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BIOTECHNOLOGICALLY RELEVANT FILAMENTOUS FUNGI OBTAINED BY THE SYSTEM 'CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS AND ASSOCIATED PROTEINS'

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Abstract

Several strains of filamentous fungi have been used to produce wide spectrum of natural products including organic acids, antibiotics, other useful proteins for centuries. Besides of these innate features, due to having versatile abilities, many filamentous fungi can be used int he fields of bioproduction as host microorganisms. Despite the relevant importance and dense use of filamentous fungi in biotechnology, detailed knowledge about the molecular biology of the metabolism is not available most of them except a few model fungi. Targeting biosytnhetic genes to edit can be achieved existing tools, however, these genetic tools are inefficient and difficult to employ in wide array of filamentous fungi by the reasons such as the low editing efficiency and the therefore large amount of labor time. Recently, CRISPR/Cas9 has become growing gene-editing technology due to significant advantages over existing editing tools such as high efficiency, easy operation, the possibility of multigene editing This technology has been started to introduce to various species of filamentous fungi since 2015. The loss or gain of function of such mutant alleles is the major application of CRIPSR-Cas mediated genome engineering. In this review, state-on-art applications of the CRISPR/Cas9 technology in several filamentous fungi were summarized and the further prospects of this technology briefly discussed.

Key words: filamentous fungi, genome editing, CRISPR/Cas9 technology, strain improvement

1. Introduction

Fungal natural products, particularly recombinant proteins and metabolites, greatly contribute in agriculture, food, textile, pharmaceutical and bioremediation industries. Several species of natural producer fungi are however, not ideal hosts for bioproduction due to the lack of efficient genetic manipulation techniques, their slow growth rates, low yields, or vulnerabilities to environmental perturbations. Therefore, the heterologous expression of the synthetic pathways for natural product synthesis in microbial cells have attracted great attention (2). The utilities of these fungi as industrial cell factories and as model organisms for research are trying to be improved by the development of highly efficient genome editing systems (3).

Nuclear genome sequences and annotations of the genes of hundreds of filamentous fungi have been established by the development and advent of DNA sequencing techniques. However, most of the gene clusters, particularly for the secondary metabolites, are not known since challenges that are encountered during the application of molecular tools in filamentous fungi.

Besides, advanced developments in genome engineering in filamentous fungi for the genes included in biosynthesis of secondary metabolites, enzyme production, and to develop resistance against phytopathogens, have shown promising outcomes. The approaches those available for yeast and bacteria are not as efficient as due to the additional morphological and cellular complexities of filamentous fungi such as; multicellularity, cellular differentiations of the cells, having thick chitinous cell walls and the lack of effective expression systems (4,5). The main limiting factors are also counted as follows: (i) filamentous fungi have a more complex genetic background than those of the yeast and bacteria (ii) The efficiency of homologous recombination in fungi is very low, usually less than 5 % (iii) the limited available screening markers, and not all screening markers are effective for filamentous fungi (6).

In recent years, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and -associated proteins (Cas) constitute the CRISPR/Cas system, as a technology, has been found promising to solve the difficulties and improve the efficiency of genomic alterations in filamentous fungi (7). Genome editing by CRISPR/ Cas9 has several advantages as an editing technology due to its high efficiency and straightforward design (8). In brief, Cas9 endonuclease as a counterpart of this system is guided by a single chimeric RNA (sgRNA) to a specific locus in DNA, where it introduces a double strand break (DSB) in the sequence. The protospacer of the sgRNA that defines the target DNA, can be virtually any 17-20 bp nucleotide sequences found adjacent 5′-NGG DNA motif (the PAM). CRISPR/Cas9 based technologies rely on the cell repair mechanisms for the fixing the DSB. The DSB introduced by the Cas9 nuclease is repaired either by the error-prone non-homologous end-joining (NHEJ) mechanism or by homologous recombination (HR) if a DNA sequence with homology close to the DSB is available. The system can also be used both for the creation of gene deletions and insertions (InDels) in a specific locus or loci in target sequence(s) (9). In filamentous fungi, substantial homology lenghts (~1 Kb) with near 100 % homology are required to achieve efficient homologous replacement. One strategy to circumvent this would be to increase either the expression of genes involved in promoting HR or decrease expression of genes involved in non-homologous end joining (NHEJ). *hfd*A resulted in efficient disruption of three genes related to beta-lactam anitibiotic synthesis (10).

