American Academic Scientific Research Journal for Engineering, Technology, and Sciences
ISSN (Print) 2313-4410, ISSN (Online) 2313-4402
http://asrjetsjournal.org/

The Working Mechanism of CRISPR/Cas9 Genome Editing in Filamentous Fungi and its Industrial Applications

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Abstract

Understanding of regulation of gene and its functions sequence which is coding the gene and genome manipulation are very crucial. For this understanding conventional methods in many filamentous fungi are either non-functional or ineffective. Filamentous fungi plays major role in agriculture, industrial production and in medical field. A broad applications and fast development of system of CRISPR/Cas9 method has given the basic outline to comply this technology of editing of gene for many filamentous fungi. In this review, by focusing the applications of CRISPR/Cas9 in research as well as in industries we give the brief introduction of the CRISPR/Cas9 system. We also give the brief description of overall review of editing of genome, describe the construction of vector, off targeted effects and different methods of transformation of the plasmid

Keywords: CRISPR; filamentous fungi; regulation of gene; aspergillus oryzae; aspergillus niger; aspergillus nidulans.

1. Introduction

Aspergillus oryzae, Aspergillus niger, Aspergillus nidulans and Rhopzus oryzae are commonly used filamentous fungi for recombinase and enzymes production. Filamentous fungi commonly used for manufacturing of organic acid and antibiotics and heterologous proteins expression [1]. A. nidulans and N. crassa are filamentous fungi which are commonly used for basic research as a model organism. Filamentous fungi are multicellular microorganisms. The genome of filamentous fungi is more complicated the bacteria. By analysis of rudimentary bioinformatics, eukaryotic organisms are manipulate by simple genome of bacteria. Clustered regularly interspaced short palindromic repeats (CRISPR) are same sequence of bases present in the bacterial phage. CRISPR is essential part of immune system of bacteria. Host bacteria use it against frequent infection of similar phage [2].

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There are two major classes of CRISPR. It can be more divided into 6 types and 19 subtypes. Type 2 CRISPR case9 are commonly used system which include Trans activating crRNA (tracr RNA), RNase3, and CRISPR associated RNA (crRNA). CRISPR type 2 are more commonly used and simpler than other. After manufacturing of system of CRISPR/Cas9 in the cells of recipient, Cas9 protein are attached with the strand complex for the formation of complex tracrRNA-crRNApc-cas9. There two significant domain of Cas9 protein. First, HNH domain and second RuvC domain. After binding to the targeted sequence, under the guidance of sgRNA the domains cleave the double strands of the DNA. The stands of DNA which is complementary to the crRNA are cleaved by the NHN domain. The stand which is not complementary to the crRNA are cleaved by the RuvC domain [3]. After the break of double standard DNA take place DNA of genome start self-replication. As result of this replication non homologous ends of DNA are joint together and will produce the insertion, bases replacement and random loss. By the aid of exogenous fragments, the pathway of HR methods edit the gene of our interest.

1.1. Working mechanism of CRISPR case 9 genome editing

CRISPR is a family of repeats sequences of DNA which found in 90% of genome of archaea and 40% of genome of bacteria. Cas9 is a protein (DNA endonuclease) combine with tracrRNA and crRNA hybrid and form a simple immune system of bacteria [4]. It recognize foreign DNA, bind it and finally cut the foreign DNA. Protospacer adjacent motif (PAM) which is present at 3 terminus of targeted DNA. It help to recognize the foreign DNA. After recognize foreign DNA CRISPRcase9 will produce a break in double stands of DNA and will activate repair process of the host. Homologous recombination (HR) and Non homologous end joining (NHEJ) are two cellular mechanisms which play an important role in manipulation of genome and genome editing in precise manner. NHEJ has been observed as error prone repair mechanism. It produce the short deletion or insertions of bases in adjacent vicinity of double bond. If in coding region of genome or with in promoter a small deletion or insertion is present, it will disturb the working of endogenous gene. A double stranded pathway called homology repair depend on template DNA. I allow the foreign fragments of DNA to assimilate into genome of host. Homology repair only take place in cells which are dividing due to which this method has a limited applications [5].

