Construction of a binary vector for the expression of the *Aspergillus niger McoD* laccase gene in the industrial filamentous fungus *Aspergillus oryzae*

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Received 2 May 2018; accepted 2 August 2018

Abstract:

The filamentous fungus *Aspergillus oryzae,* also named as the *koji* mold, is a preferred host for enzyme production due to its prominent secretion ability into the culture medium. Fungal laccases are widely used in different industrial processes, especially in removing environmental pollutants. Up till now, little was known about the roles of some laccase genes from the black mold *Aspergillus niger*. The *McoD* laccase gene from *A. niger* includes three exons interrupted by two short introns. The respective coding sequence of the gene is computationally predicted to encode a secreted laccase of 563 amino acids. In this study, a binary vector carrying the *A. niger McoD* gene was successfully constructed for heterologous expression in the edible fungus *A. oryzae*. This vector was transformed into *A. oryzae* using the *Agrobacterium tumefaciens*-mediated transformation method. The transformation efficiency was relatively high in two different auxotrophic *A. oryzae* strains, including RIB40Δ*pyrG* and VS1Δ*pyrG*. All the tested transformants possess in their genomes the construct for expression of the *McoD* laccase gene under control of the strong *A. oryzae amyB* promoter. The selected transformants were examined for the laccase activity using the ABTS substrate. The results showed that in comparison to the wild-type fungal strains, the transgenic strains could oxidise ABTS to form the sea green colour, which can be seen directly on the agar plate.

Keywords: ***Agrobacterium tumefaciens*-mediated transformation, *Aspergillus oryzae,* binary vector, laccase, recombinant expression.**

Classification number: **3.5**

Introduction

Aspergillus oryzae is a safe filamentous fungus and considered as an excellent microbial host for the biopharmaceutical industry and for the production of recombinant proteins. This fungus has been widely used in the food industry in some Asian countries for thousands of years. Several commercial digestive enzymes such as proteases, lipases and cellulases have been produced in *A. oryzae* [1, 2]. *A. oryzae* is a promising fungal cell factory owing to its ability to secrete large amounts of enzymes into the culture media. Therefore, this fungus has been employed for genetic engineering to produce economically valuable enzymes at the industrial scale [3].

Laccases are widely distributed in higher plants, insects, fungi and bacteria. They form the biggest sub-group within the multicopper oxidase (MCO) family and represent a great potential in biotechnological applications such as pulp delignification, textile dye bleaching, and water or soil detoxification [4, 5]. Laccase (EC 1.10.3.2) is a multicopper blue oxidase that couples the four-electron reduction of oxygen with the oxidation of a broad range of organic substrates including phenols, polyphenols, anilines, and even certain inorganic compounds by a one-electron transfer mechanism [6]. The ability of laccases to catalyse reactions by generating water as the only by-product makes these enzymes the ‘green’ catalysts in the industry [4, 5]. However, in most fungi, laccases are produced at low levels for commercial purposes. Therefore, cloning of the laccase genes followed by heterologous expression in suitable fungal hosts may provide outstanding enzyme yields [7].

In ascomycetes, MCOs have been much less studied [8].

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The McoD laccase belongs to the cluster of the ascomycetous laccases that exist in the common filamentous fungi such as *Botrytis cinerea*, *Neurospora crassa*, *Aspergillus niger* and *Aspergillus nidulans* [9]. It is remarkable that limited information is available for the potential of the laccase-encoding *McoD* gene. This is the first time that the *McoD* gene from *A. niger* was transformed and expressed in the safe fungus *A. oryzae* using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method.

Materials and methods

Microbial strains and plasmids

In this study, *Escherichia coli* DH5α was used as a bacterial host for plasmid propagation, with *Agrobacterium tumefaciens* AGL1 as the tool for fungal transformation and *A. niger* N402 as the DNA donor. Two different auxotrophic strains of *A. oryzae* including VS1Δ*pyrG* and RIB40Δ*pyrG* were employed as the recipients for genetic transformation. The binary vector pEX2B was used as the backbone for constructing the pEX2B-McoD vector. All these materials are listed in Table 1.

Table 1. Microbial strains and plasmids used in this study.