Now, as useful genome engineering or genome editing technologies are becoming available for several genera of industriallly important filamentous fungi species, new opportunities for the discovery and biosynthesis of desired quality and quantity of natural products such as secondary metabolites, enzymes and engineering their biosynthetic pathways for industrial applications will be more possible. As a pioneer, *Aspergillus*, has made significant progress and establishment of the CRISPR-Cas 9 technology and this host holds a lot of pomise for a broad range of applications (11). However, as the system compared with other organisms, there is still need to improve in filamentous fungi.

2. Experimental Research

-Genetic manipulation approaches to facilitate the fungal strain improvement: Gene disruption gene expression

Genetic manipulations opened an avenue for basic biological research in model filamentous fungi at the beginning of 1970s. Since Mishra and Tatum (1973) first reported the successful DNA transformation of the filamentous fungus *Neurospora crassa*, the development of molecular biological research in filamentous fungi has been so rapid (12). High efficiency manipulation approaches have been facilitated the fungal strain improvement as well as the elucidation of fungal molecular mechanisms. The first genetic transformation in *Aspergillus*

was achieved in 1983. Over the past three decades, scientists have attempted to develop various tools or strategies to improve genetic manipulations in filamentous fungi (5)**.** Earlier efficient systems were based on creating selection markers in genes. One selection marker recycle method which is called the 'Cre/LoxP' system, has allowed multiple genes manipulation in filamentous fungi. The Cre/LoxP system was adapted from P1 bacteriophage and was composed with a recombinase named Cre and two corresponding consequent *LoxP* sites. In this sytem, *LoxP* sites are deleted by creating a selection marker in a gene using Cre that digest at the two consequent *LoxP* sites. Similarly a 'FLP/FRT system, composed of FLP recombinase with corresponding FRT sites, is also used for marker recycling in filamentous fungi, but is derived from the yeast *S. cerevisiae* (13). Those two methods offer possibilites for multigene manipulation need two steps to achieve gene recycle. For example, the LoxP sites as well as a marker gene need to be integrated into the host genome first, then the Cre needs expression to finish the gene deletion process. A precise selection process of the second step is necessary for successful application of the two methods. However, these working steps to excise the marker cassette leaves scars in the genome with unknown potential effects. One of the breakthroughs of genetic manipulation is the development of gene targeting by Homologous Recombination (HR). HR-mediated gene targeting produces highly precise alterations and the desired recombination events ocur. However, its infrequency (1 in 10^6–10^9 cells), have been indicated an enormous challenge for large-scale applications of gene-targeting studies. Altough some fungi such as *Saccharomyces cerevisiae*, *N. crassa*, and *Aspergillus* spp. may have a relatively high HR efficiencies, gene disruption can not be simple for many non-model fungi due to this challange. Therefore, to overcome these challenges and enable targeted and efficient modification of a variety of eukaryotic and particularly mammalian species, a series of programmable nuclease-based genome editing technologies that are meganucleases (derived from microbial mobile genetic elements), zinc finger (ZF) nucleases (based on eukaryotic transcription factors), transcription activator-like effectors (TALEs- from *Xanthomonas* bacteria) and most recently the RNA-guided DNA endonuclease Cas9 (from the type II bacterial adaptive immune system CRISPR) have been developed in recent years (14,15).

-Toolbox for the CRISPR/Cas9 systems in filamentous fungi: promoter platforms for transcription and expression strategies

Of the current generation of genome editing technologies, the most rapidly developing is the class of RNA-guided endonucleases -widely known as Cas9- from the mostly bacterial adaptive immune system CRISPR, which can be targeted to virtually any genomic location of choice by a short RNA guide (15). From the economic perspective of filamentous fungi involved in biotechnological processes, such as *Trichoderma reesei*, *Aspergillus niger* and *A. oryzae*, gene expression is also an important issue. Novel hosts are preferred those with superior native characteristics such as high resistance to extreme conditions, specific metabolic traits and efficient protein secretion. However the lack of the suitable expression systems and other obstacles related to gene editing systems for filamentous fungi are the main problems encountered in gene manipulation. In this context, CRISPR/Cas9 based systems as versatile platforms for precision genome editing in wide range of organisms offer obvious advantages over other systems. Programming of Cas9 to recognize new targets is much easier than other systems that function by inducing DSBs TALENs or zinc-finger nucleases. A controllable and conditional CRISPR/Cas9 system has been established first in *Trichoderma reesei* use to disrupt *ura5* gene in 2015, since then various versions have been developed in several filamentous fungi (5,16-18).

