HETEROLOGOUS PHYTASE EXPRESSION IN THE FOOD FILAMENTOUS FUNGUS *Aspergillus oryzae* USING THE ADDED RICE HUSK CULTIVATION MODEL

Thai Hanh Dung[1,2], Tran Van Tuan[1,2,*]

[1]Department of Microbiology, Faculty of Biology, University of Science,
Vietnam National University, Hanoi
[2]Genomics Unit, Key Laboratory of Enzyme and Protein Technology,
University of Science, Vietnam National University, Hanoi

ABSTRACT

Aspergillus oryzae, a safe filamentous fungus, is widely used in food and enzyme production. In this study, we examined a cultivation model using rice husks as carrier to assess the capacity of recombinant protein production in *A. oryzae*. The model was first tested with the *A. oryzae* strain expressing the *DsRed* reporter gene. The expression of *DsRed* was easily detected by the pink color of the fungal mycelium on culture media and under a fluorescence microscope. The model was then evaluated with the *phyA* gene encoding a phytase from the fungus *Aspergillus fumigatus*. The *phyA* expression cassette regulated by the *amyB* promoter was permanently integrated into the genome of *A. oryzae* via *Agrobacterium tumefaciens*-mediated transformation with the *pyrG* nutritional marker. The results showed that transgenic *A. oryzae* strains carrying 2−3 copies of the *phyA* gene in their genomes exhibited a significant increase in phytase activity on agar medium supplemented with phytate. With rice husks added, these transgenic strains could secrete the recombinant phytase into the culture and phytase activity of the crude enzyme solution increased by 4.3 times compared to the original *A. oryzae*. The established cultivation model and the transgenic strategy in this study represent a potential approach for being exploited in the production of secreted recombinant enzymes for animal feeds.

Keywords: *Aspergillus oryzae, Agrobacterium tumefaciens*-mediated transformation, DsRed, recombinant phytase, rice husk.

INTRODUCTION

Aspergillus oryzae, a safe filamentous fungus, has been widely used in Asian countries for the production of traditional foods and beverages such as soy sauce, miso and sake (Barbesgaard et al., 1992; Machida et al., 2008). *A. oryzae* has the ability to express and secret a large amount of recombinant enzymes into the culture media as reported for aspartic protease and lipase from *Rhizomucor miehei* (Christensen et al., 1988; Huge-Jensen et al., 1989). Currently, the industrial production of some recombinant enzymes using *A. oryzae* as the host has been approved. *Rhizomucor miehei* lipase expressed in *A. oryzae* has been used for humans in Denmark (Barbesgaard et al., 1992).

Phytase is one of the most prominent enzymes for animal feed, accounting for about 60% of the enzyme market for cattle and poultry nutrition, which costs approximately $350 million per year. Phytase is present as an additive in about 70% of commercial animal feeds. Phytase (also known as myo-inositol hexakisphosphate phosphohydrolase) hydrolyzes phytic acid (or phytate), releasing inorganic phosphorus that is essential for animal metabolism. Monogastric animals, which do not possess phytases, are unable to metabolize phytate from grains - the main ingredients of industrial animal feeds. Unmetabolized phytate may be released by the animals into the environment as waste, causing pollution (Haefner et al., 2005; Rao et al., 2009). Although phytases have been isolated from various microbial sources, only phytases from certain microorganisms with attractive characteristics including the high rate of phytate hydrolysis, acidic pH tolerance and heat stability during feed pelleting process are widely used in the industrial production (Rao et al., 2009). Phytase from the filamentous fungus *Aspergillus fumigatus* has the ability to recover its activity level after heat treatment whereas many other phytases are usually inactivated during feed pelleting process (Pasamontes et al., 1997). However, the direct use of *A. fumigatus* for enzyme production is not allowed because this fungus is an opportunistic human pathogen (Varga et al., 2011). As a result, different strategies based on genetic engineering have been used to generate recombinant fungal strains with safety assurance for industrial-scale enzyme production (Punt et al., 2002).

Recently, our research group has succeeded in establishing a highly efficient genetic transformation system for the food filamentous fungus *A. oryzae* using the bacterium *Agrobacterium tumefaciens* as a DNA transfer tool (Nguyen et al., 2016; Nguyen et al., 2017). The *A. tumefaciens*-mediated transformation (ATMT) method has many advantages, especially the direct use of fungal spores as the transformation material and the target gene located in T-DNA is stably integrated into the fungal genome (Michielse et al., 2005). In this study, we transferred the *phyA* gene encoding a phytase from the opportunistic human pathogen *A. fumigatus* into the genome of the food fungus *A. oryzae* using the ATMT method. Instead of common antibiotic resistance genes, the *pyrG* gene was used as a nutritional marker for selecting fungal transformants. Therefore, the transgenic *A. oryzae* strains expressing the phytase gene would be safe for recombinant production.