Strains, plasmids	Description	Source
E. coli DH5α	F^- *endA1 hsdR17 supE44 thi-1* λ^- *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)-U169 Φ80d*lacZ*ΔM15	[10]
A. tumefaciens AGL1	C58, *recA::bla*, pTiBo542ΔT-DNA, Mop^+, Cb^R	[11]
A. niger N402	The wild-type strain used as DNA donor	[12]
A. oryzae RIB40	The wild-type strain used as a negative control	[13]
A. oryzae VS1	The wild-type strain used as a negative control	[14]
A. oryzae RIB40Δ*pyrG*	The uridine/uracil auxotrophic strain used for genetic transformation	[14]
A. oryzae VS1Δ*pyrG*	The uridine/uracil auxotrophic strain used for genetic transformation	[14]
pEX2B	This plasmid harbours the *A. oryzae pyrG* marker and the *DsRed* gene under control of the *A. oryzae amyB* promoter	[14]
pEX2B-McoD	This plasmid harbours the *A. oryzae pyrG* and the *A. niger McoD* laccase gene under regulation of the *A. oryzae amyB* promoter	This study

Media for cultivation

Potato dextrose agar (PDA) medium (with supplements of 0.1% uracil and 0.1% uridine) was used for cultivating the auxotrophic *A. oryzae* VS1Δ*pyrG* and *A. oryzae* RIB40Δ*pyrG* strains.

M+met medium comprising 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 and pH 5.5 [15] was used as the selective medium for fungal transformation.

The induction medium (IM) supplemented with 0.05% uracil, 0.05% uridine and 200 µM acetosyringone (AS) was used for co-cultivation between the *Agrobacterium* cells and fungal spores. The liquid IM contains MM salts (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 $FeSO_4.7H_2O$), 40 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM glucose and 0.5% (w/v) glycerol. The solid IM contains only 5 mM glucose and 2% agar [16-18].

Preparation of spore suspensions

The VS1Δ*pyrG* or RIB40Δ*pyrG* strain was cultivated on the PDA plate that was supplemented with 0.1% uracil and 0.1% uridine for 3-5 days at 30°C. Sterile distilled water was added to the plate and the spores were released from the fungal layer by scraping the plate surface with a sterile glass spreader. The obtained liquid was filtered using sterile Miracloth (Calbiochem, Germany) and the filtrate was centrifuged at 5000 rpm for 10 min. The spore pellet was washed twice with sterile distilled water prior to being resuspended in sterile distilled water to gain the respective spore suspension. The spore concentration was calculated and adjusted to 10^6 spores/ml using a Thoma counting chamber.

Genomic DNA extraction

The extraction of fungal genomic DNA was performed as previously reported [19]. The obtained genomic DNA samples were dissolved in the TE buffer and treated with 3 µl RNase A (Qiagen, Hilden, Germany) for 30 min at 60°C to remove RNA.

Analysis of the McoD gene from A. niger

The sequence of the *Aspergillus niger McoD* gene

with the accession number An11g03580 was extracted from the *Aspergillus* genome database (http://www.aspergillusgenome.org). The deduced McoD protein was used for the detection of a signal peptide using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP) [20]. The conserved domains of McoD were detected by the web tools InterPro (https://www.ebi.ac.uk/interpro) and Pfam (https://pfam.xfam.org). The comparative phylogeny of McoD and its homologues from other filamentous fungi was performed with the MEGA6 software [21] using the neighbour-joining method with 1000 bootstrap replicates.

Construction of the binary vector pEX2B-McoD

The *McoD* gene was amplified from genomic DNA of *A. niger* N402 by PCR using the following primer pair including AnMcoD-F (GGG CTT AAG ATG CAC TTG CAT ACT ATC CTG G) and AnMcoD-R (GGG GAG CTC TTA GAT ACC AGA ATC ATC CTC CTC). To ensure the accuracy of DNA replication by the PCR, Phusion® high-fidelity DNA polymerase (Thermo Scientific, USA) was used. The PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, and extension at 72°C for 1 min 40s, with a final extension at 72°C for 10 min. The PCR product was purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified product was digested with the restriction enzymes *Afl*II and *Sac*I, and ligated into the binary vector pEX2B [14] at the respective restriction enzyme sites to replace the *DsRed* gene. The ligation mixture was then used to transform the competent *E. coli* DH5α cells. The recombinant plasmid was purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) and further confirmed by digestion with *Bam*HI (Thermo Scientific, USA) to indicate the presence of the *McoD* gene.

