Development of Genome Editing Technology and Its Applications to Plants

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Abstract. As a core tool of modern life science and agricultural biological technology, genome editing technology has evolved for three generations from zinc finger nucleases (ZFNs) and transcription activator-like effector nuclease (TALEN) to CRISPR-Cas system, which has improved the editing accuracy, efficiency and applicability significantly. In this study, the principle mechanism, technological characteristics of three generations of gene editing tools as well as their typical applications to medical treatment and plant breeding were reviewed systematically. Furthermore, the working principle and unique applications of new CRISPR derivation systems (Cas12 and Cas13) were discussed and its potentials in polygenic editing, RNA regulation and high-sensitivity molecular detection were exhibited. Through comparison of differences of various edition systems in targeting mechanism, editing objects and application scenes, this study emphasizes on the important values of CRISPR family tools in functional expansion and platform building. Moreover, research results provide theoretical foundation and technological references for the transferred applications of gene editing in accurate medicine and sustainable agricultural development.

Keywords: genome editing; CRISPR-Cas9; CRISPR derivation system; disease-resisting plant breeding; nucleic acid test

1 Introduction

Gene editing technology is of important significance to modern medicine and crop improvement. Three generations of gene editing technologies from the protein-DNA targeting mechanism of zinc finger nucleases (ZFNs) and transcription activator-like effector nuclease (TALEN) to the RNA guided editing paradigm of CRISPR-Cas9 system have been breaking accuracy and efficiency boundaries of gene operations through differentiated design. Recently, the CRISPR derivation systems (e.g. Cas12 and Cas13) further expanded the dimensions of gene editing technology. In this study, technological evolution of three generations of editing tools was reviewed systematically and multifunctional applications of different genome editing technologies to disease-resisting plant breeding and fast pathogen detection were introduced, aiming to provide theoretical references to innovation of agricultural biotechnology and sustainable development.

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2 Development and Applications of Genome Editing Technology

2.1 ZFNs and Their Applications

ZFNs are a type of editable enzymes which identify and cut DNA. It is mainly composed of DNA domain (Zinc Finger Protein, ZFP) and DNA cutting domain (generally FokI). Specifically, ZFP is responsible for recognition of specific DNA sequences. It is composed of several Cys2His2 zinc finger proteins and each can identify 3 basic groups. FokI is responsible for cutting DNA. Dimer is formed when ZFP binds with nucleotide sites, which can cut DNA at the target site after activation and initiate the DNA repair mechanism (e.g. non-homologous end joining or homologous recombination) to achieve the goal of gene editing.

Assisted by ZFNs, people developed more treatments to diseases. For example, ZFNs are applied to treatment of aids. The accessory receptor of HIV is CRR5 and ZFNs can make CCR5 gene editing to CD4+T cells to treat aids. The damage of CCR5 might increase the survival rate of CD4+T cells. Experiment showed that people with CCR5-delta32 deletion homozygote can resist HIV infection and presented slower progress in aids[1]. The applications of ZFNs reflect the tremendous potentials of gene editing technology to treatment of various diseases like HIV.

2.2 TALEN and its Applications

TALEN system is transformed from TALE proteins of phytopathogen and it is composed of three critical functional domains: 1) N-end structural domain: it contains the nuclear location signal (NLS) and is responsible for guiding proteins into cell nucleus. 2) Central structural domain: it is formed by several TALE (transcriptional activator-like effector) repeated units in series and each repeated unit can make specific identification and binding with one specific DNA basic group, thus realizing accurate targeting at the target sequences. 3) C-end structural domain: it connects to the FokI endonuclease to realize directional genome editing.