MATERIALS AND METHODS

Microbial strains, plasmids and PCR primers

The strains and plasmids used in this study are listed in Table 1.

Primers for PCR amplifications listed in table 2 were synthesized by Integrated DNA Technologies (IDT) Inc. (Singapore). The primer pair (PhyA-F/PhyA-R) employed for the amplification of the *phyA* gene was designed with Primer3 based on the *A. fumigatus phyA* sequence (accession number: Afu4g08630). The primer pair (pyrG-RT-F/pyrG-RT-R) for the amplification of the *pyrG* marker was based on the *pyrG* gene sequence (accession number: AO090011000868). These two

sequences were extracted from the http://www.aspergillusgenome.org. *Aspergillus* genome database at the link:

Table 1. Microbial strains and plasmids used in this study

Strain/ plasmid	Description	Reference
Escherichia coli DH5α	F⁻ *endA1 hsdR17 supE44 thi-1* λ⁻ *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)-U169 Φ80d*lacZ*ΔM15	Nguyen et al., 2016
A. tumefaciens AGL1	C58, *recA::bla*, pTiBo542ΔT-DNA, Mop⁺, Cb^R	Nguyen et al., 2016
A. fumigatus VTCC 31414	The wild-type *A. fumigatus* isolated in Vietnam	IMBT, Vietnam National University, Hanoi
A. oryzae RIB40	The wild-type *A. oryzae* originated from Japan	Machida et al., 2008
A. oryzae AUT1-PlD	*niaD⁻ sC⁻ adeA⁻* Δ*argB::adeA⁻* Δ*tppA::argB* Δ*pepE::adeA aut1⁻* Δ*ligD* Δ*pyrG*	Zhu et al., 2013
A. oryzae AUT1-R1	The AUT1-PlD strain expressing the *DsRed* fluorescent reporter gene	Nguyen et al., 2017
pEX2B	The binary vector with the *pyrG* marker and the *DsRed* reporter gene under the regulation of the *A. oryzae amyB* promoter	Nguyen et al., 2017
pEX2B-*phyA*	The binary vector with the *pyrG* marker and the *A. fumigatus phyA* gene under the regulation of the *A. oryzae amyB* promoter	This study

Table 2. Primers used in this study

Primer name	Primer sequence (5'–3') with the underlined sites for restriction enzymes	Product size	Reference
ITS1	TCCGTAGGTGAACCTGCGG	597 bp	White et al., 1990
ITS4	TCCTCCGCTTATTGATATGC		
PhyA-F	GGG<u>CACGTG</u>ATGAAAAAGCTATAT AATGGCCGG (*Pml*I)	1.51 kb	This study
PhyA-R	GGG<u>GGATCC</u>TCAACTAAAGCACTCTC CCCA (*Bam*HI)		
pyrG-RT-F	CGGAAACACAGTCCAAAAGC	196 bp	This study
pyrG-RT-R	CAAAGATCCCTTGGAGGTCA		

Cultivation media

Potato dextrose agar (PDA) medium was used for cultivating the wild-type strains *A. oryzae* RIB40 and *A. fumigatus* VTCC 31414.

DPY medium (2% glucose, 1% peptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, pH 5.5) with required supplements (0.1% uracil, 0.1% uridine) was used for collecting spores of the AUT1-PlD strain. The AUT1-PlD strain, which was genetically engineered from *A. oryzae* RIB40, is a hyper-producing strain for recombinant protein production (Zhu et al., 2013).

M+met medium (0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.002% $FeSO_4$, 2% glucose, 0.15% methionine, pH 5.5) was used as a minimal medium for selection of fungal transformants (Zhu et al., 2013).

The induction medium (IM) was supplemented with 0.05% uridine, 0.05% uracil and 200 μM acetosyringone (AS). The liquid IM contained salts of minimal medium (MM salts), 40 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 10 mM glucose, 0.5% (w/v) glycerol. The solid IM contained

only 5 mM glucose. The solution of MM salts (g/l) consisted of 2.05 g K_2HPO_4, 1.45 g KH_2PO_4, 0.15 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.6H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.0025 g $FeSO_4.7H_2O$ (Nguyen et al., 2016).

