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Review Paper

Research advances of genetic manipulation methods in *Aspergillus oryzae*

Hui Huang, Yunlong Sun, Yali Niu, Bin Zeng and Zhihong Hu*

Jiangxi Key Laboratory of Bioprocess Engineering and Co-Innovation Center for In-vitro Diagnostic Reagents and Devices of Jiangxi Province, College of Life Sciences, Jiangxi Science and Technology Normal University, Nanchang 330013, China.

*Corresponding author. E-mail: Tel: +86-15797865372; Fax:(+86) 0791-88537850.

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ABSTRACT

Aspergillus oryzae is an important industrial micro-organism. An increasing number of researchers are beginning to utilize genetic engineering techniques to improve its fermentation performance and product yield. However, unlike other filamentous fungi, *A. oryzae* showed inherent resistance to the common antibiotics, hence, its genetic manipulation is more difficult than that of common fungi. In the past years, the genetic modification of *A. oryzae* is mainly dependent on protoplast-mediated transformation (PMT). Until 2016, the *Agrobacterium tumefaciens*-mediated transformation (ATMT) system was developed. This review summarized the research advances of genetic manipulation as well as, genome editing methods.

Key words: *Aspergillus oryzae*, selection marker, genetic manipulation, protoplast-mediated transformation, *Agrobacterium tumefaciens*-mediated transformation.

INTRODUCTION

As an important industrial micro-organism, *Aspergillus oryzae* becomes more and more important in modern biotechnology industries such as enzyme and recombinant protein production (Yamada et al., 2014). An increasing number of researchers are beginning to utilize genetic engineering techniques to improve its fermentation performance and product yield (Ichishima, 2016). However, unlike other filamentous fungi, the genetic manipulation was difficult as the common used selection marker and transgenic methods are not suitable in *A. oryzae*. In recent years, the multiple selection markers and ATMT developed in *A. oryzae* genetic manipulation, which greatly facilitated the functional gene research of *A. oryzae*. This review summarized the research advances in transgenic methods as well as, gene editing technology in *A. oryzae*.

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

The first issue to be considered in undertaking genetic manipulations is selection markers. On one hand, a suitable selection marker determines whether the transformation can be performed, and on the other hand, it can reduce the probability of false-positive transformants and minimize the screening workload (Degefu and Hanif, 2003).

Auxotrophic genes and drug resistance genes are the most commonly used selection markers for *A. oryzae* genetic manipulation (Kitamoto et al., 2005). The main auxotrophy marker gene used is the fungus-derived orotidine-5-monophosphate (OMP) decarboxylase, which can convert orotidine into uridine (the precursor of uracil), encoded by the *pyrG* gene. Wild-type *A. oryzae* cannot grow in a media supplemented with 5-fluoroorotic acid (5-FOA), as OMP decarboxylase can convert non-toxic 5-FOA into the toxic product-5-fluorouracil, which inhibits growth. When *pyrG* is mutated, the mutant can grow in the media supplied with 5-FOA and uridine/uracil (Du et al., 2014).

The *pyrG* mutant can be screened by 5-FOA and uridine/uracil supplementation, while the uridine/uracil auxotroph can then be used as the selection marker for genetic transformation (Du et al., 2014). 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

Table 1: Other auxotrophic genes used as selection markers for genetic transformation of *A. oryzae*.

Strains	Origin of stain	Selection markers/Selection mechanisms	Transgenic methods	References
niaD300	Mutagenesis from RIB40	Nitrate reductase gene (*niaD*)/*niaD* only grow in the media with NO_2^- as sole nitrogen, the transformants can grow in the media with NO_3^- as sole nitrogen	PMT	(Unkles et al., 1989)
FN-16 Δ*amdS*	Mutagenesis from FN-16	Acetamidase-encoding gene (*amdS*)/Transformants can grow in the presence of sucrose and CsCl but the growth of untransformed strains was very restricted	PMT	(Gomi et al., 1992)
NS4	Ultraviolet mutagenesis from niaD300	ATP sulfurylase gene (*sC*)/The *sC* mutants are SeO_4^- resistant and CrO_4^- sensitive, which cannot use NO_3^- and SO_4^- as sole nitrogen and sulfur sources	PMT	(Yamada et al., 1997)
PTR26	Mutagenesis of nitroguanidine from HL1034	Pyrithiamine (PT) resistance gene (*ptrA*)/The transformants can grow on the media supplied with pyrithiamine	PMT	(Kubodera et al., 2002)
SE29-70	HowB425 Δ*pyrG*	5-aminolevulinate synthase (*hemA*)/Deletion of *hemA* resulted in a lethal phenotype that could be rescured by the supplementation of 5-aminolevulinic acid (ALA) or by complementation of wild-type *hemA*	PMT	(Elrod et al., 2000)
NSR13/NSR1	Ultraviolet mutagenesis from NS4	Adenine genes (*adeA/adeB*)/*AdeA/adeB* mutants failed to grow without adenine, minimal medium supplemented with adenine restored their growth	PMT	(Jin et al., 2004)

markers (Table 1) for genetic transformation of *A. oryzae* (Nguyen et al., 2017; Jin et al., 2004).

The common antibiotics used as selection agents for fungal transformation are hygromycin B, geneticin (G418), bleomycin, and phleomycin (Suzuki et al., 2009; Shoko, 2011). However, *A. oryzae* shows inherent resistance to these antibiotics. Therefore, antibiotics are seldom used as selection agents for *A. oryzae* genetic manipulation. Wild-type *A. oryzae* is very sensitive to the vitamin B_1 antagonist pyrithiamine (PT). The PT-resistance gene-ptrA was cloned from the PT-resistant *A. oryzae* mutant and used as a selection marker for transformation (Kubodera et al., 2002). This provides an effective resistance screening marker that facilitates the screening of transformants and greatly promotes molecular biology research in *A. oryzae*.