Genetic transformation of A. oryzae using ATMT method

ATMT transformation was performed as previously described [14, 18]. Briefly, the binary vector pEX2B-McoD was transformed into the *A. tumefaciens* AGL1 cells by electroporation method [22]. A single AGL1 colony carrying pEX2B-McoD was inoculated in a conical flask containing 20 ml of liquid LB (Luria-Bertani) supplemented with kanamycin (100 µg/ml) on a rotary shaker with 200 rpm at 28°C for 17h. The bacterial culture (1 ml) was diluted with the liquid IM (9 ml) containing 200 µM acetosyringone (AS) to obtain an OD_{600} value ranging from 0.2 to 0.3. The diluted culture was further incubated for 6h at 28°C, 200 rpm to reach an OD_{600} value ranging from 0.6 to 0.8. A mixed volume including 100 µl of the induced AGL1 suspension and 100 µl of the *A. oryzae* spore suspension (10^6 spores/ml) was spread onto the 90 mm filter paper (Sartorius, Germany) placed on the IM agar plate supplemented 200 µM AS, 0.05% (w/v) uracil and 0.05% (w/v) uridine. The plate was incubated at 22°C in the dark for 60h. After the co-cultivation period, the filter paper was transferred to the M+met plate containing cefotaxime (300 µg/ml) to eliminate the *Agrobacterium* cells. This plate was incubated at 30°C for 5-7 days until the fungal transformants appeared.

Analysis of fungal transformants

Fungal transformants were purified and examined for mitotic stability by single spore isolation for three successive generations. The purified transformants were then grown for genomic DNA extraction. PCR was employed to confirm the existence of the *A. niger McoD* gene in the *A. oryzae* genome using the specific primer AnMcoD-F/AnMcoD-R. To screen the transformants expressing the *McoD* gene, some obtained transformants were grown on the M+met agar medium containing 2% maltose as the inducer of the *amyB* promoter. After incubating at 37°C for 48h, 4 mM of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) in the McIlvaine buffer (also known as citrate-phosphate buffer, pH 4.6) [23] was dropped directly onto the fungal mycelium. The oxidation of the substrate ABTS by a suitable laccase results in the stable blue/green cation radical $ABTS^{\cdot+}$ [24]. Fungal transformants expressing successfully the *McoD* laccase gene can oxidise ABTS to generate the sea green colour.

Results and discussion

Structural analysis of the A. niger McoD laccase gene

The *McoD* gene indicated by the locus An11g03580 in the *Aspergillus niger* genome database and by the accession number XP_ 001394357 in the GenBank database has a total length of 1806 bp including three exons (881 bp, 483 bp and 328 bp) interrupted by two introns (52 bp and 62 bp). The coding sequence of *McoD* encodes a predicted protein of 563 amino acids with three conserved Cu-oxidase domains