TALEN technology also can assist treatments of diseases. Some study has pointed out that TALEN technology can decrease rejection reaction of organ transplantation. GTA1 edited α-1,3-galactosyl transferase can add α-Gal into ends of glycoproteins and glycolipids, forming α-Gal epitope. It is an important antigen on pig cell surface and participates in recognition and reaction of immune system. Theα-Gal epitope is identified and cut by designing TALE protein and FokI nuclease based on TALEN technology, and then mRNA is injected into germ cells of pig, thus getting the transgenic pigs through embryo transplantation. According to detection, pigs with gene knockout can grow and develop normally, without obvious rejection reaction during heterogeneous organ transplantation [2].

2.3 CRISPR/Cas9 System and its Applications

CRISPR-Cas9 system comes from the natural immune mechanism of bacteria. CRISPR transcription generates pre-crRNA upon the invasion of virus. The pre-crRNA and tracrRNA form a complex through complementary matching of basic groups, and then processed into mature crRNA-tracrRNA dimer under the action of proteins like RNase III. The dimer guides Cas9 proteins to identify the target DNA sequences. Among them, complementary matching between interval zone of crRNA and target DNA is formed, while Cas9 determines the cutting site accurately by identifying PAM sequences (NGG). Once they are bound successfully, HNH of Cas9 and RuvC nuclease structural domain cut the DNA double strands at the target sites respectively, forming double strand breakages.

Jinek et al. improved flexibility of the design by simplifying double-stranded RNA into single-stranded RNA (sgRNA) which contains target sequences (complement with target DNA) and the support structure (binding with Cas9), and facilitated its extensive applications[3]. The cutting activity of Cas9 protein relies on sgRNA guidance and PAM sequence identification. After cutting, it triggers the DNA repair mechanism of cells, resulting in random insertion/deletion mutation through non-homologous end joining or realizing accurate editing based on homologous recombination (HDR).

With respect on studies on treatment of cardiovascular diseases, the CRISPR-Cas9 system showed promising application prospects. PCSK9 edited proteins can make specific binding with low-density lipoprotein receptor on the hepatic cell surface to promote its internalization and lysosome degradation, thus weakening the ability of liver to clear low-density lipoprotein cholesterol in the cycle. As a result, the serum cholesterol level increases. Based on this molecular mechanism, researchers designed gRNA sequence of specific target mice Pcsk9 genes by choosing SaCas9 as the gene edition tool and transferred the CRISPR-SaCas9 system into liver tissues of mice through the relevant virus vector. The serum LDL-C level in mice with Pcsk9 knockout declined, and the normal physiological functions of liver were not affected after gene edition[4]. This study offers theoretical supports to treatment of cardiovascular diseases (e.g. hypercholesteremia) in human. Nevertheless, the long-term safety, edition efficiency stability and possible ethnical disputes of the CRISPR-Cas9 system still have to be evaluated systematically before clinical applications. These problems determine the transferred medical value and social acceptance of the technology directly.

2.4 Comparison of Three Generations of Genome Editing Technologies

Through comparison among core characteristics of three generations of gene edition technologies (ZFNs, TALEN and CRISPR/Cas9) (Table 1), it found that CRISPR/Cas9 system showed remarkable advantages in the field of gene edition. ZFNs and TALEN depend on the targeting mechanism of protein-DNA interaction, while CRISPR/Cas9 identifies targets through RNA-DNA complementary matching and its sgRNA design is both flexible and high-efficiency (wide target range and short building periods of several days). The CRISPR/Cas9 system not only has the highest edition efficiency, but also supports synchronous polygenic edition and can match with several delivery

systems (e.g. virus vector and nano-particles), lowering the technological threshold significantly. Although there are moderate risks of missing in the early CRISPR/Cas9 system, its specificity has been improved greatly by the development of high-fidelity variant (e.g. SpCas9-HF1). Differently, ZFNs is replaced gradually due to the limitations in target sequences and delivery difficulties. Although TALEN has high specificity, its applications are still restricted by the long building time and single-gene edition capacity. To sum up, CRISPR/Cas9 has promising application prospects in gene therapy, agricultural breeding and other fields because of its programmability, high efficiency and polygenic edition capability. It has become the first choice for fundamental studies and applications.

