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Adaptation of the *Agrobacterium*-mediated transformation method to the *ligD*-deficient *Aspergillus oryzae* NsPlD1 strain

Thai, H. D. [1, 2] and Tran, V. T. [1, 2*]

[1]Genomics Unit, National Key Laboratory of Enzyme and Protein Technology, Vietnam; [2]Department of Microbiology, Faculty of Biology, University of Science, Vietnam National University, Hanoi (VNU), 334 Nguyen Trai, Thanh Xuan, Hanoi, Vietnam.

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Abstract *Aspergillus oryzae* is widely used to produce traditional fermented foods in Asian countries. It is also exploited as a host for homologous and heterologous protein production. The *ligD*-deficient *A. oryzae* NsPlD1 strain is a uridine/uracil auxotrophic and is commonly employed for gene targeting and recombinant expression. However, studies on the NsPlD1 strain were mainly supported by protoplast-mediated transformation. In the present study, we adapted the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method using two auxotrophic selection markers to the *A. oryzae* strain. Notably, a histidine auxotrophic mutant was constructed by deleting the *hisB* gene in the NsPlD1 strain. Under the appropriate conditions, the ATMT yields of the auxotrophic NsPlD1 strain reached 130 ± 10 transformants per 10^7 spores with the *pyrG* marker and 73 ± 18 transformants per 10^6 spores with the *hisB* marker. Furthermore, we successfully expressed the red fluorescent protein (DsRed) from *Discosoma* coral in the NsPlD1 strain with the ATMT method. Our work provided an additional option for genetic manipulation in the *A. oryzae* strain based on the *pyrG* and *hisB* auxotrophic markers.

Keywords: *Aspergillus oryzae*, *Agrobacterium tumefaciens*-mediated transformation, *PyrG* marker, *HisB* marker, Genetic manipulation

Introduction

The multicellular fungus *Aspergillus oryzae* is considered one of the most potent fungal species in the biotechnological industry. However, suitable tools for genetic transformation in this fungus are still limited. This bottleneck has become more problematic since the U.S. Food and Drug Administration has banned antibiotic-resistance genes as the selectable markers in food-related microorganisms, including *A. oryzae* (Newsome *et al.*, 2014). Therefore, auxotrophic *A. oryzae* strains and respective selectable markers are vital for genetic transformation systems that can be exploited for basic and applied

[*] **Corresponding Author:** Tran, V. T.; **Email:** tuantran@vnu.edu.vn

research fields. The *pyrG* gene, encoding orotidine-5'-monophosphate (OMP) decarboxylase, is a homolog of yeast *ura3* that is required for uridine/uracil biosynthesis in fungi (Nguyen *et al.*, 2017). This gene has been demonstrated to be a suitable selection marker for *Agrobacterium tumefaciens*-mediated transformation (ATMT) in *A. oryzae* (Nguyen *et al.*, 2016; Sun *et al.*, 2019). Besides *pyrG*, the *hisB* gene, a homolog of yeast *HIS3*, which encodes the imidazole glycerol-phosphate dehydratase required for histidine production, has been indicated to be an effective selectable marker in *Aspergilli* (Fiedler *et al.*, 2017; Thai *et al.*, 2021). Integrating an expression cassette for luciferase into the *A. niger hisB* locus resulted in better enzymatic activity than the *pyrG* locus integration. As a result, the exploitation of *hisB* as a selection marker for gene transfer and its genomic location for heterologous expression is very promising (Fiedler *et al.*, 2017). Additionally, a combination of *pyrG* and *hisB* as selectable markers using ATMT allowed the functional investigation of target genes or co-expression of two different genes in a single strain of *A. oryzae* (Thai *et al.*, 2021).

The auxotrophic *A. oryzae* NsPlD1 strain with the genotype *niaD*$^{-}$ *sC*$^{-}$ Δ*pyrG* Δ*ligD* was constructed from the well-known laboratory strain RIB40 (Maruyama and Kitamoto, 2008). This strain has been utilized as a host for kojic acid production and enhancement of bovine chymosin yields (Yamada *et al.*, 2014; Yoon *et al.*, 213). The *ligD* gene is involved in non-homologous chromosomal integration, and this gene was deleted in *A. oryzae* NsPlD1 (Maruyama and Kitamoto, 2008). The *ligD*-deficient NsPlD1 strain was exploited for gene disruption to improve heterologous protein production and secretion (Yoon *et al.*, 2010, 2011). However, genetic manipulations in this fungal strain were mainly based on the protoplast-mediated transformation method (Huang *et al*., 2013; Yamada *et al.*, 2014).