1.2. Cas9 expression

The significant constituent of CRISPR/ Cas9 is cas9 protein. It contain about 1400 amino acids and has endonuclease functions. Firstly in archaea and bacteria CRISPR/ Cas9 was discovered. In fungi, when system of CRISPR/ Cas9 is used at both end of the cas9 gene both signals of nuclear localization and optimized codons are added [6]. Promoter strength in driving the process of transcription is very significant factor. It affect the ability of gene expression exogenously. Selection of the promoter has a great importance for activity of CRISPR/ Cas9 system. In filamentous fungi, the promoter use to drive level of expression of cas9 protein are constitutive promoter. The promoter use for the expression of protein are comparatively stable. Promoters in A.nidulans such as gpdA promoter (G-3-P dehydrogenase synthesis gene) promoter trpC promoter (tryptophan synthesis gene) are use in filamentous fungi [7]. Some studied reported expression of foreign genes of Cas9 protein has negative effect on recipient organisms. Therefore it is significant to confirm that expression of Cas9 protein has

no negative effect by testing the growth of expression strain, resistance to stress, development and many other biological features [8].

1.3. Guide RNA expression

1.3.1. Regulation of gRNA by promoter of RNA polymeraseIII and polymeraseII

gRNA which is present in CRISPR/Cas9 lack poly a tail as well as the cap structure. Mostly gRNA is regulated by the U6 promoter of RNApolymerase III. It is persistence and have base priority in driving process of transcription [9]. The analysis of bioinformatics reported that from yeast to mammal's genes of U6 small nuclear RNA (snRNA) are much conserved. In many filamentous fungi system of CRISPR/Cas9 having U6 promoter for transcription of gRNA is used [10]. The system of CRISPR/CAS9 was also optimized and efficiency of expression of tRNA promoter was tested. There are four tRNA promoter i.e. ptRNAGlyTCC, ptRNATyrGTA, ptRNAGlyGCC and ptRNALeu-TAA. The efficiency of these promoters is very high than others U6 promoter. It shows that promoter of tRNA can be useful for expression of gRNA as well. The efficiency of various promoter of tRNA are depended of stain. In various cases, efficiency of the genome editing which is based on tRNA remains debatable. For effective expression of gRNA the gene of 5SrRNA is used as a promoter. For expression of the sgRNA 38bp nucleotides sequence present at upstream site of 5SrRNA gene are fused with sgRNA sequence to make cassette expression of sgRNA [11]. There are many limitaions of transcription of gRNA by U6 and U3 promoters. In most organisms these promoter are transcribe by using RNApolIII. First, U3snRNA and U6snRNA are called housekeeping gene that means they are expressed ubiquitously. Therefore they are not able to produce tissue specific as well as cell specific gRNA. Second, Due to unavailability of polymerase 3 at commercial level the use of U3 and U6 promoter in traditional Invitro gene transcription of sgRNA is not propitious [12]. Ribozymes have nucleases activity and it is use to construct an artificial RGR gene (ribozyme-gRNA-ribizyme U6snRNA). At 3′ terminal there is a hepatitis D virus (HDV) and at 5′-terminal there is a hammerhead (HH). Under control of RNA polymerase II in yeast this system was introduce. To complete the cleavage of targeted DNA transcription of ADHI gene by pol II. Ribozyme-gRNA-ribozyme is constructed by using the terminator of trpC (TtrpC) and by using promoter of gpdA (PgodA). It was applied in the many filamentous fungi very successful manner.

1.4. Delivery of CRISPR/Cas9 into cells of fungi

The system is delivered to cell by using the vector carrying the expression cassettes of sgRNA and Cas9. Conventionally these cassettes (Cas9 and sgRNA) are carried into double and single vector. Zhang and his colleagues (2016) confirmed effectiveness of these two approaches of expression in model organism Aspergillus fumigatus. He reported that the efficiency of the vector of single expression was significantly higher than the double expression vector. It is because of the transformation of vector in cells of fungi cannot be exactly undercontrol. There two methods for the transformation of CRISPR/Cas9 in to cells of fungi. 1) Agrobacterium mediated transformation (AMT) 2) polyethylene glycol (PEG) transformation. Many studies reported Poly ethylene glycol is most convenient methods for transformation of cassettes of gRNA and Cas9 into cells of fungi [12]. For conventional editing of genome the most common method is Argobacterium mediated transformation.

But for the transformation CRISPR/Cas9 Argobacterium is not a frequently use method. Many studies reported that Argobacterium is most convenient method for genome editing of many fungi.

