

Research and prospect of CRISPR system in marine biological system

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Abstract. Gene editing is a widely used and promising technology in biology field. Its ability to precisely edit the endogenous genes of organisms is a major boost to the research process in the biological sciences. Among these gene editing technologies, CRISPR is a more effective system and it is in a wider range of applications. The CRISPR system has advantages over Zinc finger nuclease (ZFNs) technology and transcription activator-like effector nuclease (TALENs) technology, including higher efficiency, gentler construction and a decreased risk of off-target effects. Currently, the treatment of diseases, the creation of animal models, and the genetic breeding of plants all make extensive use of CRISPR technology. Despite its wide request in model construction, its application in marine organisms is just beginning. This review systematically introduces the classification, development of gene editing technology, and summarizes the current status and development prospects of the application of CRISPR system in marine organisms.

Keywords: CRISPR system; Marine biological system; Gene-editing.

1. Introduction

Gene editing refers to the precise manipulation of genes at the genomic level through targeted insertions, mutations deletions, knockouts, multi-locus mutations and small deletions, with simultaneous mutation and small fragment deletion at the genome level [1]. The most basic technique of gene editing is homologous recombination-mediated gene targeting, which can accurately modify specific genes. However, it has disadvantages of low efficiency, time consuming and off-target in practice, which reduce the practical application of this technique. In order to achieve targeted mutation, insertion, or knockout of the target gene, gene editing technology first activates the system of cellular DNA repair by introducing targeted DNA double-strand breaks (DSBs), which can stimulate the process of gene editing. Recent years have seen the development of two new generations of editing technology: transcription activator-like effector nucleases (TALENs) and zinc finger nuclease (ZFN) technology [2]. The development of plant and animal models for gene therapy, among the most cutting-edge applications of gene editing technology, and the study of gene function have all demonstrated its enormous potential [3,4].

The ocean is a treasure trove of human resources, which not only provides a certain source of food, but also has great economic benefits. However, marine pollution is becoming more and more serious, and the number of marine organisms is decreasing due to uncontrolled marine fishing. The task of protecting marine biodiversity is urgent, and applying traditional genetics to marine genetic breeding research is a difficult task.

The purpose of this article is to provide a source for the field of marine genetic engineering while also presenting the fundamentals of the CRISPR system and drawing attention to the growth and preservation of marine biological resources.

2. The fundamentals of the CRISPR-Cas system

2.1. CRISPR-Cas system

Gene editing is essentially the use of restriction endonucleases to modify the genetic sequence of an organism and create stable mutations. These modifications include insertions, deletion mutations, knockouts, simultaneous multi-locus mutations, and small deletions. However, due to the limited

variety of natural nucleic acid endonucleases, scientists are eager to find new methods of gene editing [1].

The CRISPR/Cas system is composed of crRNA (CRISPR-derived RNA), trans-activating crRNA (tracrRNA) and Cas proteins. Based on the full sequence in Cas gene and the sequence similarity and site structure among Cas proteins, the CRISPR-Cas system is currently classified into two categories, including 6 types and 19 subtypes [2].

Over recent years, CRISPR systems have been classified according to their Cas-encoded complexes, and the types I-V have been combined into "Type 1" and "Type 2". Type 2 systems have Cas9 effector complexes, including Type II and Type V, while Type 1 systems have several subunit crRNA effector complexes, including Type I, Type III, and Type IV [5,6]. Depending on the genes that make up the Cas protein manipulator, these three varieties of CRISPR were further divided into subtypes I-A, I-B, I-C, I-D, I-E, I-F, I-U, II-A, II-B, and II-C, as well as III-A, III-B, III-C, and III-D [7]. Subtype I-A is a derivation of subtype I-B, while subtype I-C is an additional derivative of I-B that lacks Cas6.

All type III motifs feature the marker gene Cas10, and subtype III-A motifs typically have Cas1, Cas2, and Cas6 genes while type III-B motifs typically don't. Type III is also all inclusive. Subtypes III-A and III-descendants B's are subtypes III-C and III-D.

The type II CRISPR system derived in bacteria is the source of the CRISPR/Cas9 technology, which was initially introduced in 2013. subtype II-A contains the additional Csn2 gene that is the marker gene for this subtype, while subtype II-B lacks Csn2 having Cas4, and subtype II-C has only three proteins Cas1, Cas2, and Cas9. subtype V mainly contains Cpf1 [7]. The specific classification is shown in Figure 1[8].