Phytase screening medium (PSM) contained 1% glucose, 0.4% sodium phytate, 0.2% $CaCl_2.6H_2O$, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$, 0.001% $MnSO_4.H_2O$.

For a solid medium, 2% agar was added. All media were autoclaved before use.

Spore preparation

Fungal strains were cultivated on agar plates for 3−5 days at 30°C. Sterile distilled water was added and a sterile glass spreader was used to scrap the agar surface. The liquid was collected and filtered through Miracloth (Calbiochem, Germany). The filtrate was then centrifuged at 5000 rpm for 10 min. Collected spore pellets were washed twice with sterile distilled water and resuspended in sterile distilled water to obtain the final spore suspension. Fungal spore concentration was measured using a Thoma counting chamber and adjusted to 10^6 or 10^7 spores/ml for further experiments.

Total DNA extraction

The extraction of total DNA from fungal mycelium was based on the procedure from our previous study (Nguyen et al., 2016) or using the commercial E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, USA).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from fungal mycelium using ANAPURE® RNA Mini Kit (ANABIO R&D JSC, Vietnam). RNA samples were digested with DNase I (Qiagen, Germany) at 37°C for 30 min to remove total DNA. The enzyme DNase I was then inactivated at 75°C for 15 min. The RNA samples were immediately kept in ice. The purity of the RNA samples was then examined with PCR using the primer pair ITS1/ITS4 (Table 2) specific for the ITS region of ribosomal DNA. The purified RNA

was converted into cDNA using ProtoScript® First Strand cDNA Synthesis (New England Biolabs, USA).

Construction of the binary vector for expressing the *phyA* gene from *A. fumigatus*

The full-length coding sequence of the *phyA* gene was amplified from the *A. fumigatus* cDNA sample with PCR using the specific primer pair PhyA-F/PhyA-R (Table 2). Phusion® high-fidelity DNA polymerase (Thermo Scientific, USA) was used to ensure the accuracy of PCR amplification. The obtained PCR product was purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR product was digested with *Pml*I and *Bam*HI and ligated into the binary vector pEX2B, which was also digested with the same restriction enzymes. The resultant plasmid was transformed into the competent *E. coli* DH5α cells for propagation. The recombinant plasmid was purified with Wizard® Plus SV Minipreps DNA Purification System (Promega, USA). The existence of the *phyA* expression cassette was confirmed by PCR using the specific primer pair PhyA-F/PhyA-R and by digestion with suitable restriction enzymes as well as by DNA sequencing. The constructed binary vector was named pEX2B-*phyA*.

Transfer of the *phyA* expression cassette into the *A. oryzae* AUT1-PlD strain using *A. tumefaciens*

The binary vector pEX2B-*phyA* was transferred into the *A. tumefaciens* AGL1 cells by the electroporation method. A single *A. tumefaciens* colony harboring the vector was inoculated in a conical flask containing 20 ml of the liquid Luria-Bertani (1% peptone, 0.5% yeast extract powder, 0.5% NaCl) supplemented with kanamycin (100 mg/l) through shaking at 200 rpm and 28°C for 15-17 h. One milliliter of the culture was mixed with 9 ml of induction medium (IM) and 200 μM acetosyringone (AS). The bacteria were additionally incubated for 6 h at 28°C, 200 rpm until the OD_{600nm} reached 0.6-0.8. A mixture of 100 μl of the AUT1-PlD

spore suspension (10^7 spores/ml) and 100 µl of the induced AGL1 suspension was spread on 90-mm cellulose filter paper (Sartorius, Germany), which was placed on the IM agar plate consisting of 200 µM AS, 0.05% uridine (w/v) and 0.05% uracil (w/v). The plate was kept in darkness for 60 h at 22°C. After that, the filter paper was transferred to a new plate containing M+met medium supplemented with cefotaxime (300 mg/l) for fungal selection. This plate was incubated at 30°C for 5−7 days until fungal transformants appearing on the filter paper membrane.

Examination of the transformants

The existence of the *phyA* gene from *A. fumigatus* in the genome of the obtained *A. oryzae* transformants was confirmed by PCR using the specific primer pair PhyA-F/PhyA-R (Table 2). For examining the recombinant phytase expression, fungal transformants were directly grown on PSM supplemented with 0.15% methionine. After 3−5 days, a zone of clearance surrounding the transformants was observed.

