BIO Web of Conferences **111**, 03025 (2024) https://doi.org/10.1051/bioconf/202411103025
ICBB 2024

CRISPR/Cas System: Mechanisms, Applications, and Limitations

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Abstract. The clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease 9 (CRISPR/Cas9) gene editing system plays a role in the inhibition of immunity in many bacteria and archaea equips with various advantages such as high efficiency, diversity, and modularity. It is now widely used to improve quality and quantity of crops to satisfy global food demand. Although these prospects are tempting, deeper understanding is still required to improve their efficiency and safety. Therefore, an overview of this special system is important. In this review, the current knowledge of different types of CRISPR/Cas system as well as their mechanisms, applications in crop breeding and limitations is briefly introduced to provide fundamental understanding and guidance for future utilization.

1. Introduction

The world is still struggling to meet the Zero Hunger target. According to Fao [1], about 3.2 billion people in the world were undernourished in 2023. Furthermore, these statistics still showed an upward trend and if this trend continues to raise, the number will rise to 840 million in 2030. Besides, food insecurity is also exacerbated by several issues including climate change [1], overexploitation of natural resources and ecosystem services, loss of agricultural land [2], food usage [3] and so on. If we cannot find a solution to change current situations, the prevalence of food insecurity at moderate or severe level shall raise and increasing number of people in the world shall fail to get access to enough food.

For the past decades, several steps have already been taken to increase food productivity by crop breeding, fertilizer application, as well as change of culture conditions. However, those traditional actions seemed a mockery in the face of such enormous challenges. Thus, scientists have to turn their attention to somewhere else to find the solution, the genome editing technology. Gene editing tools have significant advantages compared with traditional ways including high accuracy, less consumption issues and less regulatory procedures [4]. This technology begins with the finding of transposon, which was first found in 1951 by Barbara [5] and has been proven to be served as a DNA sequence that can change position within a genome. Luckily, this problem is solved with the appearance of engineered nucleases (also known as first generation gene editing tools) [6]. Those engineered nucleases have power to induce targeted DNA double-strand breaks (DSBs) and further achieve error-prone nonhomologous end joining (NHEJ) and homology-directed repair (HDR) [7], with the former being the primary pathway for the repair of DSBs in somatic plant cells.

Nevertheless, first generation gene editing tools such as Zinc-finger nucleases (ZFNs) and Transcription activator-like effectors nucleases (TALENs) equip with a verity of shortcomings such as tedious procedures, high costs, long turn-over time, as well as easy to off- -target and higher toxicity. Fortunately, with the innovation and understanding of biotechnology, second generation genome editing technology emerges as the times require. Clustered regularly interspaced short palindromic repeats (CRISPR) gene editing system equips with various advantages including high efficiency, diversity, and modularity. Nowadays, several reports have pointed out that CRISPR/Cas system can be used to regulate the genomes of plants, to achieve breakthroughs in agricultural fields [8].

This review will begin by introducing the principle of different genome editing technology before demonstrating why CRISPR/Cas9 is a better choice to guide the future genetic engineering as well as its application in crop breeding.

2. Different CRISPR/Cas systems and their applications in crop breeding

CRISPR/Cas is an adaptive immunity system which present in many archaea and bacteria. It requires only a short guide RNA (gRNA) sequence that recognizes the target loci based on base pairing. By cleaving the target DNA and generating DNA double-strand breaks (DSBs), the endonuclease activity of Cas can cause gene modification and trigger in vivo DNA repair mechanisms that result in mutations including insertions, deletions and replacement [9].To be specific, CRISPR means clustered regularly interspaced short palindromic repeats, and between those repeats, spacer sequence (a sequence works for integrating short sequence of virus or plasmid DNA homologous into the CRISPR loci) is regulatorily located.

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During immunization, the long primary transcript of a CRISPR locus is generated and processed into short CRISPR RNAs (crRNAs). After that, the foreign DNA or RNA is targeted by crRNAs and cleaved by Cas protein within the proto-spacer sequence [8].