All of the CRISPR/Cas9 systems require functional host transcription and translation systems for the expression of the foreign Cas9 and sgRNA. For that, appropriate promoters must be

chosen and optimized promoters are required as first. Another important aspect is expression strategy of the sgRNA. Three expression strategies have been established for the expression of sgRNAs *in vitro* and *in vivo*. First, sgRNAs were generated by *in vitro* transcription and subsequently used to co-transform the protoplasts together with a *in vivo* or *in vitro* (as second strategyCas9, as in the *T. reesei*, *A. niger*, and *A. fumigatus*. The Cas9 and sgRNA transcribed together *in vitro* subsequently form the Cas9-sgRNA ribonucleoprotein (RNP) complex to edit target genes. The first CRISPR/Cas9 system in *Trichoderma reesei* using specific codon optimization and *in vitro* RNA transcription was established by Liu et al. (5). The establishment of a genome-editing system can be used to develop *T. reesei* as super cell factory for ligninolytic enzyme preparations and other heterologous proteins.

As the the third strategy, both the Cas9 and sgRNA can be generated *in vivo* also. However, in this case there is an issue of the stability of sgRNAs. An efficient promoter that can facilitate gRNA transcription *in vivo* is a bottleneck of adoption of CRISPR/Cas 9 system. Therefore, it is important to find reliable promoters to drive gRNA transcription *in vivo* that can result in efficient genome editing to expand its utility as a model and industrial organism. gRNA expression has a key importance for this strategy since RNA Polymerase III promoters could not be recognized by most of the filamentous fungi. Therefore, the most common way to express gRNA *in vivo* was to use two ribozyme sequences, those of 5′ terminal hammerhead (HH) and 3′ terminal hepatitis delta virus (HDV), located on the flank of the gRNA. Self splicing ability of tRNA has been used to seperate multiplex gRNAs in plants and recently in filamentous fungi. Song et al. (19) have shown that tRNA promoter-mediated gRNA expressions are reliable and efficient in genome editing in *A. niger*. Except $tRNA^{Gln2}$, the tRNA promoters tested in this study lead to high rate of mutation (82-97 %). In endogeneous U6 promoter driven platforms, the mutation rate is highly variable among different fungal species and ranges from 10-100 % sometimes failes to transcribe. These studies show that the type of promoter used to transcribe gRNA has a high impact on CRISPR/Cas9 activity and represents an important technical limitation in development of this editing system (21). In order to find a more efficient promoter, Zheng et al. (21) have established a new sgRNA expression strategy using *5S rRNA* gene of *A. niger* which is a basic cellular component that is highly conserved and abundant in cells. They fused with the sgRNA sequence to construct a sgRNA expression cassette and showed that CRISPR/Cas9 system of *5S rRNA* gene promoter showed a gene disruption efficiency close to 96% which is significantly higher than existing *PhU6* (Homo sapiens RNU6-1 gene) or *PanU6* promoter systems. Consistent with the higher gene disruption efficiencies, the intracellular sgRNA transcription levels driven by the 5S rRNA internal promoter were found significantly higher than that driven by the diverse U6 promoters as shown with quantitative reverse transcription PCR (RT-qPCR) data and demonstrated high gene disruption/insertion/deletion efficiency, even for DNA fragments up to 48 kb (Fumonisin B1) with 100% efficiency. This easy to construct and deliver CRISPR/Cas9 system based on this strategy can be broadly applicable in other eukaryotes.

To drive the expression of Cas9 protein, three common promoters -*trpC* promoter of *A. nidulans* tryptophan synthesis gene *trpC*, the *gpdA* promoter of the *A. nidulans* glyceraldehyde 3 phosphate dehydrogenase gene *gpdA*, and *TEF1* the promoter of the *A. nidulans* translation elongation factor 1α- have been used (19). In addition, *xlnA*, *Ham34*, *amyB*, *niiA*, *Otef* (modified *tef*1) and *hsp70* have also been used successfully (3,22). Cas9 was either integrated in the genome, or expressed from a plasmid containing the AMA1 (extrachromosomal, autonomous maintenance in *Aspergillus*) sequence or the *U. maydis* ARS element (20).