Determination of the *phyA* copy number in the transformants with real-time PCR

Total DNA was isolated from the selected *A. oryzae* transformants using E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, USA). DNA quantifications by real-time PCR were carried out with IQ5 real-time PCR system (Bio-Rad, USA). HotStart-IT SYBR Green qPCR Master Mix (Affymetrix, USA) and the primer pair pyrG-RT-F/pyrG-RT-R for the *pyrG* gene (Table 2) were included. The $2^{-\Delta Ct}$ formula was used for determining the *phyA* gene copy number, in which ΔCt corresponds to Ct (in the strain of interest)-Ct (in the reference strain) (Schmittgen & Livak, 2008).

Extraction of crude enzyme solution

One milliliter of a spore suspension (10^7 spores/ml) harvested from the transformants was added to a 250 ml autoclaved flask containing 10 ml of liquid M+met and 4 g of dried clean rice husks. Rice husks were used as the carrier in order to provide ventilation for fungal growth. After incubation at 30°C for different amounts of time, 30 ml of sodium acetate (200 mM, pH 6) was added. The mixture was shaken at 200 rpm for 20 min. The crude enzyme supernatant was collected by filtration using Miracloth (Calbiochem, Germany) and centrifugation at 5000 rpm, 4°C for 15 min. The crude enzyme solution was kept at 4°C for later use or used directly for phytase activity assays.

Phytase activity assay

Phytase activity was determined according to Heinonen & Lahti (1981) (Heinonnen & Lahti, 1981) with some modifications. The reaction included 125 µl of the crude enzyme solution, 125 µl sodium phytate (15 mM) and 125 µl sodium acetate buffer (200 mM, pH 5.5). The reaction mixture was incubated at 55°C for 30 min, and terminated by adding 0.5 ml of 15% TCA. The color formation was achieved by transferring 125 µl of the mixture into 1 ml of AAM solution (10 mM ammonium molybdate, 100% acetone, 5 N sulfuric acid, 1:2:1). After 2 min, 100 µl of 1 M citric acid was added and the absorbance at 355 nm was determined after termination. The non-transgenic strain AUT1-PlD was selected as the control and each experiment was repeated independently three times. One unit (U) of phytase activity is defined as the amount of enzyme required for catalysis to produce one micromole of inorganic phosphate per minute under standard assay conditions. A standard curve was constructed using KH_2PO_4 at different concentrations.

RESULTS AND DISCUSSION

***A. oryzae* AUT1-PlD represents an excellent host for heterologous expression**

A. oryzae AUT1-PlD used in this study is a hyper-producing strain for recombinant protein production. This strain was genetically engineered from the wild strain *A. oryzae* RIB40 (Zhu et al., 2013). The *DsRed* expressing transformant (AUT1-R1) formed the pink mycelium, which could be observed even with the naked eye (Nguyen et al., 2017). This strain was selected for evaluating the suitability of the rice husk

