Progress in Research Regarding Genetic Manipulation in *Aspergillus Oryzae*

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Abstract: *Aspergillus oryzae* is a kind of important industrial microorganism, used in food fermentation, condiment production, brewing, recombinant protein and enzyme production. Currently, genetic engineering techniques are increasingly being used to improve fermentation performance and product yield of *A. oryzae*. However, unlike other filamentous fungi, such as *monascus ruber* or *Aspergillus niger*, *A. oryzae* has inherent resistance to the common antibiotics, which renders genetic manipulation difficult. In the past, the genetic transformation of *A. oryzae* has mainly relied on protoplast-mediated transformation. The *Agrobacterium tumefaciens*-mediated transformation system, developed in 2016, and the CRISPR/Cas9 gene editing system, has also been successfully applied in *A. oryzae*. In this review, we have summarized the progress in research on genetic manipulation and gene editing methods in *A. oryzae*.

1 INTRODUCTION

Aspergillus oryzae, an important production strain certified safe by FDA and WHO, has been used in traditional food fermentation, flavoring production, and brewing in Asia for a long time; in addition, its importance in modern biotechnology industries, such as those producing recombinant proteins and enzymes, has increased (Wang, 2021). *A. oryzae* genome was sequenced in 2005 (Machida M, 2005). Currently, genetic engineering techniques are increasingly being used to improve its fermentation performance and product yield (Fleissner A, 2010). However, unlike other filamentous fungi, such as *monascus ruber* or *Aspergillus niger*, genetic manipulation in *A. oryzae* was difficult as the commonly used selection marker and transgenic methods cannot be used in this organism. Recently, multiple selection markers and *Agrobacterium tumefaciens*-mediated transformation (ATMT) have been developed, which have facilitated genetic manipulation in *A. oryzae*. Gene editing refers to the precise cutting, insertion or mutation of specific sites in the genome of recipient cells, to realize the specific modification of the genome. As a key reverse genetics research method, gene editing technology is an important approach for functional genome research and genetic modification. It significantly promotes the development of synthetic biology and has important applications in fungal genetics and breeding and it is a research hotspot of fungal synthetic biology (Kumar A, 2021). The known gene editing systems have been used in *A. oryzae*. However, as *A. oryzae* possesses multinucleate mycelia and conidia, homozygous gene-edited strains are hard to be obtained using traditional gene editing systems, which is unlike that in *monascus ruber* and *Aspergillus niger* (Kitamoto K, 2015). In this review, we have summarized the progress in research regarding selection markers, transgenic methods, and gene editing technology in *A. oryzae*.

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2 SELECTION MARKERS USED FOR GENETIC ENGINEERING OF *A. ORYZAE*

The use of suitable selection markers is important for successful genetic manipulations, as markers determine the feasibility of transformation, reduce the probability of obtaining false-positive transformants, and minimize the screening workload. *A. oryzae* has inherent resistance to the common antibiotics used as fungal transformation selectors, such as hygromycin B, geneticin (G418), phleomycin and bleomycin (Suzuki S, 2009). Therefore, antibiotics are seldom used as selection agents for *A. oryzae* genetic manipulation.

Auxotrophic and drug resistance-related genes are most commonly used for *A. oryzae* genetic manipulation. The fungal orotidine-5-monophosphate (OMP) decarboxylase, a commonly used auxotrophy marker that can convert orotidine into uridine (the precursor of uracil), is encoded by *pyrG*. Wild type *A. oryzae* cannot grow in a medium containing 5-fluoroorotic acid (5-FOA), as OMP decarboxylase can convert non-toxic 5-FOA into toxic 5-fluorouracil, which inhibits growth. *pyrG* mutants can grow in medium containing 5-FOA and uridine/uracil (Jiang, 2013). Therefore, the *pyrG* mutant can be selected using 5-FOA and uridine/uracil supplementation in the medium, and uridine/uracil auxotrophy can be used as the selection marker for genetic transformation (Nguyen KT, 2016). Other auxotrophic genes, such as *argB* (encoding ornithine carbamoylase), *niaD* (encoding nitrate reductase), *adeA* (encoding aminoimidazole nucleotide synthetase), and *adeB* (encoding phosphoramidyl carbazole carboxylase) have also been used as selection markers (Table 1) for genetic transformation of *A. oryzae*. Wild type *A. oryzae* is sensitive to pyrithiamine (PT), the researchers obtained the PT-resistance gene *ptrA* from a PT-resistant *A. oryzae* mutant and used it as a selection marker for transformation (Kubodera T, 2002). This provides an effective resistance screening marker that facilitates the screening of transformants and promotes molecular biology research in *A. oryzae*.