The efficient application of the CRISPR/Cas9 system requires the heterologous expression of the Cas9 gene fused to nuclear localization signal (NLS) as well as simultaneous expression of

the sgRNA. In order to improve gene editing efficiency, researchers prefer to optimize the Cas9 and NLS genes based on the codon usage of the targeted filamentous fungi themselves, or alternatively to directly select a stronger NLS to efficiently target fusion protein into the cell nucleus (5,23).

In general, transcription of the Cas9 gene by strong constitutive promoters to improve the efficiency of genome editing, may lead to the uncontrollability of the CRISPR/Cas9 system in filamentous fungi and thus contribute to possible off-target effects (24). In order to realize the purpose of controllability of this system, some inducible promoters such as *Pcbh1* and *PniiA* have been utilized to inhibit Cas9 expression under repressing conditions for minimal off-target effects (8). The control of gene expression of Cas9 was achieved in *A. fumigatus* akuBK80 by an inducible doxycycline dependent tet^{ON} promoter for functional reconstitiution of of *tynC* (25).

There have been many different approaches developed to deliver the CRISPR-Cas9 system into an organism from plasmids harboring different genes and cassettes purified Cas9 protein along with the synthesized gRNA. The traditional approach of transfecting the CRISPR-Cas9 system through expression plasmids can be challenging and has certain limitations. To make the process of integrating the CRISPR-Cas9 system more rapid, Cas9 proteins/gRNA ribonucleoprotein complexes were directly introduced into the cell and thus removing the need for cellular transcription and translation of Cas9.

To facilitate RNA guided mutagenesis in a broad range of filamentous fungi, four different CRISPR-Cas9 plasmids have been constructed each containing a commonly used fungal marker, AFUM_*pyrG*, *AN_argB*, ble^R or hyg^R, as well as the AMA1 sequence, which has been shown to support replication in many different fungal species (7).

Most of the currently developed CRISPR/Cas9 systems have been utilized primarily for functional characterization of genes some other applications, including investigation of the subcellular localization of proteins and gene regulation, have been recently reported in filamentous fungi (14). However, there are also other approaches such as genome minimization. Genome minimization might enable the generation of secondary metabolite cluster-free strains, which would be not only safer for food and industrial applications, but also a very useful chassis for discovery and production of novel bioactive secondary metabolites. Alternatively, iterative and highly efficient precision gene editing experiments would enable deletion of entire gene families, such as secreted proteases, which might enhance industrial production of useful proteins (20).

Penicillium chrysogenum is filamentous fungus which is known for its capability to produce ß-lactam antibiotics in high titers and a wide variety of other secondary metabolites, several of which are potential interest for novel applications in pharma, agriculture, food or feed industries (8). Genome editing to redirect primary metabolism and mend impaired regulation mechanisms of the producer are possible ways to increase productivity. However, in order to incorporate novel genetic elements into *P. chrysogenum* is not easy since typically relevant long (at least 700 bp) flanking regions are required. Using the CRISPR/Cas9 technology, correct integration for selection markers with homology recombination flanks as short as 60 bp. Thus, short HR flanks can be used when marker based genome editing with CRISPR/Cas9 system is performed.

Marker-free gene editing and multiple gene editing/modifications in filamentous fungi are also required for achieving indsutrial production of enzymes and secondary metabolites. Some reseacrchers have established approaches that are based on AMA-1 autonomous replicating

plasmid. For instance, the CRISPR/Cas9 system for *A*. *nidulans* which is based on an AMA1 based autonomously replicating vector carrying genes encoding Cas9 and the sgRNA is allowed for efficient gene deletion/integration with little or no adaptation and can potentially be used in many fungi (26, 27). AMA-1 based replicating plasmid based recycling technique involving *Aoace-2* conditional expressing cassette was established in *A. oryzae* strains (28). These strains are usually preferred for Japanese traditional food fermentation and production of recombinant enzymes. In this study, a new genome-editing vector, ppAsAcas9, containing the AMA1 sequence for autonomous plasmid replication to improve the efficiency of CRISPR/Cas9-mediated mutagenesis have been constructed and enabled highly efficient mutagenesis.

On the other hand, the deletion of *ku70* or *ligD* by homologous recombination has been shown that dramatically facilitated the genetic manipulation of *A. oryzae*. However, the deletion of *ku70* or *ligD* by homologous recombination would be more laborious in *A. oryzae* industrial strains because their multinucleate conidia make it difficult to isolate homokaryotic transformants. Therefore, a versatile and convenient technology for genetic engineering is much more efficient molecular breeding of industrial strains (3).

