. Typically, TALENs are designed to bind sequences that are longer than those of ZFNs, which results in the issue of larger TALENs molecules. Studies have shown that larger TALENs molecules could potentially influence their delivery, consequently reducing their specificity [19]. While the off-target effect of TALENs is less than that of ZFNs, it remains a concern, and appropriate actions should be implemented in real applications to decrease its incidence rate.

The emergence of CRISPR-Cas9 provides a more convenient method for genome editing. Compared with ZFNs and TALENs, CRISPR-Cas9 only requires the design of a gRNA sequence to guide the Cas9 protein to the cutting site, without the need for manually designing proteins[20]. The process of artificially synthesizing RNA is much simpler than synthesizing proteins, so the CRISPR-Cas9 system is easy to construct. Besides, the CRISPR-Cas9 system exhibits high cleavage efficiency, with the capacity to multiple genes simultaneously[21]. These traits of the CRISPR-Cas9 system are unique and not present in other genome editing tools. However, it does have a significant concern, namely the off-target effect. Because recognition in the CRISPR-Cas9 system is mediated by RNA, this increases the possibility of its off-target effects[22]. Reducing the off-target effects of CRISPR-Cas9 is a crucial focus in gene therapy, as it frequently results in significant repercussions. Therefore, how to improve and optimize the CRISPR-Cas9 system to reduce its off-target effects has become the key to be further developed.

CRISPR-Cas9 has a distinct technical advantage in genome editing and is usually the preferred genome editing tool. However, in certain situations, such as when exceptional high specificity is needed and in systems where CRISPR-Cas9 cannot work effectively, ZFNs and TALENs can often perform better.

2.3 Application of genome editing in yeast

As a simple unicellular eukaryote, yeast holds significant importance for humans. For thousands of years, humans have utilized yeast for alcohol production, baking bread, and other food preparations. As our comprehension of biology has advanced, the applications of yeast have been further expanded. For example, yeast has increasingly become a common model system for examining gene expression regulation in eukaryotic organisms[27]. By modifying a specific gene in its genome, corresponding changes can be detected, which in turn uncovers the role of that gene and provides further insight into gene expression regulation.

The expansion of yeast applications is inseparable from the development of genome editing. Currently, genome editing, especially the CRISPR-Cas9 system, is widely used in yeast. Other genome editing tools, such as ZFNs and TALENs, have evident limitations when applied to yeast due to their reduced efficiency, in contrast, the CRISPR-Cas9 system is not only more effective but also easier to design. *Saccharomyces cerevisiae*, primarily employed in the food and alcohol fermentation processes, is the most commonly studied and applied variety of yeast, and it holds significant relevance to human productivity. In addition to fermentation, *Saccharomyces cerevisiae* can also be used for studying gene expression in eukaryotes and as a cell factory for certain products. Hence, *Saccharomyces cerevisiae* is also the most frequently chosen strain for genome editing. Through genome editing, it is possible to specifically modify one or several genes linked to the fermentation process, consequently changing the metabolic pathways within yeast. This can be used not only to improve yeast's fermentation capacity and enhance production efficiency but also to modify the flavor of food, improving the quality of the products[28]. In addition to *Saccharomyces cerevisiae*, other types of yeast are used for genome editing to varying degrees. These yeast species, less common than *Saccharomyces cerevisiae*, are consequently termed non-conventional yeasts and include types such as *Pichia*, *Kluyveromyces lactis*, and *Kluyveromyces marxianus*[29]. Compared to *Saccharomyces cerevisiae*, these non-conventional yeasts possess different metabolic pathways and unique natural properties[30]. Their modification through genome editing can facilitate the production of the intended products more readily. This is of significant importance for the development of metabolic engineering and synthetic biology.

In addition to the aforementioned uses in food production and as a cell factory, yeast has many other applications. For instance, as a eukaryotic organism, yeast contains many genes that are homologous to humans. Variations in these genes in humans may lead to disease.