3 TRANSGENIC METHODS USED FOR *A. ORYZAE* GENETIC ENGINEERING

The transformation method is the second-most important factor affecting the outcomes of genetic engineering. Usually, filamentous fungi are transformed by two methods, protoplast-mediated transformation (PMT) and *Agrobacterium tumefaciens*-mediated transformation (ATMT). PMT is usually mediated by polyethylene glycol (PEG)-$CaCl_2$, and involves the formation of particles [which include PEG, divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}), and exogenous DNA] on the surface of protoplasts; subsequently, the particles are absorbed into the protoplast via endocytosis (Liu, 2012). The preparation of highly efficient protoplasts plays a pivotal role in PMT, as the state of protoplasts considerably affects transformation efficiency. The advantage of PMT is that introduction of exogenous genes into protoplasts is relatively straightforward. However, it also has several limitations, including complicated procedures for preparing and cultivating protoplasts and the low regeneration frequency of the transformed protoplasts (Nguyen KT, 2016). ATMT can not only transform plants, but can also be used to transform bacteria, animals, and fungi. In fungi, the first successful genetic manipulation via ATMT was performed in yeast (Bundock P, 1995). Currently, ATMT is being successfully used for transforming *A. nidulans, Neurospora crassa*, *A. awamori*, *A. niger*, *Monascus* sp., and other filamentous fungi (Chen, 2011). ATMT is easier to perform than PMT (Idnurm A, 2017), as it only requires the co-cultivation of spores and *Agrobacterium tumefaciens* harboring the target gene, followed by selection in screening media. The target gene is randomly inserted into the genome and inherited stably by the newly divided cells. Furthermore, the transformation efficiency is high. However, attempts toward establishment of an ATMT system in *A. oryzae* have been unsuccessful until Nguyen et al. first established ATMT using uracil auxotrophy as a screening marker in *A. oryzae* in 2016 (Nguyen KT, 2016). We also developed a dual selective marker ATMT system using uridine/uracil auxotrophy and pt resistance genes as selection markers (Sun, 2019). The establishment of ATMT system considerably promoted the identification of functional genes of *A. oryzae*. The methods and selectable markers used for transformation of *A. oryzae* are shown in Table 1.

4 GENE EDITING TECHNOLOGY IN *A. ORYZA*

4.1 Homologous Recombination for Gene Editing in *A. oryzae*

Early gene editing technology mainly takes advantage of homologous recombination (HDR) to replace the target genes. Therefore, adding homologous arms on both sides of foreign DNA sequences can realize the accurate integration of foreign sequences (Komor A, 2017). However, in eukaryotes, the frequency of homologous recombination is very low, and foreign DNA sequences are more likely to be randomly integrated into other sites on the genome, resulting in off target effect. Knock out genes required for non-homologous end joining (NHEJ), such as *ku70*, *ku80*, *kusA* and *ligD*, was very effective for increasing the HDR efficiency (Jiang, 2013; Kwon MJ, 2019).

