A Dual Selection Marker Transformation System Using *Agrobacterium tumefaciens* for the Industrial *Aspergillus oryzae* 3.042^S

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Received: November 19, 2018
Accepted: December 10, 2018

First published online
December 14, 2018

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^SSupplementary data for this
paper are available on-line only at
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pISSN 1017-7825, eISSN 1738-8872

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Currently, the genetic modification of *Aspergillus oryzae* is mainly dependent on protoplast-mediated transformation (PMT). In this study, we established a dual selection marker system in an industrial *A. oryzae* 3.042 strain by using *Agrobacterium tumefaciens*-mediated transformation (ATMT). We first constructed a uridine/uracil auxotrophic *A. oryzae* 3.042 strain and a pyrithiamine (PT)-resistance binary vector. Then, we established the ATMT system by using uridine/uracil auxotrophy and PT-resistance genes as selection markers. Finally, a dual selection marker ATMT system was developed. This study demonstrates a useful dual selection marker transformation system for genetic manipulations of *A. oryzae* 3.042.

Keywords: *Aspergillus oryzae*, uridine/uracil auxotrophy, pyrithiamine, *Agrobacterium tumefaciens*-mediated transformation

Aspergillus oryzae has a long history in traditional food fermentation, condiment production, and brewing industries in Asia [1, 2]. Its industrial popularity has increased over recent years, particularly for recombinant protein production, where an increasing number of researchers have utilized genetic engineering techniques to improve fermentation performance and product yield [3, 4]. Selection markers and transformation methods are key considerations for genetic manipulations. Since *A. oryzae* shows inherent resistance to common antibiotics [5, 6], auxotrophic genes are often used as selection markers [7–12]. PT is a vitamin B1 antagonist, and the PT resistance gene-*ptrA* which has been cloned from a PT-resistant *A. oryzae* mutant can also be used as a selection marker [13, 14]. PMT and ATMT are the main methods used for transforming filamentous fungi [15]. Compared with PMT, ATMT is easier to conduct and the transformation efficiency is high [15, 16]. Attempts to

establish an ATMT system in *A. oryzae* were unsuccessful until 2016, when Nguyen *et al.* (2016) established ATMT in *A. oryzae* using uracil auxotrophy as a selectable marker [17]. Herein, we constructed a dual selection marker transformation system using the ATMT method in an industrial *A. oryzae* 3.042 strain.

Our sensitivity tests suggested *A. oryzae* 3.042 is sensitive to 5-FOA (Fig. S1A). Therefore, 5-FOA was to be used for construction of the uridine/uracil auxotrophy mutant, as in a previous study [18]. The binary vector pAoG (supplementary materials), was used to delete the *pyrG* gene (uridine/uracil auxotrophy gene) in *A. oryzae* 3.042. Transformation of pAoG was performed using a method described by Nguyen *et al.* (2017) with minor revisions (supplementary materials). Five transformant colonies were selected for phenotypic and PCR identification (the primer sequences are listed in Table S1). Results suggested

Fig. 1. The deletion of the *pyrG* gene in *Aspergillus oryzae* 3.042.

(A) The transformants obtained from the 5-FOA medium were confirmed on the CDA, CDA + uridine + uracil, and CDA + uridine + uracil + 5-FOA media. (B) The scheme of the different *A. oryzae* strain cultures in (A). (C) The *pyrG* deletion mutants were confirmed by PCR analysis using two different primer pairs. 1# to 5# represent the different colonies.

Fig. 2. The identification of pEX1 and pEX1-*ptrA* transformants.

(A) Plate showing pEX1 transformation after cultivation for 5 days on the screening medium. (B) Plate showing pEX1-*ptrA* transformation 60 h after cultivation for 3 days on the screening medium. (C) Plate showing pEX1-*ptrA* transformation 48 h after cultivation for 5 days on the screening media. (D) The transformants were confirmed by PCR using the specific primer pairs for the *GFP* reporter gene. The co-cultivation concentration of spores was 10^5 /ml.

the *pyrG* was successfully knocked out (Figs. 1A and 1B).

Sensitivity tests revealed *A. oryzae* 3.042 is as sensitive to PT as other strains (Fig. S1A). Therefore, we replaced the *pyrG* cassette of pEX1 (the plasmid used for ATMT in *A.*

oryzae RIB40 [17]) with a *ptrA* cassette to construct the pEX1-*ptrA* vector (Fig. S1B). The pEX1 and pEX1-*ptrA* constructs were transformed into *A. oryzae* 3.042 *ΔpyrG* and *A. oryzae* wild type. For the pEX1 construct, an average of

10 transformants per plate were obtained; the transformation efficiency was over 100 transformants per 10^5 spores (Fig. 2A). For the pEX1-*ptrA* construct, background growth was strong, which complicated the selection of positive transformants (Fig. 2B). This may be because PT cannot inhibit hyphae growth, which occurred in some spores that had germinated during co-cultivation. Consequently, co-cultivation time was decreased from 60 h to 48 h to avoid spore germination, thereby decreasing background growth. The resulting transformants were easier to select, with an efficiency of approximately 30 transformants per 10^5 spores (Fig. 2C). Transformed colonies were confirmed by the amplification of the *GFP* reporter gene (Fig. 2D).