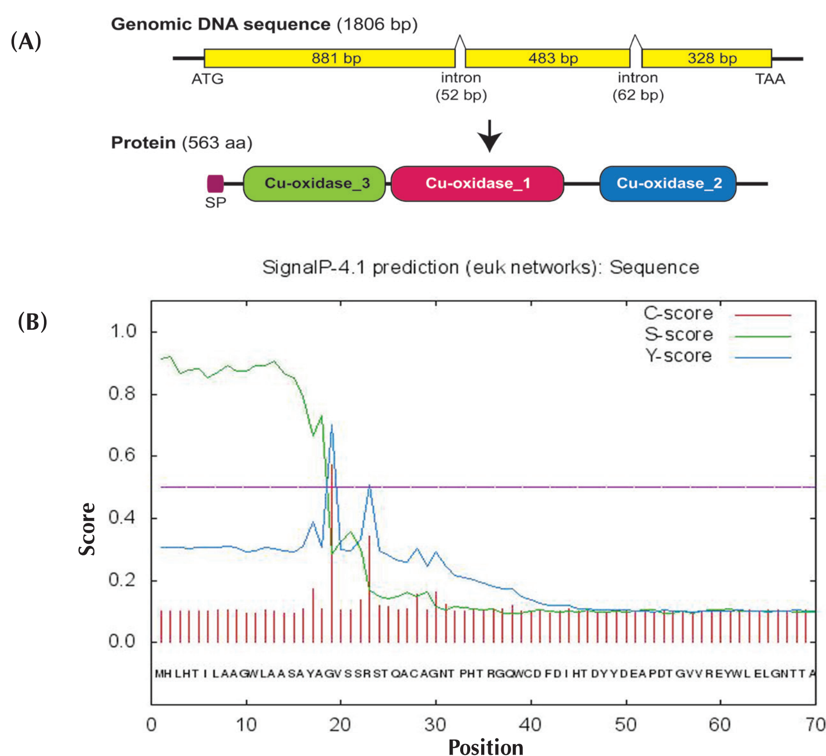

Fig. 1. Structural analysis of the *McoD* gene from *A. niger*. (A) The structure of the *McoD* gene and the respective encoded protein possessing a signal peptide (SP) and typical conserved domains for laccase family. **(B)** The signal peptide of the deduced McoD protein was detected with SignalP 4.1 [20].

(Fig. 1A). The deduced protein sequence is also indicated by the bioinformatics tool SignalP 4.1 to possess a putative signal peptide with the cleavage site between amino acid positions 18 and 19 at the N terminus (Fig. 1B).

In fungi, laccases play different physiological roles during the fungal life cycle. These enzymes display a huge potential for a variety of biotechnological applications due to their broad substrate range [4, 25, 26]. The black mold *A. niger* possesses 16 different genes encoding putative laccases in its genome [9]. Among them, McoD belongs to the group of the ascomycetous laccases. Although McoD is conserved in some other *Aspergillus* species, surprisingly, it does not exist as a homologue in the genome of *A. oryzae* (Fig. 2). Therefore, *A. oryzae* can be used as an excellent host for the heterologous expression of the *McoD* gene, as it appears that there is no activity of the McoD laccase in this filamentous fungus.

Successful construction of a binary vector for expressing the A. niger McoD laccase gene

Based on the respective sequence for *McoD* (An11g03580) from the *A. niger* genome database, this gene was successfully amplified by PCR using the specific primer pair AnMcoD-F/AnMcoD-R. The obtained PCR product was digested with two restriction enzymes, *Afl*II and *Sac*I. This digestion generated the compatible sticky ends enabling the ligation of the DNA insert to the binary vector pEX2B [14], which were also treated with the same enzymes *Afl*II and *Sac*I in order to remove the *DsRed* reporter gene (Fig 3A). The resulting recombinant plasmid pEX2B-McoD (Fig. 3A) was confirmed for the presence of the *McoD* gene by PCR with the specific primer pair AnMcoD-F/AnMcoD-R (data not shown). This plasmid was then purified and further confirmed by digestion with *Bam*HI, which cuts the plasmid at two different sites including one in the *McoD* sequence and the other in the plasmid backbone. The result showed that there were two DNA bands as theoretically calculated with the sizes of 10.56 kb and 1.39 kb appearing on the agarose gel (Fig. 3B). Therefore, we can conclude that the binary vector pEX2B-McoD for expressing the *A. niger McoD* laccase gene under the regulation of the *A. oryzae amyB* promoter was successfully constructed.