cultivation model. The expression of the *DsRed* reporter was regulated by the strong *amyB* promoter (Figure 1A). This promoter could be induced by starch or maltose, and repressed by glucose (Tsuchiya et al., 1992). In this study, we reported that glucose was still able to activate the function of the *amyB* promoter, but it was not as strong as the specific inducer maltose (Figure 1B). The *amyB* promoter worked better for regulating gene expression in the solid-state cultivation rather than in the liquid-state fermentation. However, the collection of secreted enzymes from agar media seemed to be complicated. Here, we constructed a cultivation model using rice husks as the carrier for fungal growth. This is a promising approach for low-cost isolation of secreted enzymes. We tested the growth of the transgenic strain AUT1-R1 on the rice husk cultivation model and the results showed a strong expression of the *DsRed* gene, which resulted in the pink mycelium on agar plates. When observed under Axioplan fluorescence microscope, the red mycelium fully covered and adhered to the rice husks (Figure 1C).
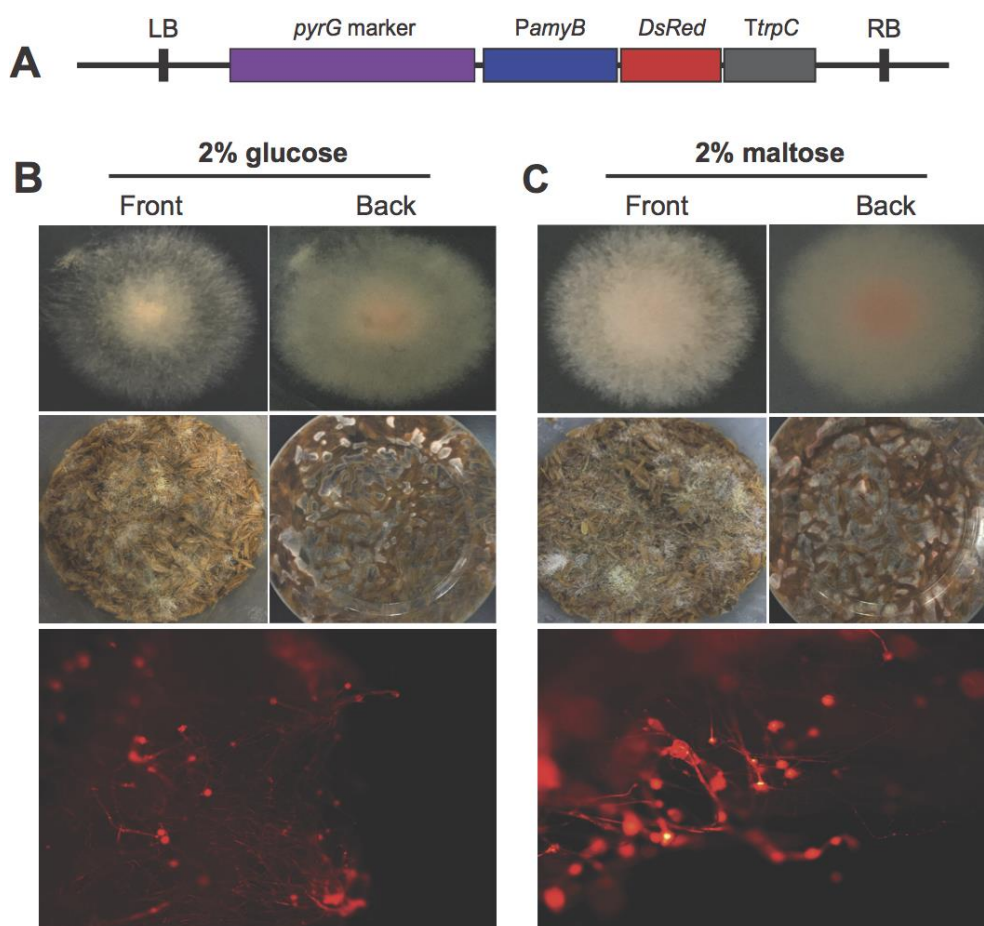

Figure 1. Heterologous expression of the *DsRed* reporter gene in *A. oryzae*. Glucose and maltose were used as the sole carbon sources to examine the ability for gene expression regulation of the *amyB* promoter. (A) The structure of T-DNA of the binary vector pEX2B. (B) The expression of the *DsRed* gene in AUT1-R1 cultivated on the agar medium and on the added rice husk medium with glucose as the sole carbon source. The mycelium of AUT1-R1 from the rice husk cultivation model was observed under the fluorescence microscope. (C) A similar experiment was carried out with maltose as the sole carbon source

The successful construction of a binary vector carrying the *phyA* expression cassette for the transformation of *A. oryzae* AUT1-PlD

The coding sequence of the *phyA* gene from the opportunistic human pathogen *A. fumigatus* was amplified from a cDNA sample and inserted into the pEX2B binary vector for genetic transformation of the safe filamentous fungus *A. oryzae*. The *DsRed* reporter gene in pEX2B was replaced with the *phyA* gene. The expression of *phyA* was controlled by the *amyB*