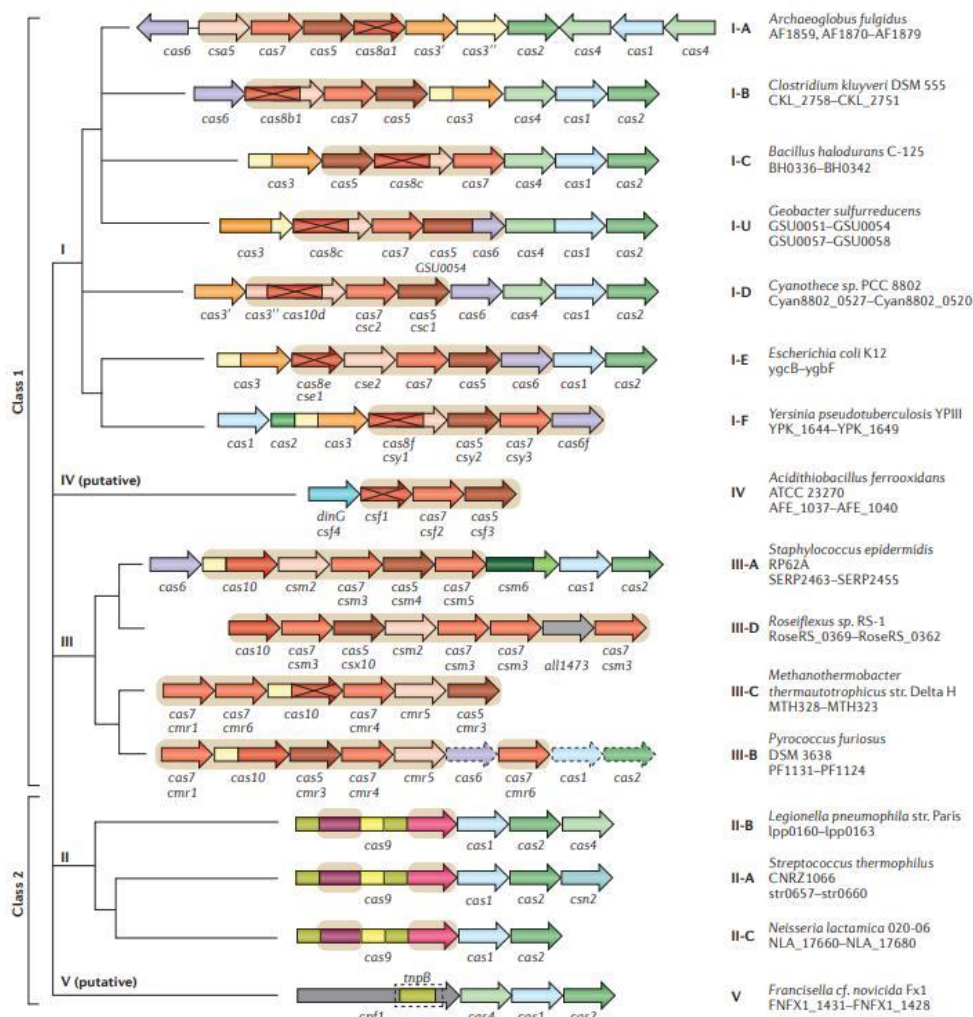


Fig. 1 Different types of CRISPR–Cas systems [8]

CRISPR/Cas9 technology, which was first unveiled in 2013, is adapted from the type II CRISPR system in bacteria. This process is divided into three major steps. The first stage is the uptake of spacer sequences: the PAM Sequence in the phage will be recognized by Cas proteins when the phage first invades, and they will then integrate the cleaved portion of phage DNA into the spacer region of the locus of the host cell. The second stage is the maturation of crRNA and tracrRNA and the formation of Cas9 cleavage complex. At this stage, some fragments of pre-crRNA transcribed and immature tracrRNA are combined in a complementary way in order to shape a double-stranded RNA region. At the same time, Cas9 protein will recognize and bind to the stem-loop structure of tracrRNA, and then RNase III will cut the double-stranded RNA region. The co-maturation of pre-crRNA and tracrRNA is completed, and the mature crRNA, tracrRNA and Cas9 form a stable Cas9 cleavage complex. The third stage is the cleavage of foreign DNA by Cas9 cleavage complex: in this stage, crRNA will recognize a strand in foreign DNA and bind to it in a complementary way. Both the HNH domain, which is positioned in the core of the protein peptide chain, and the RuvC domain, which is placed at the N-terminal of the Cas9 protein, will adhere to the double strand of DNA, respectively. The DNA is cut along two strands [1,2].

Briefly, throughout the period of invasion, the short RNAs specifically split the viral genome using the CRISPR-related nuclease Cas9. Thus, the CRISPR/Cas mechanism is a potent tool that tries to selectively mutate the genomes of eukaryotes.