Transformants were cultured on the corresponding selective media to examine their growth phenotype. Results indicated that the pEX1 transformants complemented the uridine/uracil auxotrophy and the pEX1-*ptrA* transformants demonstrate PT-resistance (Figs. 3A and 3B). Then, the GFP fluorescence was observed, and it was found that both pEX1 and pEX1-*ptrA* transformants can successfully express the *GFP* reporter gene (Figs. 3C–3H). Therefore, the pEX1

and pEX1-*ptrA* vectors can be employed for the transformation of *A. oryzae* 3.042 via the ATMT method.

Finally, a dual selection marker transformation system in *A. oryzae* 3.042 was established. Since both pEX1 and pEX1-*ptrA* encode GFP, we selected pEX2B, an alternative binary vector with a uridine/uracil auxotrophy selection marker and *DsRed* reporter gene [9]. We used the pEX1-*ptrA* and pEX2B vector to construct a dual selection marker transformation system. The pEX1-*ptrA* was transformed into *A. oryzae* 3.042 Δ *pyrG*; then, the transformant was used as the host to transform with pEX2B. The dual transformant was screened for both selection markers. Fluorescence of the GFP and *DsRed* were observed, confirming successful transformation. As shown in Fig. 4, the pEX1-*ptrA*/*A. oryzae* 3.042 Δ *pyrG* and pEX2B/*A. oryzae* 3.042 Δ *pyrG* strains display either GFP or *DsRed* fluorescence, respectively; while in dual transformants, both GFP and *DsRed* fluorescence were observed (Fig. 4). Thus, our studies revealed that uridine/uracil auxotrophy and PT-resistance can be used as a dual selection marker for ATMT in *A. oryzae* 3.042.

In summary, this study established a novel two-marker

Fig. 3. Confirmation of transformants by assaying their growth on selective media and GFP observation.

(A and B) The growth of pEX1/*A. oryzae* 3.042 Δ *pyrG* and pEX1-*ptrA*/*A. oryzae* 3.042 on different media; (C–E) and (F–H) The 3-day-old mycelia of *A. oryzae* 3.042, pEX1/*A. oryzae* 3.042 Δ *pyrG*, and pEX1-*ptrA*/*A. oryzae* 3.042 under bright field and fluorescent field microscopy.

Fig. 4. GFP and DsRed fluorescence in the transformants.

The mycelia of transformants were grown under coverslips for 3 days. Only GFP (Second column) or DsRed (third column) can be observed in pEX1-*ptrA*/*A. oryzae* 3.042 Δ *pyrG* or pEX2B/*A. oryzae* 3.042 Δ *pyrG* transformants, respectively; both GFP and DsRed can be observed in the dual transformants (fourth column).

selection system in an *A. oryzae* 3.042 strain. For the first time we demonstrate the use of ATMT to transform PT-resistance as a selection marker in *A. oryzae*. An advantage of ATMT using PT-resistance as a selectable marker in ATMT is that it can be performed on the wild-type *A. oryzae*. However, selection efficiency was less than when using the uridine/uracil auxotrophy method. The possible reasons are as follows: firstly, although PT showed strong inhibition of *A. oryzae* spore germination, it cannot inhibit mycelia growth once the spores germinated [13]; secondly, the presence of a very small amount of vitamin B1 in the culture system (such as the tissue fluid from lysing cells or vitamin B1 in culture medium) also greatly reduces the inhibitory effect [13]. Despite this, we believe our study shows the establishment of a dual selection marker transformation system using ATMT, which can simplify transformation procedures and improve the efficiency of genetic manipulations in *A. oryzae*. This could facilitate functional gene research, which can improve the fermentation performance and productivity of *A. oryzae*.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (NSFC Grant Nos: 31700068 and 31460447), Natural Science Foundation of Jiangxi Province (Grant Nos: 20181BAB214001 and 20171BAB214004), the

Open Foundation of Hubei Key Laboratory of Edible Wild Plants Conservation and Utilization (Grant No: EWPL201705) and Research Project of Jiangxi Provincial Department of Education (Grant No:GJJ180600).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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