CRISPR/Cas system can be divided into three classes based on the types of Cas, the type I systems that contain the Cas3 gene encoding protein with helicase and DNase activities, typical type II systems that contain the Cas9 gene for generating crRNA and cleaving the target DNA, and type III systems that use the Cas10 gene as marker gene. Specifically, Cas3, Ces1, Ces2, Cas7, Cas5, Cas6e, Cas1, and Cas2 genes are included in the type I systems [10]. It is noted that all of them shall one same promoter except Cas3. Type II systems can be further divided into II-A and II-B two subtypes based on the Csn2 and Csn4 gene respectively. While typical type III systems can be further classified into III-A and III-B according to the Csm2 and Cmr5 gene. (III-A contains Csm2 while III-B has Cmr5) [8].

2.1. CRISPR/Cas9

Cas9 is a special single-effector enzyme which is programmed by a single gRNA complementary to a genomic target and have power to break double-strand DNA with the help of a short trans-activating CRISPR RNA (tracrRNA). Specifically, Cas9 can bind adjacent to a Protospacer adjacent motif (PAM) sequence on the target and introduce double-strand breaks through its two nuclease domains, HNH and RuvC (Fig. 1). The class 2 type II endonuclease Cas9 which was found in Streptococcus pyogenes (SpCas9) is the first one to be widely used as a genome editing tool. Emerging research showed that Cas9 can be derived from various bacteria, such as Brevibacillus laterosporus, Staphylococcus aureus, Streptococcus pyogenes, and Streptococcus thermophilus.

As the most widely used genome editing technology, CRISPR/Cas9 is a promising tool for plant biology and the crop breeding, which has been used to precisely modify the elite cultivars. Steroidal glycoalkaloids (SGAs), plant secondary metabolites toxic to human and taste bitter, are characterized by nitrogenous steroidal aglycone and glycoside residues, of which 107 have already been identified from Solanum plants. Thus, generation of SGAs-free potato is necessary for the potato breeding. Nakayasu et al have completely abolished the SGAs α-solanine accumulation in potato hairy root by CRISPR/Cas9-mediated genome editing of the St16DOX encoding a steroid 16α-hydroxylase in SGA biosynthesis, which significantly improved the potato quality. Moreover, knock-out of the self-incompatibility gene S-RNase in diploid potatoes by CRISPR/Cas9-mediated genome editing, Huang's group successfully created the self-compatible diploid potatoes, which opens new avenues for diploid potato breeding. Specifically, a single transformation in rice through CRISPR/Cas9 technology can modify eight agronomic genes in one generation to increase population diversity. Moreover, by using CRISPR/Cas9 technology to do multiple genome edition on five JAGGAR (JAGGAR are the key factors in regulatory web of dehiscence fruit) homologous genes in oilseed rape, the mutation shows the enlarged cell size, bumpy fruit, and reduced length compared with those of the wild type. CRISPR/Cas9-based genome editing system has emerged as a powerful tool for plant functional genomics studies and genetic improvement(Fig 2).

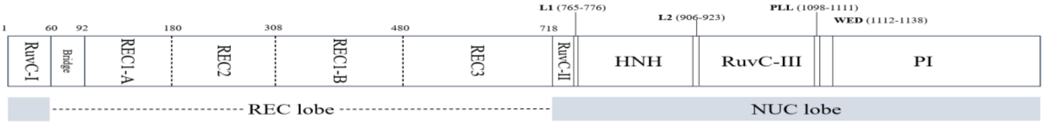

Figure 1. The structure of Cas9 protein

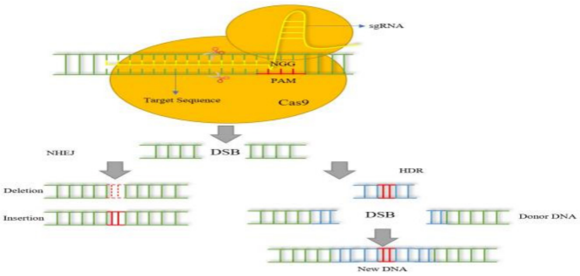

Figure 2. The mechanisms of Cas9 protein

2.2. CRISPR/Cas12

Type V Cas12a effector (also known as Cpf1) is another member of the class 2 systems which attract researchers' attentions. Compared with Cas9, Type V Cas12a effector is smaller and does not require tracrRNA to break dsDNA. It equips with RNase activity that allows itself to deal with its own g RNA array.