Table 1. Comparison of three technologies

	ZFNs	TALEN	CIRSPR/Cas9
Targeting principle	Protein-DNA interaction	Protein-DNA interaction	RNA-DNA interaction
Target design	Complicated and limited by sequences	Relatively simple and extensive target range	Simple and extensive target range
Building time	Long, ranging from months to weeks	Weeks	Days
Edition efficiency	Moderate	Relatively high	High
Specificity	Low, limited length of identified sequences, easy missing	High, low missing rate	Moderate; the missing rate can be lowered through high fidelity variant
Usability	High delivery difficulty, high technological threshold	High delivery difficulty, moderate technological threshold	Applicable to multiple delivery system and low technological threshold
Polygenic edition functions	Limited	Limited	Synchronous edition of multiple genes

3 Principle of CRISPR Derivation System and its Applications

3.1 CRISPR/Cas12 System and its Applications

As a core member of V-type CRISPR-Cas system, Cas12 has significant differences with Cas9 of II-type system on the gene edition mechanism and application scenes. Cas12 family (represented by Cas12a) only contains single RuvC-like nuclease structural domain and it generates interleaving sticky ends (DSB site locates at far PAM) of 5-7 nucleotide by continuous cutting of DNA double strands. Cas9 forms the blunt end or 1-nt protruding end (near PAM) through the synergistic effect of HNH and RuvC.

With respect on dependence on RNA, Cas12a can complete targeting only based on crRNA. The built-in RNase activity can process the precursor crRNA independently and support the compact crRNA array to realize polygenic edition of single vector. Cas9 processes the paired complex of crRNA and tracrRNA through the host RNase III. Additionally, Cas12a identifies T-rich PAM (e.g. TTTV) and can extend to the broad-spectrum PAM types through the engineering variants, while the typical PAM of Cas9 is NGG. Compared to Cas9, the Cas12 family shows unique advantages in virus vector delivery and complicated genome editing due to the smaller protein size (Fig.1).

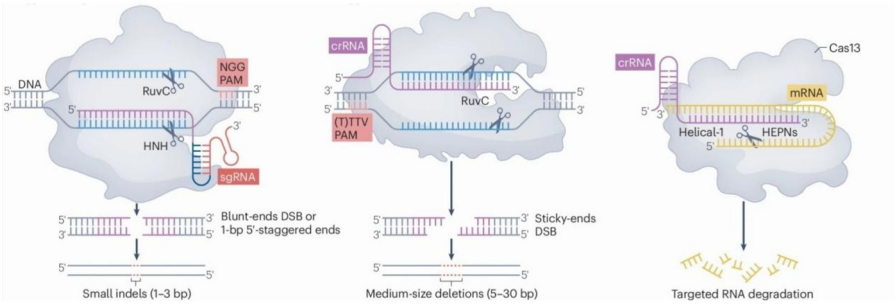

Fig. 1. Principles and comparison of Cas9 (a),Cas12a (b) and Cas13(c) [5]

Cas12 protein in the CRISPR-Cas12 system has both RNA enzyme activity and DNA enzyme activity. It can process crRNA and cut the target sites in the same time. The PAM sequences identified by the Cas 12a system are rich of A and T. The DNA double strands could be cut at the sequence stream to form sticky ends. Cas12a can cut several expression arrays of crRNA simultaneously through its nuclease functional domain to realize polygenic edition. Wang et al. used phage recombination protein RecT with Cas12a to achieve kilobase-scale multiplex editing in human cells, thus proving the high efficiency and specificity multiple genome editing capacity of Cas12a[6].