2.2. Advantages and disadvantages of CRISPR-Cas technology

ZFN is the first artificial nuclease successfully constructed by scientists, and the technology has already implemented in a number of model organisms for knockout or targeted modification of target genes [9,10]. ZFN has no specific correspondence to its target DNA sequences, and the technology requires the construction of zinc finger expression libraries to screen for zinc finger proteins that specifically recognize these targets. Moreover, due to the specificity of ZFN sequence recognition, it is still impossible to design an ideal ZFN and to ensure that each gene can find a suitable target for ZFN action.

The transcriptional activator-like effector protein (TALE) was first proposed by Moscou et al. to specifically bind host genes [11]. The mechanism of action of TALENs technology is similar to that of ZFN technology in that the nucleoli is a TALE protein that recognizes the DNA sequence and the nuclease Fok I. The TALEs protein recognizes the target gene, while the role of Fok I is to cleave the DNA. Fok I is an endonuclease that must form a dimer to be active, so it greatly reduces the probability of random enzymatic cleavage. The TALEs protein and Fok I are linked into a recombinant protein expressing 1 recombinant nuclease that recognizes the target nucleic acid sequence and exerts endonuclease activity, thus cutting off the target gene and achieving gene knockout [1].

The advantages of CRISPR-Cas9 are clear. Firstly, it is easier to construct the vector, and the targeting efficiency is higher. In general, just a few dozen bases of CRISPR sgRNA need to be constructed to match the DNA sequence, so as to mediate the cleavage of DNA sequence by Cas9 protein. Secondly, compared with the previous two generations of technologies, CRISPR-Cas9 has more editable sites and higher distribution frequency, which makes it easier to select suitable editing sites. Most importantly, CRISPR-Cas9 can simultaneously edit the genome at more than one site.

In general, the CRISPR-Cas9 system has plain components, high efficiency in mutation, convenient operation, and a lower cost. It has become the directional gene editing technology most rapidly which applied to the genomes of many organisms.

3. Applications of CRISPR technology in marine biology

Marine species have indeed been brilliantly employed to address issues across a broad range of areas. The CRISPR system-related models of marine animals have been a potent tool for examining the basic biological processes up to this point [12].

3.1. *Ciona intestinalis*

In 2014, Sasaki et al. used the CRISPR system to accomplish the first targeted gene deletion in *Ciona enterinalis*, which resulted in the first study to be published on the use of the CRISPR system in marine creatures [13]. In this study, they selected eight targets of 20 bases in length for *Hox3*, *Hox5*, and *Hox12* of the *Ciona intestinalis* and constructed the corresponding sgRNA expression frames; these expression frames were electroporated together with mRNA expressing Cas9 protein into unfertilized eggs of the vitelline ascidian, and these eggs were allowed to fertilize with sperm to start embryonic development. The results showed that the sgRNA frames of *Hox3* and *Hox5* mutated the target genes, respectively [14].

3.2. *Nematostella vectensis*

Ikmi et al. created two guide RNAs (gRNAs) that addressed NVFP-7R exons 2 and 3, and then they injected the gRNA-exon/Cas9 complex assembled into unfertilized eggs. This strategy was utilized to eliminate NvFP-7R [15], an endogenous red fluorescent protein in sea anemone (*Nematostella vectensis*). Additionally, the absence of red fluorescence from the knocked-out anemone demonstrated that NvFP-7R was not required for sea anemone development.

3.3. *Crepidula fornicata*

Perry and Henry attempted to edit the β -catenin gene of the Atlantic horn snail (*Crepidula fornicata*) by using CRISPR technology and they successfully added the fluorescent protein mCherry sequence to the 3' end of its CDS. Larvae of the Atlantic hornworm were found to contain the β -catenin protein with a red fluorescence signal. In order to start DSBs, scientists injected fertilized eggs with Cas9 RNA, a sgRNA that directs Cas9 to the appropriate β -catenin site [16].

3.4. *Petromyzon marinus*

Square et al. studied marine seven-gill eel (*Petromyzon marinus*) by using CRISPR technology [17], the TYR gene was successfully knocked out by the researchers after they administered Cas9 mRNA and gRNA targeted for the TYR gene into fertilized eggs.

3.5. Echinoidea

Lin and Su successfully knocked out the *spNodal* gene of Echinoidea by injecting mRNA expressing Cas9 protein and sgRNA designed for *spNodal* gene into the Echinoidea urchin using microinjection technique. In this study, the authors designed three gRNAs for each of the two exons of the *spNodal* gene, and obtained good knockdown results for five of the six gRNAs, producing the expected radial phenotype [18].