Recently Liu et al. (17) have successfully use the CRISPR/Cas9 system for increasing the production of cellulase in *Myceliophtora thermophila* and *M*. *heterothallica*. In this study, the genes *cre-1, res-1, gh-1* and *alp-1* responsible for synthesizing cellulase were disrupted simultaneously leading to hyper cellulase producing strains.

With inactivation of the functional parts of the HNH and RuvC domain, the Cas9 nucleases become reppressor-like proteins that can block the transcription progress, which is calles CRISPR interference (CRISPRi). If a regulatory part, a repressor or enhancer is combined with a Cas9 protein with DNA targeting ability, a single gene or a series of genes can be regulated (29).

3. Conclusions

Since 2015, the CRISPR/Cas9 system in genome editing has been established to edit the genomes of several genera of filamentous fungi such as *Aspergillus* spp., *Pyricularia oryzae*, *Neurospora crassa*, *Myceliophthora* spp., *Phytophtora sojae*, *Talaromyces atroroseus*, *Trichoderma reesei*, *Ustilago maydis*, *Penicillium chrysogenum.*

Although there are still challenges with the current CRISPR-Cas9 system, for instance the stability of sgRNAs, the rapid development of this technology will contribute to the advancement of science and the translation of this science in industry by the use of efficient targeted mutagenesis (30). There is still need for tools to facilitate introduction of mutations such as scripts to allow the identify protospacers that engineer filamentous fungi.

4. References

1. Shanmugam K, Ramalingam S, Ventakatamaran G, Hariharan GN. The CRISPR/Cas9 system for targeted genome engineering in free-living fungi: advances and oppurtunities for lichenized fungi. Front in Microbiol 2019; doi: 10.3389/fmicb.2019.00062.

2. Xu X, Liu Y, Du G, Ledesma-Amaro R, Liu L. Microbial chassis development for natural bioproduct synthesis**.** TIBTEC 2020; https://doi.org/10.1016/j.tibtech.2020.01.002.

3. Katayama T, Tanaka Y, Okabe T, Nakamura H, Fuji W, Kitamoto K, Maruyama J. Development of a genome editing technique using the CRISPR/Cas system in the industrial filamentous fungus *Aspergillus oryzae*. Biotechnol Lett 2016; 38: 637–642.

4. Jiang D., Zhu W., Wang Y., Sun C., Zhang K.Q., Yang J. Molecular tools for functional genomics in filamnetous fungi: recent advances and new strategies. Biotechnol Adv 2013; 31: 1562-1574.

5. Liu R, Chen L, Jiang YP, Zhou ZH, Zou G. Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. Cell Discov 2015; 1:15007.

6. Mei Y-Z, Zhu Y-L, Huang P-W, Yang Q, Dai C-C. Strategies for gene disruption and expression in filamentous fungi. Appl Microbiol Biotechnol 2019; 103: 6041-6059.

7. Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH. A CRISPR/ Cas9 system for genetic engineering of filamentous fungi. PLoS One 2015; 10:e0133085.

8. Pohl C, Kiel JAKW, Driessen AJM, Bovenberg RAL, Nygard Y. CRISPR/Cas9 based genome editing of *Penicillium chrysogenum*. ACS Synthetic Biol 2016; 5 (7): 754-764.

9. Sander JD and Joung JK. CRIPSR-Cas systems for editing, regulating ad targeting genomes. Nat. Biotechnol 2014; 32(4): 347-355.

10. Kück U, Hoff B. New tools for genetic manipulation of filamentous fungi. Appl Microbiol Biotechnol 2010; 86: 51-62.

11. Krappmann S. CRIPSR-Cas 9, the new kid on the block of fungal molecular biology. Med Mycol 2017; 55: 16-23.

12. Shi T-Q, Liu G-N, Ji R-Y, Shi K, Song P, Ren L-J et al. CRISPR/Cas9 based genome editing of the filamentous fungi: state of the art. Appl Microbiol Biotechnol 2017; 101(20): 7435-7443.

13. Kopke K, Hoff B, Kück U Applications of *Saccharomyces cerevisiae* FLP/FRT recombination system in filamentous fungi for marker recycling and construction of knockout strains devoid of heterologous genes. Appl Environ Microbiol 2010; 76(14):4664-4674.