Besides, Cas12 may attack all surrounding single-stranded DNA/RNAs after target cutting and it can bind with self-extinguishing report modules easily due to its high-efficiency trans-cutting activity to produce amplified output signals. As a result, Cas12 can be applied to nucleic acid test, cut and trigger activity of the probe, and emit detectable fluorescence. The Doudna team developed a detection system DETECTR by combining the constant-temperature amplification technology and CRISPR-Cas12a, which was applied for fast and simple real-time detection of micro DNA in samples[7]. Recently, a detection system PHD (pseudo hybrid DNA–RNA) have been developed by Qiao et al. to enhance CRISPR-Cas12a's capability in RNA detection, and identify RNA biomarkers and viral DNA[8].

3.2 CRISPR/Cas13 System and its Applications

As the core member of VI-type CRISPR-Cas system, Cas13 is a type of RNA-guided RNase. It can combine with specific RNA sequences and realize cutting and degradation through crRNA getting, providing a new tool for gene expression regulation independent from DNA edition. Different from DNA editing enzymes like Cas9 or Cas12, Cas13 catalyzes the cutting of single-stranded RNA (ssRNA) through two high-order HEPN (higher eukaryotes and prokaryotes nucleotide-binding) structural domains, and it plays the critical role in virus resistance, RNA interference and post-transcriptional control (Fig.1). Specifically, Cas13d subtype (CasRx) has been extensively applied to gene silence and virus RNA suppression due to the high catalytic efficiency, compact protein size and low missing rate. It is important to note that collateral activity of Cas13 is presented after Cas13a is activated, that is, nonspecific cutting of other RNA molecules near the target RNA. Hence, Cas13a can be applied to nucleic acid test.

Cas13 guides cutting by identifying protospacer flanking sequence (PFS) of RAN targets or through the RNA structural features rather than the traditional DNA-PAM (adjacent motifs of the interval sequence), making it more flexible. Specifically, CasRx has more flexible targeting capacity because it only relies on the complementary matching of crRNA and target RNA, without the need of strict PFS. The breakage of target RNA after cutting triggers the RNA degradation mechanism of cells or realizes accurate calibration of RNA basic groups by designing the repair module.

In studies on anti-virus therapy, CRISPR-Cas13a system shows breakthrough potentials. For instance, researchers designed crRNA of specific targeting conservation area for the genome RNA of COVID-19, and delivered the Cas13a system to the infected cells through lipid nanoparticles[9].

3.3 Comparison of Cas9, Cas12a and Cas13d

Through a comparison on functional characteristics of three effect proteins (Cas9, Cas12a and Cas13d) in the CRISPR system, it disclosed the significant differentiations of target molecular types and functional applications. Cas9 and Cas12a both target at double-stranded DNA, but they have completely different action mechanism. Cas9 cuts DNA by relying on the double structural domains of HNH and RuvC, forming the 3' flat end. The PAM sequence is 3'-NNG-5' and crRNA processing requires assistance of tracrRNA, showing low flexibility of target design. Cas12a only contains the RuvC structural domain and produces the 5' sticky end after cutting. Its independent ability in pre-crRNA processing simplifies the experimental process significantly and it has more advantages in polygenic edition and high-sensitivity nucleic acid test due to its unique trans-cutting single-stranded DNA activity.

Cas13d targets at the single-stranded RNA through the double HEPN structural domains and its PFS sequences prefer A/U basic groups. It can complete RNA processing independently without DNA-PAM limitations and can realize RNA edition or degrade virus RNA directly. moreover, its trans-cutting single-stranded RNA activity provides a high-specificity tool for RNA virus detection. To sum up, Cas9 is still a main tool for DNA gene edition, Cas12a expanded the DNA control boundaries based on multiple

editions and molecular diagnosis potentials, and Cas13d develops a new way for gene regulation and anti-virus studies on the RNA level. As show in table 2.