TRANSGENIC METHODS USED FOR *A. ORYZAE* GENETIC ENGINEERING

The second issue to be considered is the transformation method. Usually, there are two methods for transforming filamentous fungi. One is a PMT and the other is ATMT. PMT is usually mediated by PEG-$CaCl_2$. Its main principle is to form particles, which include PEG and divalent cations such as Mg^{2+}, Ca^{2+}, and Mn^{2+} and exogenous DNA on the surface of protoplasts; thereafter, the particles are absorbed into the protoplast by endocytosis (Kim et al., 2009). The preparation of highly efficient protoplasts plays a pivotal role in the PMT method as the state of protoplasts has a great impact on transformation efficiency. The advantage of PMT is that introducing exogenous genes into

Table 1 (Continuation): Other auxotrophic genes used as selection markers for genetic transformation of *A. oryzae*.

Strains	Origin of stain	Selection markers/Selection mechanisms	Transgenic methods	References
NSAR1	Gene knock out from NSR13NSR13	The ornithine transcarbamylase (OTCase) (*argB*)/*ArgB* delection muants failed to grow in the absence of arginine, restored with complementation test	PMT	(Jin et al., 2004)
Bleomycin-resistance mutant	RIB40wild type	Bm-resistance expression cassette (*BmR*)/Disruption of *ligD* gene with *BmR* replaced using Triton X-100 and chlorpromazine to enhance the susceptibility of *A. oryzae* to Bm	PMT	(Suzuki et al., 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 could not survive in the presence of 5-FOA	PMT	(Du et al., 2014; Nguyen et al., 2017; Zhu et al., 2013; Ji et al., 2013)
RIB40/*ΔpyrG*	Gene knock out from RIB40	Cells lacking *pyrG* are uridine/uracil auxotrophic mutants and resistant to 5-FOA	ATMT	(Nguyen et al., 2017)
3.042/ΔpyrG	Gene knock out from 3.042	Pyrithiamine (PT) resistance gene and OMP decarboxylase gene/mechanisms	ATMT	(Sun et al., 2019)

protoplasts is relatively straightforward. However, there are objective deficiencies, including complicated procedures for preparing and cultivating protoplasts and the low regeneration frequency of transformed protoplasts (Nguyen et al., 2005).

Recent research has shown that the ATMT method can not only transform plants but can also be used to transform bacteria, animals, and fungi (Michielse et al., 2005). In fungi, the first successful genetic manipulation by ATMT was conducted in yeast (Bundock et al., 1995). At present, ATMT has been successfully used for transformations of *Aspergillus nidulans, Neurospora crassa*, *Aspergillus awamori*, *A. niger*, *Monascus sp*., and other filamentous fungi (Weld et al., 2006). Compared with PMT, ATMT is easier to conduct. It only requires the co-cultivation of spores and *Agrobacterium tumefaciens* carrying the target gene, followed by selection in screening media (Idnurm et al., 2017). The target gene was randomly inserted into the genome and inherited stably by newly divided cells. Moreover, the transformation efficiency was high. However, the attempts to establish an ATMT system in *A. oryzae* did not succeed until 2016, when Nguyen et al. (2016) established ATMT in *A. oryzae* using uracil auxotrophy as a screening marker for the first time.

Our laboratory also established an ATMT system by using uridine/uracil auxotrophy and PT-resistance genes as selection markers, and we developed a dual selectable marker ATMT system (Sun et al., 2019). The establishment of ATMT system greatly promotes the identification of functional genes of *A. oryzae*. Table 1 shows the methods and selectable markers for transformation of *A. oryzae*.

GENE EDITING TECHNOLOGY IN *A. ORYZAE*

The successful application of gene editing technology in *A. oryzae* mainly includes Cre/*Loxp*, TALEN and CRISPR/Cas9. Cre/*Loxp* can be used as a selection marker recycle method for multiple gene integrations and consecutive gene deletion system in *A. oryzae* (Zhang et al., 2017). For example, the 75-kb gene cluster was introduced into *A. oryzae* genome for the heterologous production of a novel cyclic peptide compound using the Cre/*Loxp* system (Yoshimi et al., 2018).

TALEN and CRISPR/Cas9 technologies have been reported in yeast, fish, plants and mammals. In recent years, they have also been reported in filamentous fungi such as *Rinderomyces oryzae*, coarse pulmonomycetes, *Aspergillus fumigatus* and *Aspergillus structuralis*. In 2017, Mizutani team successfully knocked out the sC and *ligD* genes in *A. oryzae* using TALEN techniques by PMT (Mizutani et al., 2017). Katayama team successfully knocked out the *pyrG* gene in 2015 by CRISPR/Cas9 technology and constructed a uracil-deficient *A. oryzae* strain (Katayama et al., 2015), while in 2017, the team knocked out the *ligD* gene by CRISPR/Cas9 technology, which improved the efficiency of gene editing of *A. oryzae* (Nakamura et al., 2017).

FUTURE PERSPECTIVES

The genome sequencing of *A. oryzae* was completed in 2005. And so far, the genomic sequence of six (6) different *A. oryzae* strains were also completed. As *A. oryzae* become more and more widely used in industry, it is very necessary to conduct genetic modification to improve traits or to produce new products. The development of transgenic technology, especially the ATMT technology will promote the research of molecular and cell biology in *A. oryzae*. Combining with the gene editing technology, it will greatly promote the identification of functional genes of *A. oryzae* and lay a theoretical and technical foundation for genetic modification.

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