Therefore, by artificially editing these genes in yeast, disease models can be constructed, providing new insights for treating corresponding human diseases[31]. Thus, genome editing plays a crucial role in the practical application of yeast. It not only paves the way for advancements in conventional yeast applications but also unveils numerous new fields of application, empowering yeast to generate even more substantial benefits for humanity.

3. Tup1 and construction of yeast Tup1 mutants

3.1 Tup1

Tup1 is a transcriptional corepressor widely found in yeast. It often interacts with other proteins to form complexes within yeast, which plays a regulatory role in transcription. The protein that Tup1 most frequently associates with is Ssn6, forming the Tup1-Ssn6 complex. The structure of this complex is typically composed of one Ssn6 subunit and four Tup1 subunits. The N-terminal of Tup1 interacts with the TRP domain of the Ssn6 protein, thus binding to Ssn6; the C-terminal is composed of seven consecutive WD40 domains (Figure 1). In most cases, Tup1-Ssn6 acts as a transcriptional repressor within yeast, but in a few cases, it can also act to activate gene transcription[32]. The function of Tup1-Ssn6 is critical and is associated with various metabolic pathways in yeast. Studies have shown that the Tup1-Ssn6 complex is closely related to cellular morphological changes, oxidative stress, and asexual reproduction in yeast[33-34]. Therefore, Tup1 plays a key role in yeast, participating in the regulation of gene expression, and maintaining the normal life activities of yeast.

While the detailed mechanism of yeast Tup1 transcriptional repression is still unclear, some progress has been made. Studies have shown that when the Tup1-Ssn6 complex functions as a transcriptional repressor, it is usually recruited to the promoters of relevant genes by transcription factors[35]. Then it can exert a transcriptional repressing effect by recruiting histone deacetylases and stabilizing the structure of the nucleosome[36]. Studies indicate that other fungi possess homologs of Tup1, and proteins analogous to Tup1 are found in more complex eukaryotes. These include Groucho in fruit flies, Grg in mice, and TLE1 in humans[37]. Therefore, in-depth research into the yeast Tup1 gene is beneficial for understanding the specific regulatory mechanisms that are not yet clarified. It can also help deepen our understanding of gene expression regulatory networks in eukaryotes and reveal the functional mechanisms of similar proteins in more complex eukaryotes. Moreover, such research can aid in constructing relevant disease models, thus promoting the development of human medical practice.

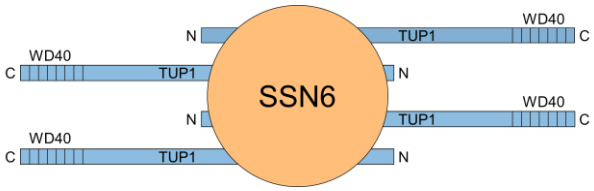

Figure 1. Tup1-Ssn6 Complex

3.2 Construction of yeast Tup1 mutant using genome editing

In yeast, Tup1 is associated with the repression of many genes. Therefore, when mutations occur in the yeast Tup1 gene, rendering Tup1 nonfunctional or altering its structure, multiple metabolic pathways in the yeast are often affected. Such a mutation is not lethal to yeast but is pleiotropic, leading to various affected phenotypes[38]. For instance, studies have shown that mutations in the Tup1 gene of *Saccharomyces cerevisiae* may enable it to assimilate and utilize mannitol, a characteristic absent in the wild type[39]. Therefore, mutations in the Tup1 gene can result in various different outcomes, some of which might produce strains beneficial to humans. However, research on Tup1 mutants is currently limited, and the functional mechanisms of the Tup1 protein are not entirely clear. Furthermore, the frequency of natural mutations in the Tup1 gene is very low, making naturally occurring yeast Tup1 mutants extremely rare, which poses obstacles to related research. In order to further clarify the mechanism of yeast Tup1 in gene expression regulation and to discover more applications of Tup1 mutants, the artificial construction of mutants could be a viable way to efficiently produce yeast Tup1 mutants.