4.2 ZFNs, TALENs and CRISPR/cas9 System for Gene Editing in A. oryzae

Studies have found that the double strand breaks (DSBs) that occur at specific DNA sites on the genome can greatly improve the efficiency of homologous recombination (Porteus, 2003). Therefore, in recent years, researchers have successively developed artificial specific endonuclease to cleave double strand breaks at genome-specific DNA sites. Currently, the most widely used nucleases including: clustered regularly interspaced short palindromic repeats (CRISPR), transcription activator-like effector nuclease proteins (TALENs) and zinc-finger nucleases (ZFNs) (Rajat, 2014). CRISPR/cas9 system is the most widely used technology at present (Kitamoto K, 2015; Suzuki S, 2009). In the first two techniques, specific DNA-binding proteins fused with endonucleases to cleave the double-stranded DNA (dsDNA), resulting in DNA DSB at specific sites (Rajat, 2014). However, the CRISPR/Cas9 technique takes advantage of single guide RNA (sgRNA) to guide Cas9 endonuclease to cleave the DNA double strand, generating DSBs at the desired site (Zheng, 2017). Then, the DSB is repaired via NHEJ or HDR. The NHEJ error-prone features can be utilized to trigger the mismatching of nucleobase pairs, resulting in the non-deterministic editing of the target gene. For HDR, the desired DNA sequence can act as the template to replace the target gene, thus to realize accurate gene editing.

ZFNs were first used in animal cells. However, as the design of ZFNs is complex, the cost of ZFNs is high, and the editing efficiency depends on the sequences of target DNA, which limits the development of this technology. Until now, ZFNs have not been used in *A. oryzae*. TALENs is the second generation gene editing technology and it has been successfully applied in many species (Joung JK, 2013). Recent research showed it can also work in *A. oryzae*. For example, *sC* and *ligD* can be successfully knocked out in *A. oryzae* by transient expression of high-efficiency platinum-fungal TALENs (PtFg TALENs) (Mizutani O, 2017). CRISPR/Cas9 is considered a breakthrough in the field of gene editing due to its versatility and efficiency, as only the Cas9 endonuclease and the corresponding gRNA have to be induced in vivo. In many species, CRISPR/Cas9 system has been successfully applied. In 2016, the CRISPR/Cas9 system was successfully used to edit the genes of *A. oryzae* (Katayama T, 2016). The plasmids expressing the gene encoding Cas9 (codon optimized) nuclease and sgRNAs were transformed into *A. oryzae* strain via PMT, and the target genes were successfully knocked out by exploiting the error-prone property of NHEJ. However, the mutation efficiency of transformants was only 10% to 20%, and the most common induced mutations were 1 bp deletions or insertions (Katayama T, 2016). The low efficiency of traditional CRISPR/Cas9 system in *A. oryzae* may due to its multinucleate mycelia and conidia.

4.3 Optimization CRISPR/Cas9 System in A. oryzae

Researchers have attempted to improve the efficiency of the CRISPR/Cas9 system in *A. oryzae*. The optimization mainly focused on three aspects: first, using NHEJ related gene deletion strains (*Δku70*, *Δku80*, *ΔkusA* and *ΔligD*) to increase HDR for accurate gene editing. Sometimes, it needs to replace, insert or delete the target gene by HDR. Therefore, using CRISPR/Cas9 system in NHEJ deficiency mutant strain can increase the HDR efficiency. This is a useful strategy for many species. However, it has not been reported in *A. oryzae*, which may due to the reason that other strategies were enough to realize accurate gene editing by HDR in *A. oryzae*. The second strategy is to increase the expression levels of Cas9 and gRNA using different promoters or autonomous replication plasmid. To increase the Cas9 and sgRNA levels, strong promoters were selected for their expression.