The study aimed to apply the ATMT method based on the *pyrG* auxotrophic markers to the *ligD*-deficient *A. oryzae* NsPlD1 strain. The *hisB* gene was also deleted in this special fungal strain using the *pyrG* selection marker to generate a histidine auxotrophic mutant with the genotype *niaD*$^{-}$ *sC*$^{-}$ Δ*ligD* Δ*hisB*. This histidine auxotrophic strain was then employed for genetic transformation and heterologous expression via the ATMT method.

Materials and methods

Agrobacterium tumefaciens AGL1 was used for fungal transformation, and the uridine/uracil auxotrophic *A. oryzae* NsPlD1 was recruited as the recipient. The binary vector pAoH was employed to delete *hisB* in *A. oryzae* NsPlD1. Two binary vectors, pEX2B and pEX2D were utilized for evaluating

genetic transformation. All microbial strains and plasmids are listed in Table 1. Primers for PCR amplification were synthesized by Phusa Genomics (Can Tho, Vietnam) and are listed in Table 2.

Table 1. Microbial strains and plasmids used in this study

Microbial strains, plasmids	Description	Reference
***Ag. tumefaciens* AGL1**	A modified strain of C58 with the genotype *recA::bla*, pTiBo542ΔT-DNA, Mop^+, Cb^R	Lazo *et al.* (1991)
***A. oryzae* RIB40**	A wild-type strain	Machida *et al.* (2005)
A. oryzae* RIB40Δ*pyrG	A uridine/auxotrophic strain of RIB40	Nguyen *et al.* (2017)
***A. oryzae* NsPlD1**	A derivative strain of RIB40 with the genotype *niaD*$^-$ *sC*$^-$ Δ*ligD* Δ*pyrG*	Maruyama and Kitamoto (2008)
pAoH	The binary vector used for *hisB* deletion in the Δ*pyrG* strain	Thai *et al.* (2021)
pEX2B	The binary vector carries the *A. oryzae pyrG* marker and the *DsRed* gene under the regulation of the *A. oryzae amyB* promoter	Nguyen *et al.* (2017)
pEX2D	The binary vector harbors the *A. oryzae hisB* marker and the *DsRed* gene under the control of the *A. oryzae gpdA* promoter	Thai *et al.* (2021)

Table 2. Primers for PCR analysis used in this study

Primer	Primer sequence (5' – 3')	Product size	Reference
DsRed-cf-F	ATGGCCTCCTCCGAGG	678 bp	Our primer collection
DsRed-cf-R	CTACAGGAACAGGTGGTGGC		
AohisB-P5	CGAAAACCGTACATCGGACT	1630 bp	Thai *et al.* (2021)
AohisB-P6	CCGGCTTTCAACTCCAAATA		
AohisB-F	GCGTACAGCCTCCCTCACTA	625 bp	
AohisB-R	AGAGTCACACCCGCTTCTTG		

DPY medium (2% glucose, 1% peptone, 0.5% yeast extract powder, 0.5% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 2% agar, pH 5.5) was used for spore preparation. M+Met medium (Zhu *et al.*, 2013) (0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002% $FeSO_4.7H_2O$, 2% glucose, 0.15% methionine, pH 5.5) was employed for cultivating *A. oryzae* NsPlD1 and other derivative strains. This medium was supplemented with 0.1% uridine and 0.1% uracil or with 0.1% histidine when required. The induction medium (IM) was supplemented with 0.05% uridine and 0.05% uracil or with 0.02% histidine to support the germination of fungal spores during the co-culture process (Thai *et al.*, 2021). Acetosyringone (200

μM) was added to the IM to induce gene transfer by *Ag. tumefaciens*. The solid media contained 1.6% agar.