Genome editing provides a promising platform for the construction of yeast Tup1 mutants. With the development of genome editing, its application in yeast has evolved significantly, leading to substantial outcomes. It is expected that applying genome editing techniques to the artificial creation of yeast Tup1 mutants could also yield positive outcomes. Given this, I propose an example of constructing yeast Tup1 mutants using CRISPR-Cas9. First, a plasmid can be artificially designed that contains the sgRNA sequence and the Cas9 protein. The sgRNA needs to be designed based on the Tup1 sequence, and the Cas9 protein is used to specifically cut the Tup1 sequence. Then, this plasmid can be introduced into wild-type yeast, where the sgRNA and Cas9 protein will be expressed. Once the Cas9 protein, guided by the sgRNA, completes cutting the Tup1 gene, the gene will get a DSB. This will activate the yeast's HDR or NHEJ mechanisms to repair the break site. At this point, a mutant sequence can be introduced to change the original Tup1 sequence or create a deletion mutation in Tup1. In this way, an artificially constructed yeast Tup1 mutant is obtained (Figure 2).

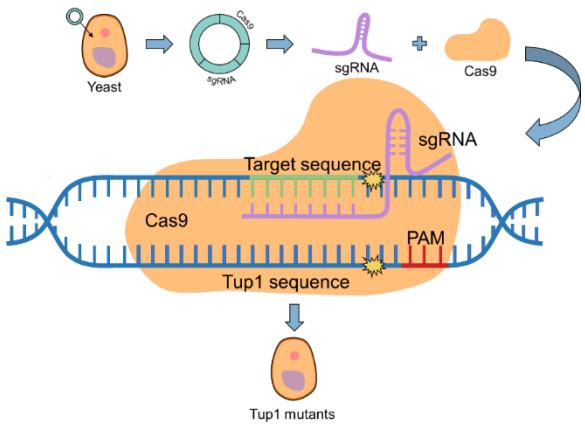

Figure 2. Construction of Yeast Tup1 Mutants Using CRISPR-Cas9

4. Discussion

The rapid development of genome editing has brought new possibilities to the field of biology. Based on different principles, several genome editing tools have been developed. Different genome editing tools have their respective advantages and disadvantages and are compared in detail in this article. In yeast, the advancement of genome editing has consistently broadened its scope of uses, introducing new areas of study and allowing yeast to provide more benefits for humans. Of course, besides yeast, genome editing has also greatly promoted the development of other fields. This is because genome editing has universal applicability and even has the potential to edit any gene of any species. However, certain shortcomings still exist when applying genome editing in practice. For instance, each genome editing tool has certain limitations. This may pose certain risks, so researchers should consider carefully which genome editing tools to use in order to minimize negative impacts. Furthermore, the biosecurity issues caused by genome editing have also become a topic of discussion[40]. As genome editing develops, different genome editing tools will continue to be optimized and new ones will be implemented, progressively resolving existing challenges. As for Tup1, a transcriptional corepressor in yeast, it has been somewhat understood in terms of its structure and function through years of research[41]. But this understanding is not profound, and many mechanisms are waiting to be discovered. Yeast and humans are eukaryotes and share many similar proteins. The human TLE1 is a similar protein to Tup1, and they have many structural and functional characteristics in common. And TLE1 is involved in various processes of human development and is associated with disease onset[42]. This emphasizes the importance of research on Tup1. The emergence and development of genome editing provide a powerful tool for Tup1 research. A method to construct a yeast TUP1 mutant using CRISPR-Cas9 as an example is also mentioned in this paper. This is certainly an efficient way to construct Tup1 mutants artificially. With the help of genome editing, research on yeast Tup1 will progress rapidly, creating even greater value for humanity.

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