Cas12g is another effector that has been well reported as it not only be considered as the smallest Cas12 effector with nearly 800 amino acids, but also possesses with a variety of DNA and RNA cleavage activities. A ternary complex of target-specific gRNA and tracrRNA are asked for Cas12g to work to induce single-stranded RNA cleavage. Once processing, Cas12g would cleave single-stranded DNA and RNA in trans nonspecifically. This discovery is particularly exciting as Cas12g might be used for RNA editing as the collateral RNA cleavage could be exploited for the virus's detection as well as reveling the presence of certain mutations.

With regard to Cas12h and Cas12i, these two special effectors show strong nickase activity towards the DNA strand which not paired with the gRNA and do not need a tracrRNA to cleave single-strand DNA. Same as Cas12g, Cas12i is also described as a highly specific platform for genome editing. Further studies are required to achieve this goal.

In a parallel effort, Cas12b works similarly compared with Cas9, they both require sgRNA and tracrRNA to work and show the optimal cleavage activities. However, Cas12b only works at high temperature (48°C) which is too high to use in most cases and shows the mainly nicking activity under physiological temperature, Fortunately, with the help of specific mutations technology, introduction of mutation in DNA binding area would bring the target strand closer to the RuvC domain and lead to a cut in both DNA strands. Thus, the final engineered Cas12b is able to introduce double-strand breaks with high specificity under 37°C.

Apart from CRISPR/Cas9, CRISPR/Cas12 is currently winning broader adoption and widely used in agriculture crops including rice, tobacco, maize, soybean, tomato, and wheat. Endo's group introduced directed mutagenesis in rice and tobacco and get 47.2% and 28.2% mutation rate respectively by codon optimized FnCas12a binary vectors using Cas12a. Yin et al. successfully create loss-of-function alleles of OsEPFL9, a gene for stomatal density control in rice, using LbCas12a which increases the efficiency of water usage 8 times. It is also notable that zero off-target mutation is achieved by whole-genome sequencing analysis in the LbCas12a-edited rice. Therefore, even for now Cas12a seems less efficient, it does have a bright future in plant biology.

2.3. CRISPR/Cas13

Cas13 has nonspecific collateral trans-cleaving activity and has three experimentally characterized subtypes, Cas13a, Cas13b, and Cas13d. The discovery of Cas13 effectors begins with the research of class 2 CRISPR systems.

Three novel class 2 CRISPR systems called C2c1, C2c2, and C2c3 were discovered using bioinformatic technology. C2c1 and C2c3 are classified as type V-B Cas12b and type V-C Cas12c as they all contain RuvC-like endonucleases like Cas12a. With regards to C2c2, C2c2 is classified as Cas13 for its unique properties, the putative effector is assigned to a novel type, class 2 type VI.

Notably, Cas13a is the first class 2 effector found to work as a single RNA-gRNA-targeting protein. Two "higher eukaryotes and prokaryotes nucleotide-binding" (HEPN1 and HEPN2) domains (further proved to be associated with RNase activity) which are located on the outer surface are revealed by analysis of the Cas13a protein sequence. Further studies showed that HEPN exposes catalytic site functions with unspecific RNA cleavage activities in bacterial cells and this special HEPN site is available to all RNAs in a solution. It is also found that Cas13a is guided by a crRNA (contain a 28-nt spacer sequence) and has power to process its own pre-crRNA without the help of tracRNA. Helical1 domain is working to catalyze the crRNA maturation. Further analysis of Cas13 system's sensitivity to single and double mismatches shows that there is a mismatch sensitive "seed" region which is opposed to the 5′-seed regions found in type I and II systems in the central of the crRNA.

CRISPR/Cas13 is currently used in RNA manipulation including viral interference, RNA detection in organisms as well as RNA knockdown and producing virus resistance in plants. To be specific, Aman's group successfully introduce viral RNA interference against the Turnip Mosaic Virus (TuMV) RNA genome by Leptotrichia shahii Cas13a (LshCas13a) in Arabidopsis thaliana and Nicotiana benthamiana. Abudayyeh et al. found that inactive LwaCas13a has power to bind RNA transcripts while active Leptotrichia wadei Cas13a (LwaCas13a) can be used for mRNA knockdown in rice protoplasts. Later on, East-Seletsky et al. developed an RNA detection tool based on Cas13 system. What's more, CRISPR/Cas13 is then applied for RNA virus targeting in various plants including potato (potato virus Y), rice (rice stripe mosaic virus) and tobacco (tobacco mosaic virus). It is noted that CRISPR/Cas13 can also be used for non-coding RNA targeting in plants at the RNA level which furthermore producing virus resistance without changing genome in plants.