The binary vector pAoH was utilized to delete *hisB* in the *A. oryzae* NsPlD1 strain by homologous recombination. The optimal ATMT procedures were conducted as previously described for *A. oryzae*, and fungal transformants were selected on different media (M+Met, M+Met+Uri+Ura, M+Met+His) (Nguyen *et al.*, 2016; Thai *et al.*, 2021). The binary vector pEX2B carrying the *A. oryzae pyrG* and the *DsRed* expression cassette was chosen to evaluate the ATMT efficiency in the NsPlD1 strain. The binary vector pEX2D harboring the *A. oryzae hisB* marker was used for the *DsRed* expression under the control of *Aspergillus nidulans gpdA* promoter in the histidine auxotrophic NsPlD1 strain.

Fungal transformants were purified and examined for mitotic stability by single spore isolation. The purified transformants were then inoculated for genomic DNA extraction. PCR analyses using GoTaq® Green Mastermix (Promega, Wisconsin, USA) with specific primers (Table 2) were conducted to confirm the transformants at the DNA level, as reported by Thai *et al.* (2021). To examine the DsRed expression, each DsRed-tagged transformant was grown on a sterile microscopic slide containing a droplet of M+Met agar medium containing 2% maltose as the inducer of the *amyB* promoter. The slide was observed under a fluorescence microscope, as described by Nguyen *et al.* (2016).

Results

Evaluating the uridine/uracil auxotrophic phenotype of the NsPlD1 strain

We examined the growth of the NsPlD1 strain in comparison to the wild-type strain RIB40 on the M+Met minimal medium or M+Met supplemented with uridine and uracil (M+Met+Uri+Ura). The wild-type RIB40 strain grew normally on M+Met and M+Met+Uri+Ura with the typical yellow mycelium. In contrast, the NsPlD1 strain could not grow on M+Met, and its growth was only recovered when uridine and uracil were added to M+Met (Figure 1). These results confirmed that the NsPlD1 strain is uridine/uracil auxotrophic and can be used as a recipient for genetic transformation.

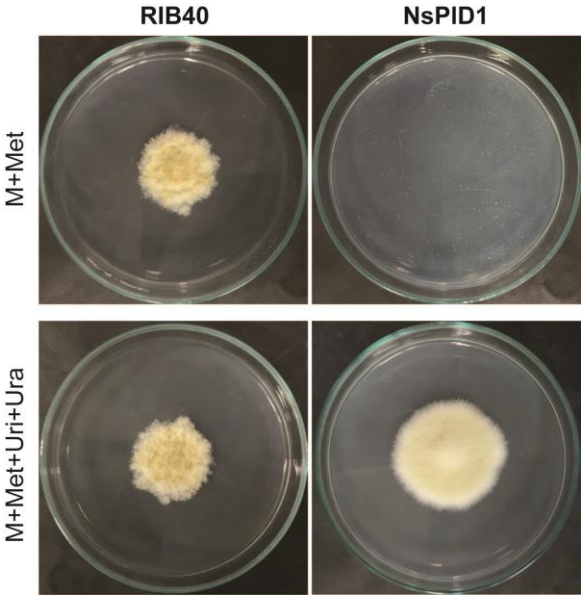

Figure 1. The uridine/uracil auxotrophic status of the *A. oryzae* NsPlD1 strain, the wild-type strain RIB40 was used as the reference strain for comparison, these strains were cultivated on M+Met and M+Met+Uri+Ura at 30^{o}C for 8 days

The successful construction of a histidine auxotrophic strain based on A. oryzae NsPlD1

The successful deletion of *hisB* in NsPlD1 (genotype: *niaD*$^{-}$ *sC*$^{-}$ Δ*ligD* Δ*pyrG*) resulted in a histidine auxotrophic mutant, in which the whole open reading frame (ORF) of *hisB* was replaced with the *pyrG* marker. The obtained histidine auxotrophic strain was named NsHlD1 (genotype: *niaD*$^{-}$ *sC*$^{-}$ Δ*ligD* Δ*hisB*). The *hisB* deletion in NsHlD1 was detected by PCR using primer pairs AohisB-P5/AohisB-P6 and AohisB-F/AohisB-R (Table 2). With the primer pair AohisB-P5/AohisB-P6, one DNA fragment of 1630 bp was obtained for the NsPlD1 background strain, and a bigger band of 2404 bp resulted from the histidine auxotrophic NsHlD1 mutant. Additionally, the second primer pair AohisB-F/AohisB-R was used to confirm the loss of *hisB* ORF. The PCR results revealed that one DNA band of 625 bp was present in the NsPlD1 background strain indicating the existence of *hisB* ORF in the genome of the strain. Meanwhile, no bands could be detected for the NsHlD1 mutant due to the loss of *hisB* (Figure 2A).

