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Supplementary Data

1. Materials and methods

1.1 Strains and culture media

Escherichia coli strain DH5α [1] was used for plasmid construction, and *Agrobacterium tumefaciens* strain AGL1 [2] was used for genetic transformation. These bacterial strains were grown in Luria-Bertani (LB) medium. The wild-type *A. oryzae* strain 3.042 (CICC 40092) was obtained from the China Center of Industry Culture Collection (Beijing, China). CDA (Czapek-Dox agar) medium (2% sucrose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.05% NaCl, 0.002% $FeSO_4$, 1.6% agar, pH 5.5) was generally used. *E. coli* strain DH5α was cultured at 37°C, while *Agrobacterium tumefaciens* and *A. oryzae* were cultured at 28°C and 30°C, respectively.

1.2 The sensitivity assays for 5-FOA and PT in *A. oryzae* 3.042

A. oryzae 3.042 spores (10 µl, 10^6/ml) were plated onto CDA, CDA+DMSO (1% *v*/*v*), CDA+5-FOA (2 mg/ml), and CDA+PT (0.1 µg/ml) media, and the growth of mycelia was observed for 3 to 7 days. For selection, 5-FOA (Solarbio, Beijing, China) was dissolved in DMSO, and PT was dissolved in sterile distilled water.

1.3 Plasmid construction and primers

The vectors pAoG (used for knocking out *pyrG*), pEX1 (used for the transformation of *A. oryzae* 3.042 △ *pyrG*), and pEX2B (used for dual selection marker

transformation of *A. oryzae* 3.042 △*pyrG*) were provided by the Tran Research Group [3], and the pPRTI vector (used for cloning the *ptrA* cassette) was obtained from Takela (Dalian, China, Code No. 3621). The *pyrG* marker of the pEX1 vector was cut by the restriction enzymes *Eco*RI and *Spe*I, and the *ptrA* cassette was amplified by the primer pair *ptrA*-F and *ptrA*-R (Table 1; underlined sequences show homology with pEX1). The *ptrA* cassette was fused to pEX1 by using a one-step cloning kit (Vazyme Biotech Co., Ltd, Nanjin, China). The primers used in this study are shown in Table 1.

1.4 *Agrobacterium tumefaciens*-mediated transformation of *A. oryzae* 3.042

The *Agrobacterium tumefaciens*-mediated transformation method used here is based on previous research by Nguyen et al. (2017) with minor revisions. First, to avoid the problem of having too many colonies on culture plates, the concentration of spores in our study is 10^5/ml. Second, in order to decrease the background growth of the fungus, after co-cultivation, the spores on the cellophane were transferred to the screening media and covered with new screening media. The transformants can grow through the screening media; however, the background mycelium cannot grow through the screening media. Third, 0.5% Triton-100 (Sigma, St. Louis, MO, USA) was added to the new screening media to suppress the growth of transformant mycelia and limit cross contamination among different transformants. For PT-resistance-mediated transformation, the co-cultivation time was limited to 48 hours to decrease background growth.

1.5 Fluorescence observations

For the examination of the fluorescent reporter gene expression, the transformants were grown on sterile microscopic slides containing a drop of CDA or CDA

supplemented with PT. Then, the slides with media were covered with a coverslip and incubated in a sterile Petri plate containing some pieces of wetted filter paper for 2–3 days, at 30°C. The slides were observed under an Olympus fluorescence microscope (Olympus, Tokyo, Japan) to detect GFP signals. For the dual selection marker transformants, GFP and DsRed were observed using a ZOE fluorescent cell imager (Bio-Rad, Hercules, CA, USA).

References

1. Grant SG, Jessee J, Bloom FR, Hanahan D. 1990. Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. *Proc Natl Acad Sci U S A*. **87:** 4645-4649.

2. Lazo GR, Stein PA, Ludwig RA. 1991. A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. *Biotechnology*. **9:** 963-967.

3. Nguyen KT, Ho QN, Do L, Mai LTD, Pham DN, Tran HTT*, et al.* 2017. A new and efficient approach for construction of uridine/uracil auxotrophic mutants in the filamentous fungus Aspergillus oryzae using Agrobacterium tumefaciens-mediated transformation. *World J Microbiol Biotechnol.* **33:** 107.

Supplementary table

Table S1 Primers used in this study

Name	Sequences (5′-3′)	Product size
ptrA-F	TGACATGATTACGAATTCGGGCAATTGATTACGGGATCC	2000 bp
ptrA-R	GTCACCGGTCACACTAGTATGGGGTGACGATGAGCCGC	
GFP-F	ATGGTGAGCAAGGGCGAG	720 bp
GFP-R	TCACTTGTACAGCTCGTCCATGC	
P5	GGGAATTCATGCGAAGGTAAGTGCTTC	1800 bp for wild type and 540 bp for △*pyrG* mutant (P5 + P6)
P6	GGACTAGTTGGCTAGGCTCTGACTCG	
P7	TGTAAGGTGATGTGTGCCAG	2300 bp for wild type and 1100 bp for △*pyrG* mutant (P5 + P7)

Supplementary figure

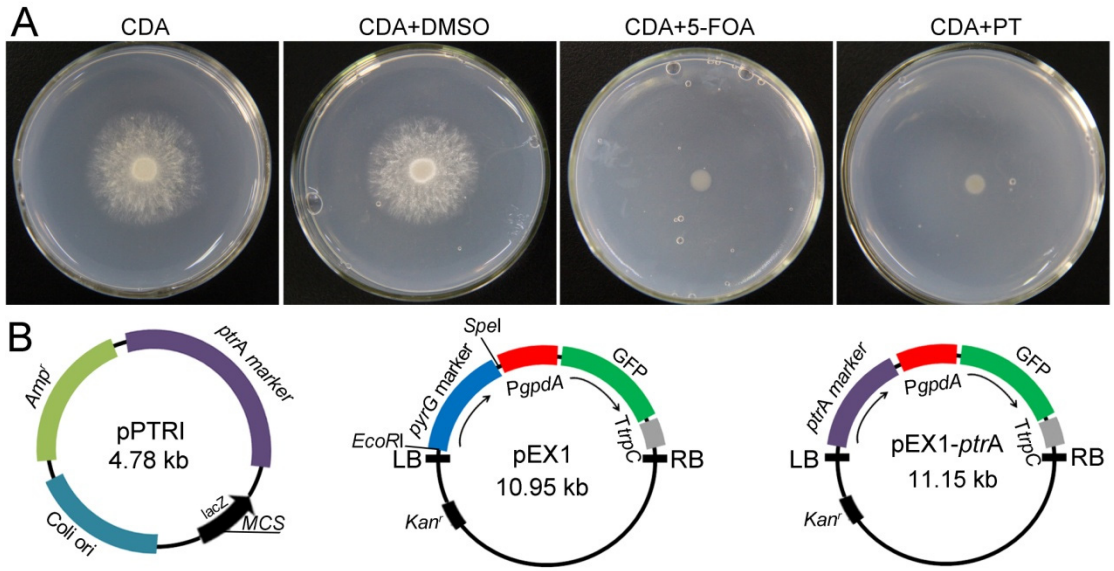

Figure S1. The 5-FOA and PT sensitivity tests and ATMT vector construction. (A) The mycelial growth of *Aspergillus oryzae* strain 3.042 on CDA medium with 5-FOA (2 mg/ml) and PT (0.1 μg/ml) for 3 days. (B) The plasmid construction scheme for using PT as a selection marker for ATMT.