2.4. CRISPR/Cas14

Cas14 is well-known as its small size (nearly half size compared with other Cas effectors) which means it could act as a stand-alone CRISPR effector. It was reported that 24 variants of the Cas14 gene were explored and they were divided into 3 subgroups, cas14a, cas14b and cas14c. What's more, a RuvC nuclease domain which irrespective of sequence diversity was also founded and presented in all Cas14. However, until now, there are still no evidence to prove that Cas14 gene exists in bacteria (Cas14 is now only found in archaea) which indicates that this special gene might preform more primitive compared with other Cas genes (especially Cas9 and Cas 12).

It is reported that when transmit Cas14 into E. coli, Cas14 enzyme would work similarly as Cas12 and Cas13, it would bind and cleave a single-stranded DNA specifically without the help of PAM sequence. This may indicate that Cas14 has power to evolve to defend against single-stranded DNA viruses or mobile genetic elements which could propagate through single-stranded intermediate structures. Note that a specific gRNA sequence is required for Cas14 enzyme to start working, thus, Cas14 would perform more specifically than Cas12 or Cas13 which furthermore increase the fidelity of single nucleotide polymorphism (SNP).

CRISPR/Cas14 might be used in several fields in the future such as viral diagnosis and microbial infections due to its high efficiency in abundant in single-stranded DNA viruses' ecosystems.

2.5. CRISPR/Cas7-11

Cas7-11 is a single-protein effector which classified in the class 1 CRISPR-Cas systems and original from the fusion of a putative Cas11 domain and multiple Cas7 subunits that are derived from subtype III-D. It is reported that DiCas7-11 (Cas7-11 from Desulfonema ishimotonii) possesses interference effect against the bacteriophages and mRNAs when expressed in E.coli. Notably, DiCas7-11 works similarly to many class 2 effectors, while unique among class 1 systems. It has power to cleave RNA at target positions without detectable non-specific activity after catalyzing pre-CRISPR RNA into mature CRISPR RNA (crRNA). It is shown that when using Cas7-11 for RNA knockdown in mammalian cells, it would have no influences on cell life while the other RNA-targeting tools (Cas13 especially) are proven to be toxicity to the cell. This study expends our understanding and provides basic information of programmable RNA-targeting tools with no side effect as well as toxicity.

3. Discussion

The revolutionary CRISPR/Cas systems have undoubtedly transformed genetic engineering, offering unprecedented precision in manipulating DNA. However, the path to maximizing the efficiency and ensuring the safety of CRISPR applications requires a deeper understanding of the underlying mechanisms.

It is noted that off-target effect remains a substantial concern. Despite the remarkable precision of CRISPR/Cas technology, unintended modifications in non-target regions can occur, potentially leading to unintended consequences. Besides, the need for a more nuanced comprehension of the molecular processes involved in CRISPR-mediated editing is crucial. Delving deeper into the cellular response to CRISPR-induced changes and understanding the repair mechanisms employed by the cell can pave the way for optimizing editing outcomes. Improving the efficiency of the homology-directed repair (HDR) pathway, for instance, is a key area where research is warranted to enhance the precision and reliability of CRISPR-mediated alterations.

Overall, a deeper understanding of the intricacies of CRISPR/Cas systems is imperative for unlocking their full potential while mitigating risks. Ongoing research endeavors should focus on refining target specificity and elucidating cellular responses. Only through a holistic and comprehensive approach can we harness the power of CRISPR technology to its fullest extent while ensuring its responsible and safe application in diverse fields.

4. Conclusion

CRISPR/Cas genome editing technology has developed rapidly in recent years with advantages including simple operation, low cost, fast detection speed and high sensitivity, it has diverse applications in the field of crop breeding and has broad prospects for future development. This also reminds us that regardless of the approach and technology, as long as it has a positive impact on the industry, it is worth our attention.

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