For example, the *A. oryzae amyB* promoter was used for Cas9 expression and the promoter of *Aspergillus Niger* U6 RNA polymerase III (PU6) was used for a heterologous expression of sgRNA (Katayama T, 2016). In addition, the use of the autonomous replicating plasmid containing *Aspergillus nidulans* AMA1 (allows for autonomous plasmid replication) can also increase the expression of Cas9 and sgRNA, thereby increasing the gene editing efficiency (Katayama T, 2019). Another way of gene editing involves transformation of the assembled Cas9-CRISPR gRNA RNP complexes in vivo (Jie, 2019). Compared with the expression of Cas9 and gRNA in vivo, this strategy has obvious advantages, as the amount or rate of Cas9 translation or gRNA transcription cannot limit the assembly of Cas9-gRNA RNP, and it can protect gRNA from degradation. Recently, this method was also successfully used in *A. oryzae*. For example, Zou et al. used vitro-assembled RNP in *A. oryzae* protoplast using chemical reagents to improve the transformation efficiency of CRISPR-Cas9 RNP (Zou, 2020). Furthermore, they also added inositol and benomyl to control the cell division and mitotic cycles, respectively, which increased the formation of mononuclear protoplasts. The mononuclear transformation of *A. oryzae* increased significantly increased from 0% to 40.0% with inositol and to 71.43% with benomyl (Zou, 2020). As using autonomous replicating plasmid greatly improved gene editing efficiency, multiple gene editing with one plasmid was realized. For example, Takuya et al. expressed two gRNA molecules from one gene editing plasmid and edited two genes (Katayama T, 2019). In addition, this approach can be used to replace or insert DNA sequences by co-transforming a circular donor DNA (Katayama T, 2019; Nodvig CS, 2018). Furthermore, a marker-free gene editing

Table 1: The selection markers and transformation method of *A. oryzae.*

Strains	Origin of stain	Selection markers/selection mechanisms	Transgenic methods	Ref.
niaD300	Mutagenesis of RIB40	Nitrate reductase gene (*niaD*)/*niaD*-bearing strains only grow in media with NO_2^- as sole nitrogen; the transformants can grow in media with NO_3^- as sole nitrogen source.	PMT	(Unkles SE, 1989)
FN-16 Δ*amdS*	Mutagenesis of FN-16	Acetamidase-encoding gene (*amdS*)/transformants can grow in the presence of sucrose and CsCl, but the growth of untransformed strains was restricted.	PMT	(Gomi K, 1992)
NS4	UV mutagenesis of *niaD300*	ATP sulfurylase gene (*sC*)/the *sC* mutants are SeO_4^- resistant and CrO_4^- sensitive, and cannot use NO_3^- and SO_4^- as sole nitrogen and sulfur sources.	PMT	(Yamada O, 1997)
PTR26	Mutagenesis of HL1034	Pyrithiamine (PT) resistance gene (*ptrA*)/transformants can grow on media supplemented with pyrithiamine.	PMT	(Kubodera T, 2002)
SE29-70	HowB425 Δ*pyrG*	5-aminolevulinate synthase (*hemA*)/deletion of *hemA* resulted in a lethal phenotype that could be rescued by the supplementation of 5-aminolevulinic acid or *hemA.*	PMT	(Elrod SL, 2000)
NSR13/NSR1	UV mutagenesis of NS4	Mutants of adenine genes (*adeA/adeB*)/*AdeA/adeB* failed to grow without adenine; minimal medium supplemented with adenine restored their growth.	PMT	(Jin, 2004)
NSAR1	Gene knocked out of NSR13	The ornithine transcarbamylase (OTCase) (*argB*)/*ArgB* deletion mutants did not grow in the absence of arginine; growth was restored after complementation.	PMT	(Nguyen KT, 2016)
Bm-resistance mutant	RIB40 wild type	Bleomycin (Bm)-resistance expression cassette (*BmR*)/disruption of *ligD* with *BmR* replacement to enhance the susceptibility of *A. oryzae* to Bm.	PMT	(Suzuki S, 2009)
AUT1-PlD/ AS11, C2/ VS1ΔpyrG	RIB40/3.042/VS1	OMP decarboxylase gene (*pyrG*)/cells lacking *pyrG* are uridine/uracil auxotrophic mutants and resistant to 5-FOA; wild-type and *pyrG* transformants did not survive in the presence of 5-FOA.	PMT	(Zhu, 2013)
RIB40/*ΔpyrG*	Gene knocked out of RIB40	Cells lacking *pyrG* are uridine/uracil auxotrophic mutants and resistant to 5-FOA.	ATMT	(Jiang, 2013)
3.042/ΔpyrG	Gene knocked out of 3.042	Pyrithiamine (PT) resistance gene and OMP decarboxylase gene/mechanisms are the same as described above.	ATMT	(Sun, 2019)

