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Application and Prospect of CRISPR/Cas9 Technology in Marine Diatoms

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Abstract. CRISPR/Cas9 technology is a third-generation artificial nuclease technology that has been successfully applied in several fields, given its simplicity and efficiency. Although it has been widely used, its application in marine organisms, especially in eukaryotic marine phytoplankton represented by marine diatoms, is just beginning. As one of the most important marine primary producers, marine diatoms have a variety of irreplaceable ecological significance and potential applications, and therefore need the support of genetic engineering technologies to advance their further development. This paper will first introduce the mechanism of action and the history of CRISPR/Cas9 technology. It will also present the application of the CRISPR/Cas9 system in the genetic engineering of marine diatoms and discuss its prospects.

Keywords: CRISPR/Cas9, marine diatom, genome editing

1. Introduction

The oceans cover more than 70% of the Earth's surface area and contain a huge number of marine organisms, including marine algae. They are diverse and are an important biological resource of the ocean. Due to their rich biodiversity, the diversity of ways they have adapted to their environment, and their irreplaceable evolutionary status, algae are ideal materials for genetic engineering research. Marine diatoms are widely distributed and are the main group of light and microalgae. As a representative eukaryotic marine phytoplankton, diatoms have a simpler structure compared to other eukaryotes. However, the difficulty in controlling their ploidy and homologous recombination systems has limited the use of RNA interference (RNAi) and homologous recombination methods in diatom genetic studies, which have lagged behind other model organisms such as cyanobacteria and green algae at the molecular level[1].

In recent years, gene editing technologies have been continuously developed and artificial nuclease technologies have emerged, including Zinc-finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and Clustered regularly- interspaced short palindromic repeats (CRISPR)/Cas technologies. These technologies have been successfully applied experimentally in many organisms. Among them, TALENs technology and CRISPR/Cas9 technology have been applied to the study of marine diatoms at the molecular level in the field. Given the complexity and high cost of the process of constructing TALEN vectors[1], the application of CRISPR/Cas9 technology in marine diatom gene editing is more promising.

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CRISPR/Cas technologies, especially CRISPR/Cas9 technology, have made progress in gene editing applications in plants, animals as well as microorganisms due to the advantages of shorter required recognition target sequences, simple design and operation, and low cost.

Based on the development process of genetic engineering and the discovery and mechanism of action of the CRISPR/Cas9 system, this paper outlines the results of the application of CRISPR/Cas9 technology in marine diatoms and discusses the problems and future application prospects of the system in marine diatom applications.

2. Development of gene-editing artificial nuclease technology

Gene editing is a genetic engineering technique capable of making highly specific alterations to an organism's genome. Ever since the structure of DNA was discovered, researchers have been pondering the possibility of making targeted modifications to the genome.

Initially, Scherer and Davis used homologous recombination in their research to edit the Saccharomyces cerevisiae gene, enabling the manipulation of genes and chromosomes in yeast[2], thus contributing to the development of reverse genetics. The subsequent rapid development in the field of gene editing, where advanced technologies continue to replace those of the past, has contributed to the advancement of molecular biology. Artificial nuclease technology is an emerging gene editing technology in recent years, which has now developed its third generation, including ZFNs, TALENs, and CRISPR technology.

2.1. Zinc-finger nucleases (ZFNs)

ZFNs are the first generation of artificial nuclease technology. It is composed of zinc finger protein (ZFP) and the shearing domain of FokI endonuclease [3].

The zinc finger (ZF) is the basic structural unit of ZFP, a protein motif found in most proteins. The ZF DNA-binding domain part generally contains three independent ZF repeat structures, each recognizing 3-6 bases, thus a ZF DNA-binding domain can recognize specific sequences of 9-18 bp in length.

FokI is a kind of restriction enzyme present in Flavobacterium okeanokoites. It was found that when combined, the two can function as site-specific nucleases thereby replacing the natural recognition structural domain for specific shearing of target genes[4].

This technology has been successfully commercialized and widely used in various biological fields, but the high design cost, time consumption, application limitations, and high off-target rate prevented its efficient large-scale application[5].

2.2. Transcription activator-like effector nucleases (TALENs)

TALENs are the second-generation artificial nuclease system that consists of a fusion of TALE proteins that specifically recognize bound DNA and FokI nucleic acid endonucleases.

TAL effector (TALE) was originally identified in a plant pathogen named Xanthomonas sp. These TALEs enter plant cells through the bacterial type III secretion system and then regulate transcription through specific promoters that target effectors, thereby promoting bacterial colony formation[6].

The TALENs produced by FokI nuclease binding to TALE function as dimers that undergo double-strand breaks (DSBs), with the binding site located on the opposite strand. The DNA binding domain is composed of monomers containing tandem repeats, two of which are highly variable, each recognizing and binding to a nucleotide in the target sequence. They are easier to design than ZFNs due to the presence of variable DNA sequences responsible for recognition. However, difficulties in the design and synthesis of related proteins, as well as in the validation of experimental results, remain obstacles to its widespread use on a large scale.

2.3. Clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas technology

CRISPR/Cas9 technology is a third-generation artificial nuclease technology derived from the bacterial type II CRISPR system.

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The CRISPR/Cas system is a specific adaptive immune mechanism of archaea and bacteria for defense against foreign phages, plasmids, etc. This system has been applied and modified as a novel gene editing tool.

The CRISPR/Cas9 complex is efficient and precise because it remains unchanged to various gene targets and requires only a short guide RNA sequence change to redirect a specific cleavage. The Cas9 proteins, belonging to the type II CRISPR system, are currently the most popular and have been applied in several scientific fields[7].