14. Wang Q, Coleman . Progress and challenges: Development and implementation of CRISPR/Cas9 technology in filamentous fungi. Comp and Struc Biotechnol 2019; J 17: 761-769.

15. Hsu PD, Lander ES, Zhang F. Development and applicatons of CRISPR-Cas9 for genome engineering. Cell 2014; June5: 157(6): 1262-1278.

16. Zhang C, Meng X, Wei, Lu L. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. Fungal Genet Biol 2015; 86: 47–57.

17. Liu Q, Gao R, Li J, Lin L, Zhao J, Sun W et al. Development of a genome-editing CRISPR/Cas9 system in thermophilic fungal *Myceliophthora* species and its application to hyper-cellulase production strain engineering. Biotechnol. Biofuels 2017; 10:1. https://doi.org/10.1186/s13068-016-0693-9.

18. Yin CM, Fan XZ, Shi DF, Gao H. CRISPR/Cas genome editing technology and its application in fungi. Biotechnol Bull 2017; 33: 58–65.

19. Song L, Ouedraogo J.-P, Kolbusz M, Nguyen TTM, Tsang A. Efficient genome editing using tRNA promoter-driven CRISPR/Cas9 gRNA in *Aspergillus niger*. PLoSOne 2018; 13(8): e0202868. https://doi.org/10.1371/journal. pone.0202868.

20. Song R, Zhai Q, Sun L, Huang E, Zhang Y, Zhu T et al. CRISPR/Cas9 genome editing technology in filamentous fungi: progress and perspective. Appl Microbiol Biotechnol 2019; 103: 6919-6932.

21. Zheng X, Zheng P, Zhang K, Cairns TC, Meyer V, Sun J et al. 5srRNA promoter for guide RNA expression enabled highly efficient CRISPR/Cas9 genome editing in *Aspergillus niger*. ACS in Biol 2019; 8(7): 1568-1574.

22. Zhang Y, Luan X, Zhang H, Garre V, Song Y, Ratledge C. Improved γ-linolenic acid production in *Mucor circinelloides* by homologous overexpressing of delta-12 and delta-6 desaturases. Microb Cell Factories 2017; 16:113. https://doi.org/10.1186/s12934-017-0723-8.

23. Fang, Y. and Tyler, B.M. The Oomycete *Phytophthora sojae* uses non-canonical nuclear localization signals to direct proteins into the nucleus. Fung Genet Rep 61 2015; (Suppl.), Abstract #165.

24. Shi T-Q, Liu G-N, Ji R-Y, Shi K, Song P, Ren L-J, et al. CRISPR/Cas-9 based genome editing of the filamentous fungi: the state of the art. Appl Microbiol Biotechnol 2017; DOI 10.1007/s00253-017-8497-9.

25. Weber J, Valiante V, Nodvig CS, Mattern DJ, Slotkowski RA, Mortensen UH et al. Functional reconstitution of a fungal natural product gene cluster by advanced genome editing. ACS Synt Biol 2017; 6(1): 62-68.

26. Nielsen ML, Isbrandt T, Rasmussen KB, Thrane U, Hoof JB, Larsen TO et al. Genes linked to production of secondary metabolites in *Talaromyces atroroseus* revealed using CRISPR/ Cas9. PLoS One 2017; 12:e0169712.

27. Sarkari P, Marx H, Blumhoff ML, Mattanovich D, Sauer M, Steiger MG. An efficient tool for metabolic pathway construction and gene integration for *Aspergillus niger*. Bioresour Technol 2017; 245 (PtB): 1327-1333.

28. Katayama T, Nakamura H, Zhang Y, Pascal A, Fujii W, Maruyama J-I. Forced recycling of an AMA-1 genome editing plasmid allows for efficient multiple gene deletion/integration in the industrial filamentous fungus *Aspergillus oryzae*. Appl Environ Microbiol 2019; 85(3): 01896-18.

29. Larson MH, Gilbert LA, Wang X, Lim WA, Weismann JS, Qi LS. CRISPR interference (CRISPRi) for sequence specific control of gene expression. Nat Protoc 2013; 8(11): 2180-2196.

30. Lau V, Davie JR. The discovery and development of the CRISPR system in applications in genome manipulation. Biochem and Cell Biol 2017; 95(2): 203-210.