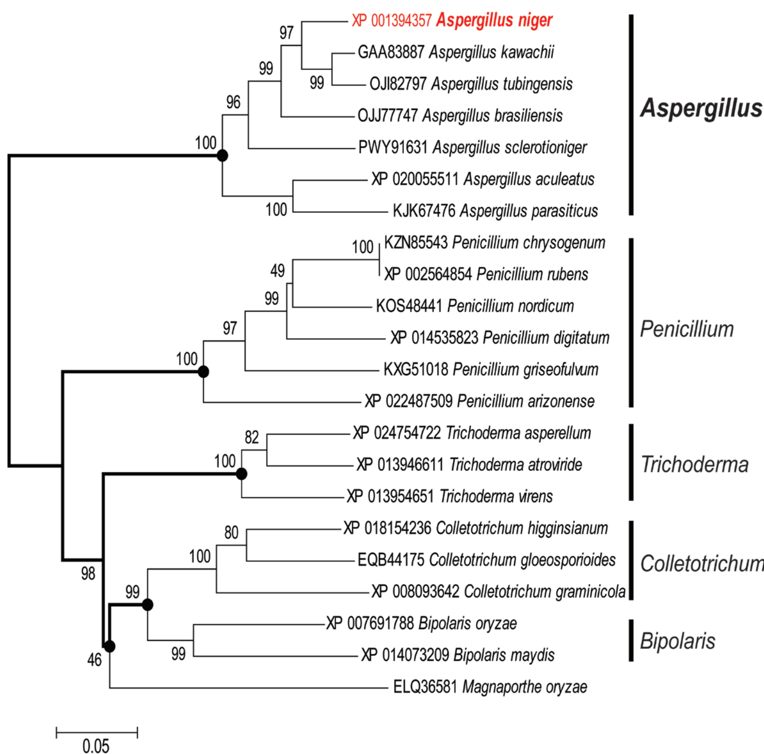

Fig. 2. Phylogenetic analysis of the *McoD* gene from *A. niger* in comparison to its homologues from other filamentous fungi. The phylogenetic tree was constructed with the MEGA6 software [21] using the neighbour-joining method with 1000 bootstrap replicates. The statistical support values at nodes of branches, genetic distance scale bar and the accession numbers from the GenBank database have been indicated.

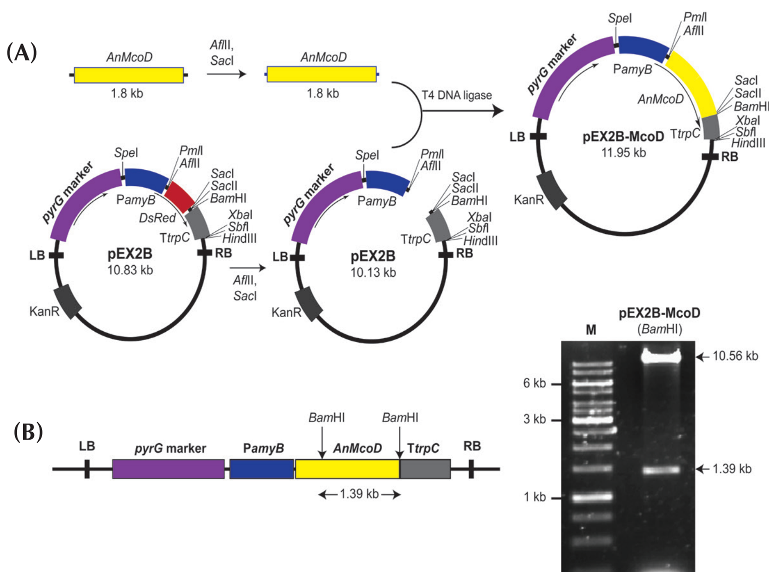

Fig. 3. Construction of the pEX2B-McoD binary vector. (A) The procedure for constructing pEX2B-McoD by replacing the *DsRed* gene in the pEX2B vector with the *A. niger McoD* laccase gene. **(B)** Confirmation of the correctness of pEX2B-McoD by digestion of this plasmid with *Bam*HI.

Successful transformation of A. oryzae using the ATMT method and the pEX2B-McoD vector

The ATMT method has been recently demonstrated to be a powerful tool for the genetic transformation of the filamentous fungus *A. oryzae* based on the uridine/uracil auxotrophy [14]. This method does not use drug resistance markers, and therefore, it is considered as a safe approach for subsequent applications. In this study, the binary pEX2B-McoD was constructed and transformed successfully into the competent *Agrobacterium tumefaciens* AGL1 cells by electroporation. The resulting bacterial colonies were screened for the presence of the plasmid by PCR with the primer pair AnMcoD-F/AnMcoD-R using GoTaq® Green Master Mix (Promega, USA). All of the tested colonies were indicated to carry pEX2B-McoD via the DNA band for *McoD* appearing on the 0.7% agrose gel (data not shown). The steps for fungal transformation using *Agrobacterium tumefaciens* were performed as previously reported [14, 18]. The procedure was summarised in Fig. 4A. During the co-cultivation step, *A. oryzae* spores germinated on the induction medium (IM) were supplemented with 0.05% uracil and 0.05% uridine at 22°C for 60h. Germination of fungal spores could facilitate the transfer and random integration of T-DNA carrying the *McoD* expression cassette and the auxotrophic *pyrG* selection marker from *Agrobacterium* cells into the *A. oryzae* genome. The mechanism of this DNA transfer event has been proved in numerous fungi [27]. The fungal transformants as prototrophic strains were selected on the M+met minimal medium [15], which was supplemented with cefotaxime to eliminate the *Agrobacterium* cells. After 5-7 days, the results showed that on an average, the transformation efficiency reached 6 transformants per plate, which corresponded to a total of 60 transformants per 10^6 spores for the auxotrophic *A. oryzae* VS1Δ*pyrG* strain. Meanwhile, 136±17 transformants per plate corresponded to a total of approximately 1360 transformants per 10^6 spores for the auxotrophic *A. oryzae* RIB40Δ*pyrG* strain (Fig. 4B).