promoter (Figure 2). Transfer of the *phyA* expression cassette from the binary vector pEX2B-*phyA* into *A. oryzae* AUT1-PlD was conducted as previously described (Nguyen et al., 2016). In this strategy, the *pyrG* gene was recruited as a selection marker and the well-known *Agrobacterium tumefaciens* AGL1 strain was used as a tool for gene transfer into the fungal genome. The utilization of the *A. oryzae pyrG* gene as the nutritional marker for genetic transformation in *A. oryzae* would help to construct fungal transgenic strains without using antibiotic resistance genes.
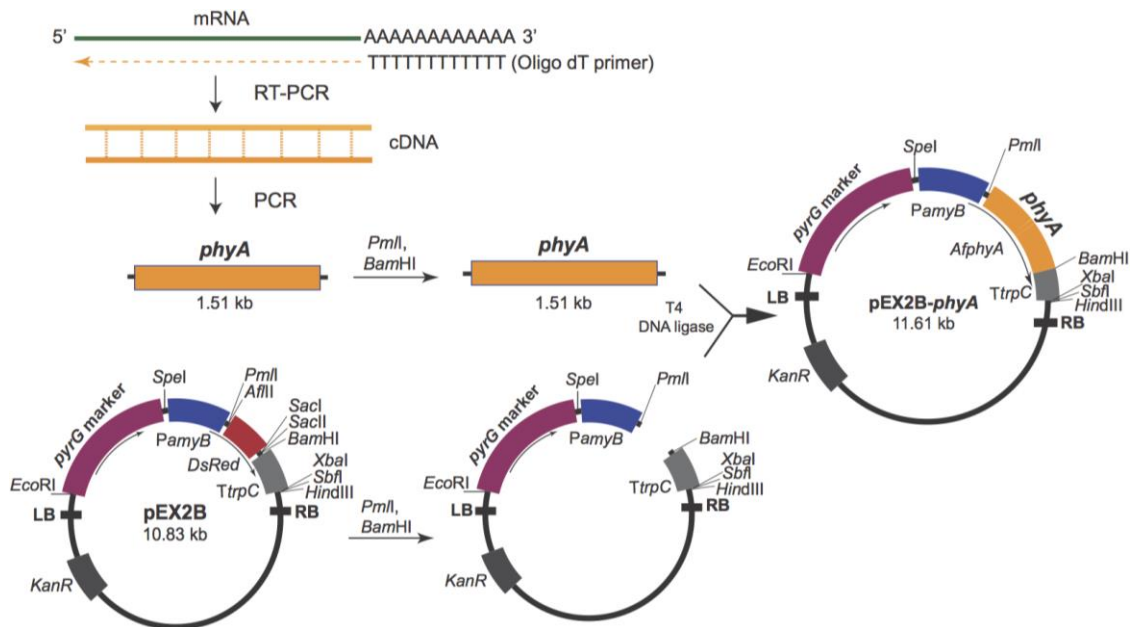

Figure 2. The diagram for the generation of the *phyA* expression construct. The *phyA* coding sequence was amplified from an *A. fumigatus* cDNA sample by PCR. The PCR products were treated with *Pml*I and *Bam*HI prior to being ligated into the respective restriction sites in the pEX2B binary vector to generate the recombinant vector pEX2B-*phyA*

Genetic transformation of *A. oryzae* AUT1-PlD using the ATMT method and the binary vector pEX2B-*phyA* resulted in 5−7 transformants per plate (Figure 3A). The obtained *phyA* expressing strains were cultivated on PSM agar plates for screening phytase activity. Bigger clearance zones of phytate degradation indicated stronger phytase activity in the transgenic strains. The non-transgenic *A. oryzae* AUT1-PlD strain was

used as the control. Three transgenic strains with the best activity for phytate degradation, designated as P1, P2, P3, (Figure 3B) were selected for DNA extraction to examine the presence of the *phyA* gene in fungal genomes by PCR using the specific primer pair PhyA-F/PhyA-R. The results indicated that the *phyA* expression cassette was successfully integrated into the genomes of the selected transformants (Figure 3C).
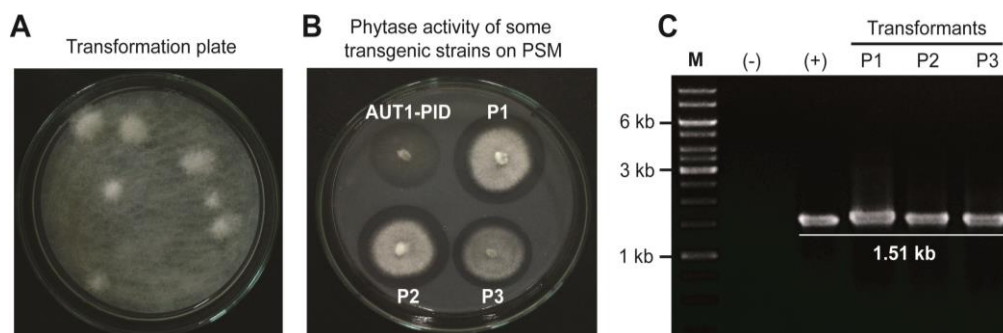
Figure 3. Transfer of the expressing construct for the *A. fumigatus phyA* gene into *A. oryzae* AUT1-PlD using ATMT. (A) Fungal transformants appeared on cellulose filter paper. (B) The ability for phytate degradation of three transgenic strains. (C) Confirmation of the *phyA* gene in the *A. oryzae* transgenic strains by PCR. M: 1 kb DNA ladder, (-) and (+) correspond to the negative and positive controls, respectively