3. History of CRISPR/Cas system development

CRISPR/Cas technology originated from an adaptive prokaryotic immune system (CRISPR-Cas) capable of generating specific and acquired immunity against mobile and exogenous genetic material. This approach subsequently evolved into a precise genome editing tool that transformed the field of molecular biology.

The CRISPRs system was first discovered in the E. coli genome, consisting of a series of repetitive sequences and some non-repetitive spacer sequences, that appeared when the bacteria came into contact with phage DNA. Later, CRISPRs have been discovered several times in phylogenetically diverse archaeal and bacterial genomes. Scientists have made predictions about their possible role in DNA-related activities.

In 2005, it was observed that many spacer sequences in CRISPRs belong to some plasmids and viruses[8] and that CRISPR sites are generated by transcription..... Thus there is speculation claiming that the CRISPR/Cas system may be used to resist invading phages, plasmids, and so on. This function of the mechanism is similar to that of the eukaryotic RNAi system. This conjecture was confirmed in 2007 when it was found that the bacterium acquired immunity to phage by integrating sequences in the phage genome into new spacer sequences of its own and that the addition or deletion of spacer sequences would alter the resistance of the bacterium to certain phages.

In 2008, it was shown that processed and modified CRISPR RNAs (crRNAs) can interfere with the workings of the bacterial antiviral immune system. Later, CRISPR technology continued to mature, with practical examples such as the cleavage operations that can be performed on RNA by CRISPR systems, and the simplicity and efficiency of type II CRISPR systems in DNA cleavage[9]. Several subsequent studies have demonstrated that CRISPR/Cas9 technology is an irreplaceable tool for gene editing, opening a period of wide application of CRISPR/Cas9 technology in multiple fields.

4. The Mechanism and Application Process of CRISPR/Cas9

The complex that functions in the CRISPR/Cas system contains three components: the crRNA, Cas9 protein, and transactivating crRNA (tracrRNA).

The overall working process consists of roughly three steps. In the first phase, the acquisition of the highly variable spacer region of CRISPR; in the second phase, the expression of CRISPR motif sequences (crRNA generation); and in the third phase, the CRISPR/Cas system plays a target interference role.

First, exogenously invaded DNA fragments are processed using Cas nucleases, and PAM sites are identified. DNA sequences near the PAM are selected as possible prototype spacer sequences and the processed DNA fragments are inserted within the CRISPR sequence.

Subsequently, pre-crRNA is transcribed from the CRISPR motif, while TracrRNA, which is complementary to it, is also transcribed. The two form a duplex by base-complementary pairwise hybridization and assemble with the Cas9-encoded protein to produce the complex. Under endogenous RNaseIII cleavage, a mature crRNA is produced.

In the final disruption of the invading DNA, the crRNA, Cas9 protein, and TracrRNA form a complex that precisely recognizes the foreign nucleic acid fraction bound to the complementary one, causing DSB of the invading DNA[10]. The PAM sequence plays a hard-to-replace role in the final interference phase. This is because PAM is the locus that distinguishes foreign invasive DNA from the host genome, where the CRISPR sequence does not contain PAM within it.

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5. Application of CRISPR/Cas9 in marine diatoms

Diatoms are microalgae with ecological importance and biotechnological potential. Therefore, we should use the tools of genetic engineering to study them at the genetic level and have the ability to perform specific manipulations on them, including the ability to replace and tag genes as well as control gene expression[11].

With the continuous development of algal genetic engineering techniques, TALENs technology, and CRISPR/Cas9 technology have now established a system of application in the field of marine diatoms, mainly focusing on two model diatoms - Thalassiosira pseudonana and Phaeodactylum tricornutum[1,11,12]. However, in practice, the complexity of TALEN constructs limits their application in the diatom field[1], compared to the CRISPR/Cas9 complex construction process, which requires fewer operations, making the application of CRISPR/Cas systems highly promising in the marine diatom field.

Nymark successfully implemented CRISPR/Cas9 technology in Phaeodactylum tricornutum. He mediated its gene mutagenesis of CpSRP54 and knocked out the other two genes. The success of this work contributed to the creation of stable targeted gene knockouts in marine diatoms[12]. In the same year, hopes' team assembled a single construct by using the Golden Gate cloning. Using two sgRNAs, they introduced a precise deletion early in the coding region of the urease gene, at a length of 37 nt. It finally resulted in cells with a high percentage of double mutations (⩽61.5%)[11].

In the course of ongoing research, various successful cases of gene mutation using CRISPR/Cas9 technology have emerged, and the application of CRISPR technology is evolving toward maturity[1].

6. Conclusion

The technology has already made some progress in its application in the field. Despite this, several issues have arisen during the practice of CRISPR/Cas9. Due to the specificity and difficulty of operating in eukaryotes, more severe off-target mutations have been shown in previous studies for application in CRISPR/Cas9 model organisms. And when predicting potential off-target sites, the results are influenced by high autosomal mutation rates and the diversity of diatom genomes. Therefore, while using CRISPR/Cas9 technology to achieve research goals, optimizing the technology as much as possible and thus achieving non-specific resolution of off-target effects is one of the important goals of future research.

Researchers have now succeeded in reducing the likelihood of off-target effects in some ways. Nawaly's team found that there is a risk of off-target effects when using intact Cas9. This possibility is amplified by the fact that sgRNAs are stably integrated into the genome of diatoms. However, if two sgRNAs could be used to edit the Cas9 nickase, this risk would be greatly reduced[1]. However, there is no concrete data to confirm this possibility and further experimental records could be used to demonstrate the reliability of this method.

We believe that CRISPR technology can be used in more diatoms shortly, as the understanding of CRISPR technology is further improved.

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