Table 2: Gene editing method in *A. oryzae.*

Strains	Selection markers	Transgenic methods	Editing methods	Optimization methods	Ref.
BCC7051/NID1/ NRRL2270ΔpyrG	*pyrG/argB*	PMT	CRISPR/Cas9	Optimized promoter (PU6, PU3 and tRNA)	(Nodvig CS, 2018; Song, 2018)
NSAR1	linear neo cassette	PMT	CRISPR/Cas9	Transcribed gRNA in vitro	(Zheng, 2017)
RIB40/RIB128/RIB915	*ptrA*	PMT	CRISPR/Cas9	Construct including an AMA1-based autonomously replicating plasmid	(Katayama T, 2019)
NSAR1	*pyrG*	PMT	CRISPR/Cas9	Assembled Cas9-CRISPR gRNA ribonucleoprotein (RNP) complexes in vitro	(Zou, 2020)
PFJo218	*pyrG*	PMT	CRISPR/Cas9	Used Single-stranded Oligo nucleotides as Gene-Targeting Substrates (GTS)	(Nodvig CS, 2018)
NSAR1	*pyrG*	PMT	CRISPR/Cas9	Chemical reagents were added	(Zou, 2020)
RIB40	*ptrA/niaD*	PMT	CRISPR/Cas9	Expressed two gRNAs from a single genome-editing plasmid	(Katayama T, 2019)
NID1	*pyrG*	PMT	CRISPR/Cas9	Transcripted two sgRNAs from a single tRNA-spacer	(Nodvig CS, 2018)
NSAR1	linear neo cassette	PMT	CRISPR/Cas9	Transcribed two sgRNAs in vitro	(Zheng, 2017)
RIB40	*ptrA/niaD*	PMT	CRISPR/Cas9	Used conditional expression of *Aoace2* in plasmid to force plasmid recovery	(Joung JK, 2013)

system has also been developed in *A. oryzae*. Takuya et al. used an inducible promoter to express *Aoace2*, a gene causing cell lysis, in the gene editing plasmid (Katayama T, 2019). Then, the transformed plasmid can be removed by inducing *Aoace2* after the target gene edition finished. Thus, this method can be used for the repeatable genetic engineering of *A. oryzae*. The optimizations of gene editing methods in *A. oryzae* are shown in Table 2.

5 CONCLUSIONS

A. oryzae genome was completely sequenced in 2005. So far, the genomes of 6 different *A. oryzae* strains have been sequenced. Owing to the development in molecular biology techniques and increase in investigations regarding *A. oryzae*, genetic transformation of this fungus for improving traits or producing new products has received an impetus. *Agrobacterium tumefaciens*-mediated transgenic technology will further promote the application of reverse and forward genetics in functional gene research in *A. oryzae*. The gene editing technology is a powerful tool for the identification of functional genes. Nowadays, the gene editing system in *A. oryzae* mainly depends on CRISPR/Cas9. And the accurate gene editing main depends on HDR as the NHEJ editing gene was non-deterministic. However, the efficiency of HDR was much lower than that of NHEJ, which limits the application of this system. Recently, the single base editor system was also developed based on CRISPR/Cas9 (Komor A, 2016). This system allows accurate and efficient mutation of cytosine (C) to thymine (T) without generating dsDNA breaks. This technique is more efficient than other gene editing techniques, and will not produce side effects, such as random insertion and deletion. Combination of this method with the existing gene editing technologies will promote the identification of functional genes of *A. oryzae* and lay a theoretical and technical foundation for genetic modification.