3.6. *Clytia hemisphaerica*

Clytia is a hydroid jellyfish with two stages, hydroid and jellyfish. After the injection of Cas9 and sgRNA, effective *double allelic* KO was observed in this species. The *GFP1/GFP2 double KO* in embryos lacking endogenous GFP fluorescence has established stable quasi-immortal strains and **successfully passed on the germline** [19].

3.7. Other editing examples

In addition to *Petromyzon marinus* [17], *Salmo salar* [20], zebrafish [21,22] and *Oryzias latipes* [23] can all be microinjected with CRISPR-dependent gene knockdowns, and the adaptability of the CRISPR system can be determined by the physiological state of the knockout genes.

In addition to the creatures mentioned above, CRISPR technology is also used in the *Phaeodactylum tricornutum*. Scientists successfully knocked out the *CpSRP54* gene of *Phaeodactylum tricornutum* by using biological DNA transfection method [24]. What is more, in 2019,

scientists used CRISPR/Cas9 system to change the rhabdomopsin *R-opsin1* protein in *Capitella teleta* and observed a significant decrease in larval phototaxis [25].

Meanwhile, CRISPR technology has also been applied in the genetic breeding of crustaceans. By using CRISPR technology, scientists have edited the *Pax6* gene in *Daphnia magna* by microinjection and shown that it is involved in eye development [26]. Additionally, *Chi4* in *Exopalaemon carinicauda* was successfully knocked down by using CRISPR/Cas9 method, and the impact of this on growth as well as development was seen [27].

Table 1. Application of CRISPR system in some marine organisms

Type	Species	Target Gene	Mutation
Fish	<i>Salmo salar</i>	<i>Slc45a2, tyr</i> [20]	Color reduced
	<i>Danio rerio</i>	<i>Atplala 1-5 pkd2</i> [21], <i>faf1</i> [22]	Detect of embryo, delay of pigment deposition
Crustaceans	<i>Oryzias latipes</i>	<i>Slc45a2, tyr</i> [23]	Sex determination
	<i>Daphnia magna</i>	<i>Pax6</i> [26]	Abnormal ocular morphology
Mollusc	<i>Exopalaemon carinicauda</i>	<i>Chi4</i> [27]	Affect growth and development
	<i>Crepidula fornicata</i>	<i>B-catenin</i> [16]	Red fluorescence
Echinodermata	<i>Strongylocentrotus purpuratus</i>	<i>Nodal</i> [18]	Bone shape
	<i>Ciona intestinalis</i>	<i>Hox3, Hox5, Hox12</i> [13,14]	No feedback
Others	<i>Capitella teleta</i>	<i>r-opsin1</i> [25]	Decreased phototaxis of larvae
	<i>Phaeodactylum tricornutum</i>	<i>CpSRP54</i> [24]	No feedback
Cnidaria	<i>Clytia hemisphaerica</i>	<i>Biallelic KO</i> [19]	No feedback

4. Conclusion

Since there are fewer cell lines in marine organisms, it is difficult to manipulate cell lines or embryos by microinjection and other methods, so the current research is limited to model organisms and a few marine organisms. Diverse tactics have been monitored to boost delivery effectiveness and lessen the off-target effect in order to increase the system's accuracy. Future research on even more marine creatures will be feasible with the sequencing of ever more marine genes. Therefore, establishing a reliable transgenic method is necessary before CRISPR technology is widely used in aquatic creatures. When the issue with transgenic techniques is resolved, an attempt can be made to create transgenic lines with steady expression, which will greatly advance the use of CRISPR technology in marine creatures.

Another direction worth exploring in the application in marine animals is the combination of optogenetics and CRISPR technology. The combination of optogenetics is the use of light to control certain biological activities in combination with bioengineering methods. In the study of CRISPR, it is possible to control the CRISPR system in time and space by using the coupling of light control elements with Cas9 protein [14].

Furthermore, CRISPR technology offers possibilities for investigating marine organisms. The following areas can be reinforced since CRISPR can perform targeted gene editing and will have a considerable impact on marine organisms' genetic breeding in the future.

1) Expanding the research and application scale of marine organisms: Compared with other species, CRISPR is more limited in the research of marine organisms, and there are problems such as enzyme activity temperature and difficulties in sampling, which need to be addressed to expand the application scope [1].

2) Analysis of marine gene functions by bioinformatics: as more marine genomes are sequenced, gene editing technologies will further disclose the role of different genes in the genetic breeding of marine organisms and even in biological evolution and development.

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