ATMT has been reported to be an effective method for gene targeting in several filamentous fungi with the transformation efficiency up to 100-250 transformants per 10^6 spores [28, 29]. In the previous study, we showed that genetic transformation of the auxotrophic *A. oryzae* strains by the ATMT method using the pEX2B vector resulted in very high transformation efficiencies with 265±13 transformants per 10^6 spores for the auxotrophic VS1Δ*pyrG* strain and 1060±143 transformants per 10^6 spores for the auxotrophic RIB40Δ*pyrG* strain [14]. In this study, using the pEX2B-McoD vector, we also obtained similar results for the transformation efficiencies of the auxotrophic VS1Δ*pyrG* strain and the auxotrophic RIB40Δ*pyrG* strain (Fig. 4B).

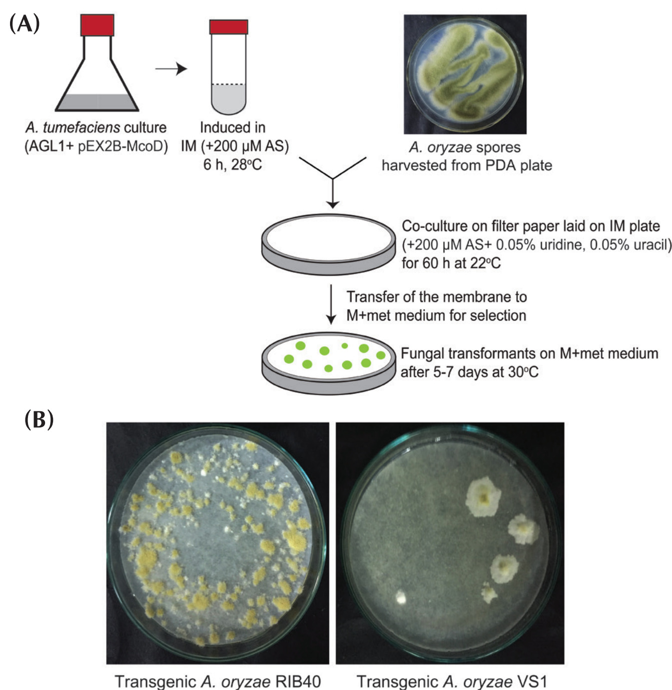

Fig. 4. Genetic transformation of the auxotrophic *A. oryzae* strains using the ATMT method. (A) The detailed ATMT procedure for *A. oryzae* VS1Δ*pyrG* and RIB40Δ*pyrG* with pEX2B-McoD. **(B)** The prototrophic *A. oryzae* transformants grew on the filter papers placed on M+met agar plates after 5 days of incubation at 30°C.

Successful integration of the McoD expression cassette into the A. oryzae genomes

In a recent publication, the overexpression of *McoD* in *A. niger* under the control of the *glaA* promoter could result in a green halo with ABTS oxidation [9]. In the constructed binary vector, *McoD* gene is regulated by the *amyB* promoter from *A. oryzae* RIB40. The *amyB* promoter is responsible for regulating the high-level expression of amylase in *A. oryzae*, and it is induced by starch or maltose [30]. Therefore, to activate the expression of the *McoD* gene under regulation of the *amyB* promoter, the M+met minimal medium was supplemented with 2% maltose as the sole carbon source. The mycelia of the transformants, which were cultivated on M+met (2% maltose) at 37°C for 48h, were tested for the ABTS oxidation ability. The transgenic strains expressing *McoD* in this study showed laccase activity towards ABTS. After treating with the ABTS substrate for 10-15 min, the fungal mycelia of all tested transformants changed to a sea green colour in comparison to the wild-type strains (Fig. 5A). This is a quick assay for screening the potential strains expressing the *McoD* laccase gene. The successful integration of the *McoD* expression cassette into the *A. oryzae* genome in the transformants was confirmed by PCR with the primer pair AnMcoD-F/AnMcoD-R. All of the tested strains were demonstrated to carry the McoD laccase gene with the size of 1.8 kb (Fig. 5B).