REFERENCES

Bundock P, Den DA, Beijersbergen A, Hooykaas PJ. (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. Embo Journal. 14:3206-3214.

Chen X, Yang J, Peng Y. (2011) Large-scale insertional mutagenesis in *Magnaporthe oryzae* by *Agrobacterium tumefaciens*-mediated transformation. Methods in molecular biology (Clifton, NJ). 722:213-224.

Elrod SL, Jones A, Berka RM, Cherry JR. (2000) Cloning of the *Aspergillus oryzae* 5-aminolevulinate synthase gene and its use as a selectable marker. Current Genetics. 38:291-298.

Fleissner A, Dersch P. (2010) Expression and export: recombinant protein production systems for *Aspergillus*. Applied microbiology and biotechnology. 87:1255-1270.

Gomi K, Kitamoto K, Kumagai C. (1992) Transformation of the industrial strain of *Aspergillus oryzae* with the homologous *amdS* gene as a dominant selectable marker. Journal of Fermentation & Bioengineering. 74:389-391.

Idnurm A, Bailey A, Cairns T, Elliott C, Foster G, Ianiri G, et al. (2017) A silver bullet in a golden age of functional genomics: the impact of *Agrobacterium*-mediated transformation of fungi. Fungal biology and biotechnology. 4:6.

Jiang D, Zhu W, Wang Y, Sun C, Zhang KQ, Yang J. (2013) Molecular tools for functional genomics in filamentous fungi: recent advances and new strategies. Biotechnol Adv. 31:1562-1574.

Joung JK, . Sander JD. (2013) TALENs: a widely applicable technology for targeted genome editing. Nature Reviews Molecular Cell Biology. 14:49-55.

Jie Q, Sun W, Lin S, Rong J, Ma L, Yi L. (2019) Cytosolic delivery of CRISPR/Cas9 ribonucleoproteins for genome editing using chitosan-coated red fluorescent protein. Chemical Communications. 55.

Jin FJ, Maruyama J, Juvvadi PR, Arioka M, Kitamoto K. (2004) Adenine Auxotrophic Mutants of *Aspergillus oryzae*: Development of a Novel Transformation System with Triple Auxotrophic Hosts. Journal of the Agricultural Chemical Society of Japan. 68:656-662.

Kumar A, Chadha S, Rath D. (2021) CRISPR-Cas9 system for functional genomics of filamentous fungi: applications and challenges.

Kitamoto K. (2015) Cell biology of the Koji mold Aspergillus oryzae. Bioscience, biotechnology, and biochemistry. 79:863-869.

Kubodera T, Yamashita N, Nishimura A. (2002) Transformation of *Aspergillus sp.* and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. Bioscience Biotechnology & Biochemistry. 66:404-406.

Komor A, Badran A, Liu D. (2017) CRISPR-Based Technologies for the Manipulation of Eukaryotic Genomes. Cell. 169:559.

Kwon MJ, Schutze T, Spohner S, Haefner S, Meyer V. (2019) Practical guidance for the implementation of the CRISPR genome editing tool in filamentous fungi. Fungal Biol Biotechnol. 6:15.

Katayama T, Tanaka Y, Okabe T, Nakamura H, Fujii W, Kitamoto K, et al. (2016) Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. Biotechnol Lett. 38:637-642.

Katayama T, Nakamura H, Zhang Y, Pascal A, Fujii W, Maruyama JI. (2019) Forced Recycling of an *AMA1*-Based Genome-Editing Plasmid Allows for Efficient Multiple Gene Deletion/Integration in the Industrial Filamentous Fungus *Aspergillus oryzae*. Appl Environ Microbiol. 85.

Komor A, Kim Y, Packer M, Zuris J, Liu D. (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 533:420-424.