The selected transgenic strains harbored more than one copy of the *A. fumigatus phyA* gene in their genomes

T-DNA of pEX2B-*phyA* carried both *pyrG* and *phyA*, which were simultaneously transferred into the *A. oryzae* AUT1-PlD strain. Therefore, the copy number of *pyrG* also reflected the copy number of *phyA* in genomes of the transgenic strains. Based on the genome database of *A. oryzae* RIB40 (http://www.aspergillusgenome.org), the *pyrG* gene has only one copy (accession number: AO090011000868) in chromosome 7. Therefore, the RIB40 strain (the original of the AUT1-PlD strain) was selected as the reference control for determining the *phyA* copy number in the transgenic strains (P1, P2, P3). The primer pair pyrG-RT-F/pyrG-RT-R

was designed for specific amplification of a piece of the *pyrG* gene with a product size of 196 bp. Analysis of real-time PCR quantifications based on Ct (cycle threshold) values and the $2^{-\Delta Ct}$ formula revealed the copy numbers of the *A. fumigatus phyA* gene in the genomes of three transgenic strains to be between 2 and 3 (Table 3).

The level of gene expression strongly relied on the copy number of the gene in the host cell (Li et al., 2013). Our results showed that three selected strains harbored 2 or 3 copies of the *phyA* expression cassette in their genomes (Table 3). The transgenic strain P2 with a high phytase activity on PSM (Figure 3B) exhibited the integration of 3 copies of the *phyA* gene in its genome (Table 3).

Table 3. The copy numbers of the *phyA* gene in the transgenic strains

Strain	Ct	Copy number ($2^{-\Delta Ct}$)
AUT1-PlD	-	0
RIB40	17.68	1
P1	16.47	2
P2	16.07	3
P3	16.75	2

The strain P2 appears to be a promising candidate for recombinant phytase production

The strain P2 and the control strain AUT1-PlD were grown in the cultivation medium

with added rice husk and supplemented with 2% maltose as the sole carbon source for induction of the *amyB* promoter. The crude enzyme solutions extracted from fungal cultures were used for phytase activity

evaluation. The obtained results indicated that the transgenic strain P2 displayed a phytase activity level 4.3 times higher than the control strain AUT1-PlD (Figure 4A). The data also showed that the amount of time suitable for optimal phytase activity of the strain P2 was approximately 6 to 7 days at 30ºC (Figure 4B). In this study, we have succeeded in expressing the *phyA* gene from the pathogenic fungus *A. fumigatus* in the hyper-producing strain *A. oryzae* AUT1-PlD. The transgenic strains can be exploited for future studies, in which the recombinant phytase will be purified and analyzed in more detail for favored characteristics such as heat stability or pH tolerance.
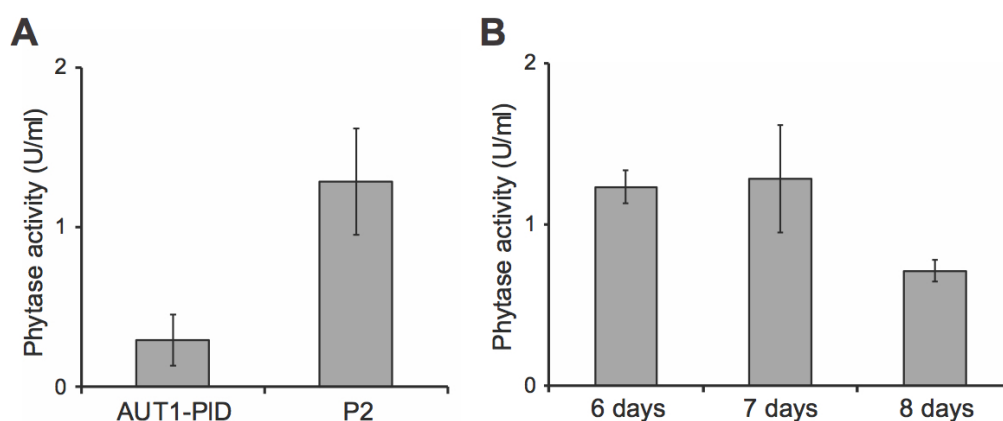