Table 2. Comparison of Cas9, Cas12a and Cas13d

	Cas9	Cas12a	Cas13d
Target molecules	dsDNA	dsDNA	ssRNA
PAM/PFS sequence requirement	3'-NNG-5'	5'-TTTV-3'	A/U base
gRNA	crRNA, tracrRNA	crRNA	crRNA
Cas protein structural domain	HNH, RuvC	only RuvC structural domain	Double HEPN structural domains
End type	3' flat end	5' sticky end	
RNA processing	Dependent on the host RNase III and tracrRNA to process pre-crRNA	Self-processing of pre-crRNA	Self-processing of pre-crRNA
Other activities	No	Trans-cutting ssDNA	Trans-cutting ssRNA
Application scenes	DNA: gene edition, knockout, etc.	DNA: polygenic edition, nucleic acid test, etc.	RNA: RNA virus detection, RNA edition, etc.

4 Applications of Genome Editing Technology to Plants

Genome editing technology shows diversified technological paths and application potentials in plant improvement. Based on the protein-DNA recognition characteristics, ZFN cuts the IPK1 gene loci of corn accurately, decreases the phytic acid content of grains while maintaining normal growth of the plant, and decrease phosphorus emission of animal feed[10]. TALEN targets at OsSWEET14 gene of rice through the modular designed TALE proteins to decrease saccharose transfer and improve rice assistance to bacterial blight [11]. As a result, the amount of sucrose leakage decreased by 70% and the disease resistance rate exceeds 85% after pathogenic bacteria. CRISPR-Cas9 system edits three TaMLO homologous gene copy in hexaploid wheat synchronously through the flexible programming of gRNA, and it realizes stable inheritance of powdery mildew resistance under the edition efficiency of 12.3%-38.5%. Moreover, it can build multiple disease-resistance barriers through collaborative edition of Pm genes[12]. These classic technologies formed the technological spectrum from single-gene accurate regulation to collaborative optimization of complicated genomes through differential combinations of target depth, edition dimension and species compatibility.

The new CRISPR system further breaks the traditional DNA edition boundary and expands the application scenes. The CRISPR/Cas12a system recognizes T-rich PAM sequences and generates staggered DNA ends with 5'overhangs, enabling efficient multiplex editing in wheat and maize. This not only establishes highly active Cas12a

base editors but also provides a robust framework for accelerating complex genome engineering in polyploid crops, particularly for multi-gene trait enhancement.[13] The breakthrough of Cas13a shifts toward the RNA operation dimension. The potato ring rot bacteria detection system developed by the NASBA isothermal amplification technology can realize specific identification of pathogenic RNA of 10 copy/μL, and shorten the detection period to less than 2 hours. Compared to conventional PCR, this detection system not only shows higher specificity and sensitivity, but also avoids the use of complicated instruments. It is applicable to fast field test [14]. Such technological expansion from DNA edition to RNA monitoring extends the application ranges of genome editing technology from basic laboratory studies to fast field tests.

5 Conclusions

With the fast evolution of genome editing technologies, human control countries over life information are expanding continuously. Three generations of tools from ZFNs and TALEN based on protein-DNA interactions to RNA-DNA/RNA guided CRISPR system complement each other in term of target selection, edition efficiency and polygenic operation capability, and lay a technological foundation for modern accurate genetic operation. In particular, the development of CRISPR-Cas system and its derivation tools not only improves operation simplicity and editing flux, but also guides the new paradigm from DNA to RNA regulation and promotes breakthroughs in multiple fields, including gene therapy, pathogen detection and crop breeding.

In future, the further development of CRISPR system will focus on following aspects: 1) improving edition specificity and efficiency, and developing Cas variants and controllable editing modules with high fidelity. 2) Expanding the target mechanism and realizing accurate control on epigenetics, RNA modification and even the single basic group level. 3) Optimizing the delivery strategy and biosafety assessment to assure its sustainable transfer in clinics and agriculture. 4) Realizing the predictable and modular gene line projects by combining AI aided design, synthetic biology and systems biology. With deep progress in interdisciplinary integration, the gene edition technology is expected to accelerate the leapfrog from mechanism study to practical applications, and bring new vitality to disease treatment, crop improvement and bio-manufacturing.

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