Liu Z, Friesen T. (2012) Polyethylene glycol (PEG)-mediated transformation in filamentous fungal pathogens. Methods in molecular biology (Clifton, NJ). 835:365-375.

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, et al. (2005) Genome sequencing and analysis of *Aspergillus oryzae*. Nature. 438:1157-1161.

Mizutani O, Arazoe T, Toshida K, Hayashi R, Ohsato S, Sakuma T, et al. (2017) Detailed analysis of targeted gene mutations caused by the Platinum-Fungal TALENs in *Aspergillus oryzae* RIB40 strain and a *ligD* disruptant. J Biosci Bioeng. 123:287-293.

Nguyen KT, Ho QN, Pham TH, Phan TN, Tran VT. (2016) The construction and use of versatile binary vectors carrying *pyrG* auxotrophic marker and fluorescent reporter genes for *Agrobacterium*-mediated transformation of *Aspergillus oryzae*. World J Microbiol Biotechnol. 32:204.

Nodvig CS, Hoof JB, Kogle ME, Jarczynska ZD, Lehmbeck J, Klitgaard DK, et al. (2018) Efficient oligo nucleotide mediated CRISPR-Cas9 gene editing in *Aspergilli*. Fungal Genet Biol. 115:78-89.

Porteus, M. H. (2003) Chimeric Nucleases Stimulate Gene Targeting in Human Cells. Science. 300:763-763.

Rajat, M., Gupta, Kiran, Musunuru. (2014) Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. Journal of Clinical Investigation.

Suzuki S, Tada S, Fukuoka M, Taketani H, Tsukakoshi Y, Matsushita M, et al. (2009) A novel transformation system using a bleomycin resistance marker with chemosensitizers for *Aspergillus oryzae*. Biochemical & Biophysical Research Communications. 383:42-47.

Sun Y, Niu Y, He B, Ma L, Li G, Tran VT, et al. (2019) A Dual Selection Marker Transformation System Using *Agrobacterium tumefaciens* for the Industrial *Aspergillus oryzae* 3.042. J Microbiol Biotechnol. 29:230-234.

Song L, Ouedraogo JP, Kolbusz M, Nguyen TTM, Tsang A. (2018) Efficient genome editing using tRNA promoter-driven CRISPR/Cas9 gRNA in *Aspergillus niger*. PLoS One. 13:e0202868.

Unkles SE, Campbell EI, Ruiterjacobs YMJTD, Broekhuijsen M, Macro JA, Carrez D, et al. (1989) The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: A convenient and general selection system for filamentous fungal

transformation. Molecular & General Genetics Mgg. 218:99-104.

Wang L, Hu T, Jiang Z, Yan Q, Yang S. (2021) Efficient production of a novel alkaline cold-active phospholipase C from Aspergillus oryzae by molecular chaperon co-expression for crude oil degumming. Food chemistry. 350:129212.

Yamada O, Lee BR, Gomi K. (1997) Transformation System for Aspergillus oryzae with Double Auxotrophic Mutations, niaD and sC. Journal of the Agricultural Chemical Society of Japan. 61:1367-1369.

Zheng YM, Lin FL, Gao H, Zou G, Zhang JW, Wang GQ, et al. (2017) Development of a versatile and conventional technique for gene disruption in filamentous fungi based on CRISPR-Cas9 technology. Sci Rep. 7:9250.

Zou G, Xiao M, Chai S, Zhu Z, Wang Y, Zhou Z. (2020) Efficient genome editing in filamentous fungi via an improved CRISPR-Cas9 ribonucleoprotein method facilitated by chemical reagents. Microb Biotechnol.

Zhu L, Maruyama JI, Kitamoto K. (2013) Further enhanced production of heterologous proteins by double-gene disruption (ΔAosedD ΔAovps10) in a hyper-producing mutant of Aspergillus oryzae. Applied Microbiology & Biotechnology. 97:6347.