Figure 4. Phytase activity measurement. (A) Phytase activity of the *phyA* expressing strain P2 compared with the AUT1-PlD strain when cultivated in the added rice husk medium for 7 days at 30ºC. (B) Phytase activity of the strain P2 at different time intervals of cultivation

CONCLUSION

This work demonstrated the possibility of heterologous expression of genes of interest from a fungal pathogen in the safe fungus *A. oryzae* using the cultivation model with rice husk as the carrier for ventilation. The obtained transgenic strains possessed a high efficiency for phytase biosynthesis in this cultivation model. Heterologous expression of phytases in *A. oryzae* using the cultivation model with added rice husks may be exploited for low-cost production of these secreted enzymes as animal supplements.

REFERENCE

Barbesgaard P., Heldt-Hansen H. P., Diderichsen B., 1992. On the safety of *Aspergillus oryzae*: a review. *Appl. Microbiol. Biotechnol.*, 36: 569−572.

Christensen T., Woeldike H., Boel E., Mortensen S.B., Hjortshoej K., Thim L., Hansen M.T., 1988. High level expression of recombinant genes in *Aspergillus oryzae*. *Nat. Biotechnol.*, 6: 1419−1422.

Haefner S., Knietsch A., Scholten E., Braun J., Lohscheidt M., Zelder O., 2005. Biotechnological production and application of phytases. *Appl. Microbiol. Biotechnol.*, 68: 588−597.

Heinonnen J. K., Lahti R. J., 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal. Biochem.*, 113: 313−317.

Huge-Jensen B., Andreasen F., Christensen T., Christensen M., Thim L., Boel E., 1989. *Rhizornucor miehei* triglyceride lipase is processed and secreted from

transformed *Aspergillus oryzae*. *Lipids*, 24: 781−785.

Li M., Zhou L., Liu M., Huang Y., Sun X., Lu F., 2013. Construction of an engineering strain producing high yields of α-transglucosidase *via Agrobacterium tumefaciens*-mediated transformation of *Aspergillus niger*. *Biosci. Biotechnol. Biochem.*, 77: 1860−1866.

Machida M., Yamada O., Gomi K., 2008. Genomics of *Aspergillus oryzae:* learning from the history of koji mold and exploration of its future. *DNA Res.*, 15: 173−183.

Michielse C. B., Hooykaas P. J. J., van den Hondel C. A. M. J. J., Ram A. F. J., 2005. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Curr. Genet.*, 48:1−17.

Nguyen T. K., Ho N. Q., Pham H. T., Phan T. N., Tran V. T., 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.

Nguyen T. K., Ho Q. N., Do L. T. B. X., Mai L. T. D., Pham D. N., Tran H. T. T., Le D. H., Nguyen H. Q., Tran V. T., 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.

Pasamontes L., Haiker M., Wyss M., Tessier M., van Loon A. P. G. M., 1997. Gene cloning, purification, and characterization of a heat-stable phytase from the fungus

Aspergillus fumigatus. *Appl. Environ. Microbiol.*, 63: 1696−1700.

Punt P. J., van Biezen N., Conesa A., Albers A., Mangnus J., van der Hondel C., 2002. Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol.*, 20: 200−206.

Rao D. E. C. S., Rao K. V., Reddy T. P., Reddy V. D., 2009. Molecular characterization, physicochemical properties, known and potential applications of phytases: an overview. *Crit. Rev. Biotechnol.*, 29: 182−198.

Schmittgen T. D., Livak K. J., 2008. Analyzing real-time PCR data by the comparative C_t method. *Nat. Protoc.*, 3: 1101−108.

Tsuchiya K., Tada S., Gomi K., Kitamoto K., Kumagai C., Tamura G., 1992. Deletion analysis of the Taka-amylase A gene promoter using a homologous transformation system in *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.*, 56: 1849−1853.

Varga J., Frisvad J. C., Samson R., 2011. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Stud. Mycol.*, 69: 57−80.

White T. J., Bruns T., Lee S. J. W. T., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18: 315−322.

Zhu L., Maruyama J. I., Kitamoto K., 2013. Further enhanced production of heterologous proteins by double-gene disruption (*ΔAosedD ΔAovps10*) in a hyper-producing mutant of *Aspergillus oryzae*. *Appl. Biochem. Biotechnol.*, 97: 6347−6357.