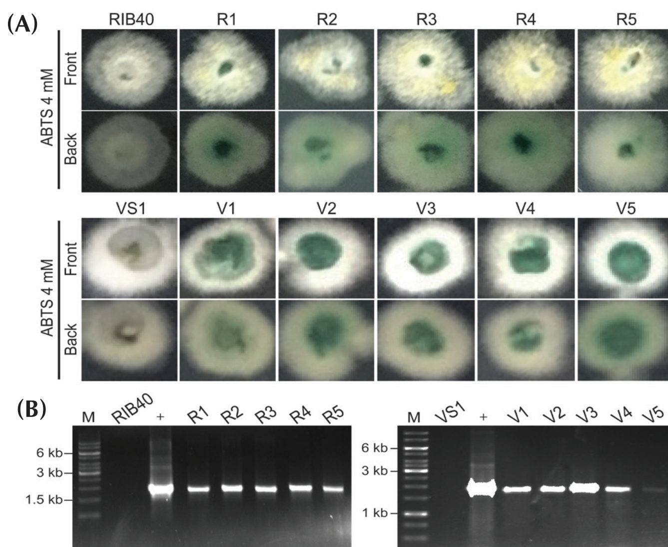

Fig. 5. Confirmation of the *A. oryzae* transformants expressing the *A. niger McoD* laccase gene. (A) Examination of some transformants expressing the *McoD* laccase gene. The selected transformants were grown on M+met (2% maltose as the sole carbon source for activating the *amyB* promoter) at 37°C for 48h. The ABTS solution was used to screen positive transformants by directly placing a drop onto the fungal mycelium. The VS1 and RIB40 wild-type strains were used as negative controls. **(B)** The above-tested transformants were confirmed by PCR for the existence of the *McoD* gene in the genome using the specific primer pair AnMcoD-F/AnMcoD-R. The genomic DNA samples extracted from the VS1 and RIB40 wild-type strains were used as templates for negative controls. The plasmid PEX2B-McoD was used as a template for positive controls.

Laccases from different fungal species may differ in their substrate specificities, and therefore, several substrates should be tested to assess a laccase activity [26]. The *A. oryzae* transformants expressing the *A. niger McoD* gene should be tested by plate activity assays for other substrates such as N, N-dimethyl-p-phenylenediamine sulphate (DMPPDA), 2,6-dimethoxyphenol (DMP) and 4-amino-2,6-dibromophenol/3,5-dimethylaniline (ADBP/DMA) to have more insights in its substrate specificity. A large number of transformants would show laccase activity against one or more than one substrate. According to Tamayo Ramos, et al. the *McoD* gene when overexpressed in *A. niger* showed its laccase activity towards ABTS, ADBP/DMA and DMPPDA, whereas it did not show activity when assayed with DMP [9]. Therefore, further studies need to be performed to examine the *A. oryzae* transformants for oxidation of these substrates by the McoD laccase activity.

Expression of a target gene in a suitable fungal host also depends on gene copy number. One copy of the gene expression cassette was sufficient for its expression, but an increase in copy number had a positive effect on the expression [31]. To identify the copy number of the target gene in a transgenic strain, real-time PCR or Southern hybridisation could be used. Furthermore, in order to determine the expression level of the *McoD* gene in potential *A. oryzae* transformants, the transcription level of the gene should be analysed by using real-time PCR with a gene-specific primer pair or by Northern hybridisation with a gene-specific probe.

Conclusions

In this study, we have succeeded in constructing the binary vector pEX2B-McoD carrying the *McoD* laccase gene from the black mold *A. niger* under the regulation of the strong *amyB* promoter. The ATMT method was employed to successfully transfer the T-DNA containing the *McoD* expression cassette from this binary vector to the genomes of two different auxotrophic *A. oryzae* strains. The *A. oryzae* strains expressing the *A. niger McoD* laccase gene were generated fruitfully by using the ATMT method with the *pyrG* auxotrophic marker. As the obtained transgenic *A. oryzae* strains possess no drug resistance gene, they are safe for exploiting potential applications of the McoD laccase in the future.

ACKNOWLEDGEMENT

We thank the members of the Genomics Unit, National Key Laboratory of Enzyme and Protein Technology (VNU University of Science, Vietnam National University, Hanoi) for their assistance and discussion.

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