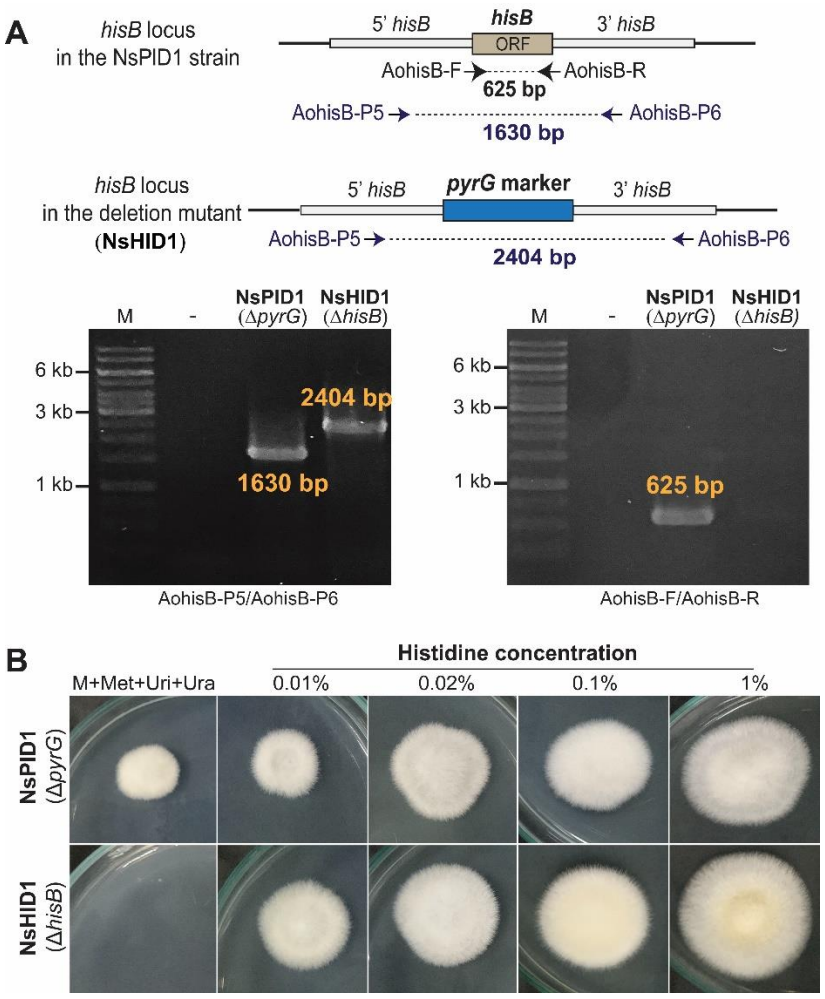

Figure 2. Construction of the histidine auxotrophic NsHlD1 strain: (A) Confirmation of the *hisB* gene deletion in NsHlD1 by PCR with two independent primer pairs, including AohisB-P5/AohisB-P6 and AohisB-F/AohisB-R. (B) Growth of NsHlD1 compared to the background strain NsPlD1 on the M+Met+Uri+Ura medium supplemented with different histidine concentrations (0.01-1%). Plates were incubated at 30^{o}C for 4 days

Furthermore, the growth of the *hisB* deletion mutant NsHlD1 was tested on the M+Met medium with or without histidine. Compared to the NsPlD1 background strain, the NsHlD1 mutant could not grow on the medium without histidine supplementation (Figure 2B). Our results corroborated that *hisB* was successfully removed from the genome of NsHlD1 by homologous recombination.

ATMT using the auxotrophic markers is effective in A. oryzae NsPlD1

We conducted the binary vectors pEX2B and pEX2D to evaluate the ability of the ATMT method for gene transfer in the uridine/uracil auxotrophic NsPlD1 strain and the histidine auxotrophic NsHlD1 strain, respectively. The optimal procedure of ATMT for these auxotrophic strains was summarized in Figure 3A.