1.5. Regulation of HR and NHEJ pathway

Cas9 generated the double standard breaks can be exposed to mutagenesis by NHEJ. If the donor DNA is delivered to cells another method of repairing called homology repair (HR) can be attained. The pathway of homology repair is different from route of repair of NHER. The repair mechanism of NHEJ with low fidelity can take place in G1 phase of the cell cycle. The homology repair mechanism has high fidelity rate and follows replication of DNA. In repair method of NHEJ, it directly attach to DSBs present at terminals of DNA. Two protein ku80 and ku70 play a vital role for the attachment of NHEJ. In homology repair mechanism, it requires to offer a target site [13]. The system of CRISPR/Cas9 use the homology repair method foe precise editing of target. The insertion of a particular sequence and mutation at targeted site can be achieved by this method.

1.6. CRISPR/Cas9 induce the mutation and off targeted effects

1.6.1. Mutation

Many studies reported that 50% of cases of CRISPR/Cas9 caused single base mutations (mostly T and A). Rest of 50% causes the small deletions of the nucleotides bases (1-50bp). The substitution of (two or more) bases is very exceptional. In filamentous fungi, mostly used system of CRISPR/Cas9 knockout only use a single gene either by insertion of single gene or by deletion of small fragment of nucleotides. If in a gene more than two sites are targeted between the targeted sites deletion of a several hundred Kbp gene fragments may take place [14].

1.6.1. Off target effects

The characteristics of system of CRISPR/Cas9 are high efficiency, simple procedure and high accuracy has broad application in medical and biological research. The main concerns are the off sides effects of CRISPR/Cas9. Off targeted effect of system are divided into two main categories. Firstly, more than 3 impatience mismatch cannot distinguish by CRISPR/Cas9 at site of DNA. Secondly, high concentration of CRISPR/Cas9 the probability of effects of off target increase. To overcome these problems scientists design the DNA plasmid which after editing of genome remove the gene of Csa9 inserted. Scientists in [15] developed a system of CRISPR/Cas9 in which sgRNA are put together with Cas9 for the formation of a stable RNP. The transformation of this complex is take place by electroporation or poly ethylene glycol (PEG) methods. Some methods which are based on sequencing techniques (next-generation) are used for detection of off targeted sites. These methods are ChIP-seq [16], GUIDE-seq [17] and Digenome-seq [18].

2. Industrial applications to enhance the production of desirable products and suppression of secondary metabolites

Filamentous fungi produce a large number of enzymes which play a very significant role in production of

different industrial products like food, detergents, textiles and feed. For the production of many fermented food and recombinant protein filamentous fungi are use. Filamentous fungi are also a vital host for secondary metabolites production in many industries. For example, the genes of biotin and four subunits of acetyl-coA carboxylase (ACC) are overexpress in photorhabdus liminescens. The overexpression of these genes in the cells of microorganisms makes the metabolical burden. To overcome this problem biotin is required as a supplement in the medium at industrial scale. So genetic engineering is requires to increase the production of these valuable metabolites from the filamentous fungi. To resolve this problem, scientists have established many new strategies to enhance the production of different products but it is appropriate to limited no of filamentous fungi. The use of genome editing method of CRISPR/Cas9 in many fungi which are important at industrial scale significantly enhance the production capability of these filamentous fungi.

Aspergillus oryzae is a filamentous fungi which is used in industry for the production of cellulose. In japan it is a very significant fungus for conventional fermentation for production of many proteins as well as enzymes. For increasing the production of these compounds and enzymes it is essential to modify the fungus by genetic engineering. Scientists in [19] developed a method of CRISPR for genetically modify the Aspergillus oryzae. They modify the fungus by using process of mutagenesis which have very low efficiency. To improve the effectiveness of genome editing method of CRISPR/Cas9 they use a plasmid carrying AMA1. It is a self-replicating nucleotides sequence which enhances the efficiency of mutation in wild strain of Aspergillus oryzae to 60-100%. The multiple copies of AMA1 plasmid was existing in fungal stain. They enhance the expression level of sgRNA and Cas9 and eventually escalate efficiency of mutation of Aspergillus oryzae [19].

3. Conclusion

Filamentous fungi played very significant role in development of drugs, biodegradation of different substances, agriculture and production at industrial scale. As technique for the sequencing of genome improve and decrease the cost. Editing of the genome by using CRISPR/Cas9 system enables the manipulation of the genome. The system of CRISPR/Cas9 technique could significantly help in utilization of filamentous fungi as biological factories. For medicine industries manipulation of genes of different secondary metabolites in genome of filamentous fungi is used for production of biological active products. The technology has some lacks like off sites an effect so there is still requirement to improve the efficiency of editing. After faulty gene repair the function of genome editing will continue which increase the risk of off sites effects.

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