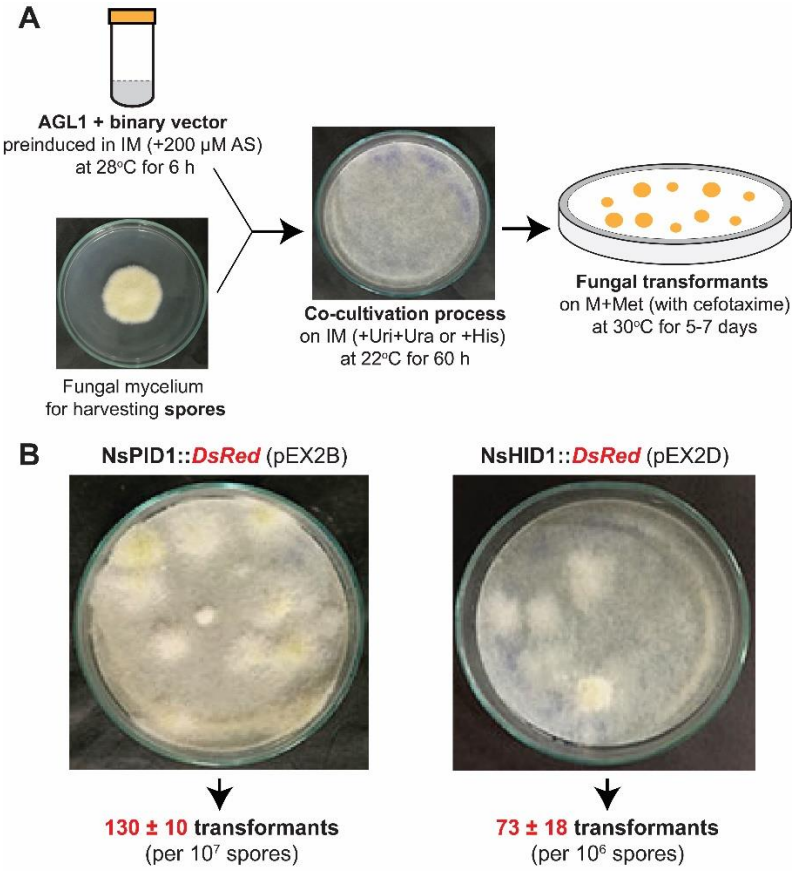

Figure 3. Genetic transformation of the uridine/uracil auxotrophic NsPlD1 strain and the histidine auxotrophic mutant NsHlD1 using the ATMT method: (A) The brief scheme of the ATMT method used the auxotrophic strains. (B) The ATMT efficiencies of the auxotrophic strains NsPlD1 and NsHlD1

Data showed that gene transfer frequencies reached 130 ± 10 transformants per 10^7 spores for NsPlD1 and 73 ± 18 transformants per 10^6 spores for NsHlD1 (Figure 3B).

Successful expression of the DsRed fluorescent protein from Discosoma coral in the histidine auxotrophic A. oryzae NsHlD1

Fungal transformants generated from the histidine auxotrophic NsHlD1 strain using the ATMT method with the binary vector pEX2D were cultured successively on the M+Met medium for three generations. It found that all the transformants were prototrophic for histidine and mitotically stable during the successive cultivation process. The integrations of T-DNA containing the *DsRed* expression cassette into genomes of the transformants were detected by PCR with the primer pair DsRed-cf-F/DsRed-cf-R (Table 2). This primer pair is specific to the *DsRed* gene. The presence of a DNA fragment of 678 bp on agarose gel confirmed the success of the gene transfer event (Figure 4A). Under an Axio fluorescence microscope (Carl Zeiss, Oberkochen, Germany), the selected transformant accumulated a strong red signal in the whole fungal mycelium. The results indicate that the heterologous expression of *DsRed* in the histidine auxotrophic NsHlD1 strain was successful (Figure 4B).

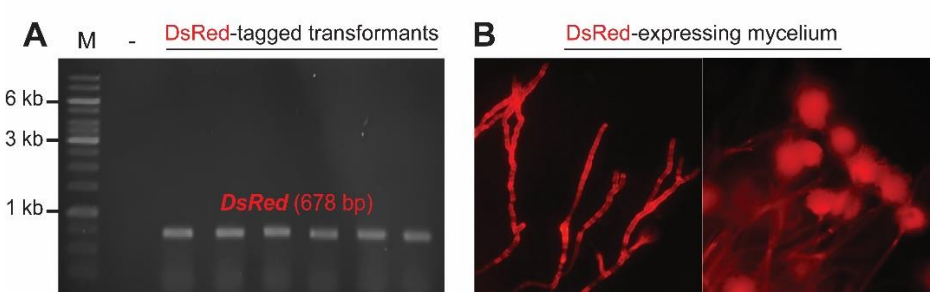

Figure 4. Analysis of the DsRed-tagged transformants: (A) Confirmation of the DsRed-tagged transformants by PCR using the specific primer pair DsRed-cf-F/DsRed-cf-R. (B) The expression of *DsRed* in the histidine auxotrophic NsHlD1 strain was examined under fluorescence microscopy using the slide culture method

Discussion

A. oryzae can proliferate vigorously on common solidified media and promote the formation of a large number of conidia which turns its mycelium yellow or yellowish-green (Tamano *et al.*, 2022). This filamentous fungus can also secrete vast amounts of industrially potential enzymes into culture media (Machida *et al.*, 2005; Zhu *et al.*, 2013). However, *A. oryzae* is intrinsically resistant to antifungal substances commonly used for genetic transformation. As a result, developing new transformation strategies based on nutritional

markers is vital for basic and applied research fields in the industrial filamentous fungus *A. oryzae* (He *et al.*, 2019; Jin *et al.*, 2021; Yamada *et al.*, 2014). Several nutritional markers such as *pyrG*, *adeA*, *niaD*, *sC*, and *argB* were effectively employed for polyethylene glycol (PEG)-mediated protoplast transformation of *A. oryzae* (He *et al.*, 2019; Jin *et al.*, 2004a, 2004b). Recently, the *pyrG* marker has been indicated to be very efficient for genetic manipulation in *A. oryzae* using the ATMT method (Nguyen *et al.*, 2006, 2017; Sun *et al.*, 2019). Additionally, *hisB* existing in the genome of filamentous fungi as an ortholog of yeast *HIS3*, was reported to be a suitable marker for genetic approaches in *Aspergillus niger* (Fiedler *et al.*, 2017). In *A. oryzae*, it proved that *hisB* is an excellent selection marker for heterologous expression and gene targeting in *A. oryzae* via the ATMT method (Thai *et al.*, 2021).

In the current study, we constructed the histidine auxotrophic strain NsHlD1 by deleting the *hisB* gene in the NsPlD1 strain using the binary vector pAoH (Table 1) and the ATMT method. T-DNA of pAoH contains the *hisB* deletion cassette with the *pyrG* marker inserted between the 5' and 3' flanking regions of the *hisB* gene (Thai *et al.*, 2021). The NsHlD1 is mitotically stable for long-term use in genetic transformation. The results showed that *pyrG* and *hisB* could be used as selectable markers for the ATMT method in the *A. oryzae* NsPlD1 background strain and its derivative strain NsHlD1. The ATMT efficiencies reached approximately 130 transformants per 10^7 spores for the uridine/uracil auxotrophic NsPlD1 strain and 73 transformants per 10^6 spores for the histidine auxotrophic NsHlD1 strain. Furthermore, heterologous expression of the DsRed fluorescent protein from *Discosoma* coral was confirmed to be successful in the NsHlD1 strain. Previously, we indicated that the efficiency of the ATMT method in the uridine/uracil auxotrophic *A. oryzae* RIB40 was very high, with 1060 ± 143 transformants per 10^6 spores (Nguyen *et al.*, 2017). However, the frequency of ATMT in the AUT1-PlD strain was relatively low, with only 116 ± 39 transformants per 10^7 spores (Nguyen *et al.*, 2016). LigD is required for the non-homologous end-joining pathway. The deletion of the *ligD* gene decreased random DNA integration and promoted homologous recombination, which facilitated the generation of targeted knockout mutants in the fungus (Maruyama and Kitamoto, 2008). Both strains AUT1-PlD and NsPlD1 possessed the null mutation in the *ligD* gene. Therefore, the reduced transformation yields in these *A. oryzae* strains via the ATMT method may be resulted from the loss of *ligD*.

In conclusion, this study provided a feasible solution for ATMT-based genetic manipulation in the *ligD*-deficient *A. oryzae* NsPlD1 strain, commonly used in the laboratory. The results of our work may also be helpful for future studies on comparing gene targeting efficiency of *Agrobacterium*-based

transformation versus protoplast-based transformation in the *ligD*-deficient auxotrophic strains.

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