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COMMENTARY

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# *Magnaporthe oryzae* as an expression host for the production of the unspecific peroxygenase *Aae*UPO from the basidiomycete *Agrocybe aegerita*

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## Abstract

The filamentous fungus *Magnaporthe oryzae* has the potential to be developed as an alternative platform organism for the heterologous production of industrially important enzymes. *M. oryzae* is easy to handle, fast-growing and unlike yeast, posttranslational modifications like N-glycosylations are similar to the human organism. Here, we established *M. oryzae* as a host for the expression of the unspecific peroxygenase from the basidiomycete *Agrocybe aegerita* (*Aae*UPO). Note, UPOs are attractive biocatalysts for selective oxyfunctionalization of non-activated carbon-hydrogen bonds. To improve and simplify the isolation of *Aae*UPO in *M. oryzae*, we fused a *Magnaporthe* signal peptide for protein secretion and set it under control of the strong EF1α-promoter. The success of the heterologous production of full-length *Aae*UPO in *M. oryzae* and the secretion of the functional enzyme was confirmed by a peroxygenase-specific enzyme assay. These results offer the possibility to establish the filamentous ascomycete *M. oryzae* as a broad applicable alternative expression system.

**KEYWORDS**
*Aae*UPO, heterologous expression, *Magnaporthe oryzae*, oxyfunctionalization, unspecific peroxygenases

## 1 | INTRODUCTION

Almost 20 years ago, the filamentous fungus *Magnaporthe oryzae* was established as a model organism for the investigation of the molecular basis of plant/pathogen interactions, infection-related differentiation processes, and fungal mitogen-activated protein kinases (MAPK) signaling by the pioneers: Dean, Talbot, Valent, and Xu (Dean et al., 2005, 2012). In the last few years, we worked intensively with this organism to unravel signal transduction mechanisms and fungicide action in a phosphorelay system called the high osmolarity glycerol (HOG) pathway (Bohnert, Antelo, et al., 2019; Bohnert et al., 2019; Jacob et al., 2014, 2015). *M. oryzae* is easy to cultivate in the laboratory, and bioinformatics pipelines as well as efficient transformation systems for directed genetic manipulation, including the possibility of generating "multistage mutants" as multiple selection markers, are established. A major advantage of *M. oryzae* compared to established yeasts' expression systems is that posttranslational modifications, such as N-glycosylation, are in the standard repertoire of filamentous fungi whereas hyper-glycosylation is not a problem (Maras et al., 1999; Punt et al., 2002; Salovuori et al., 1987). Yeasts often perform hyper-glycosylation, and invariably hyper-mannosylated glycoproteins might not be converted into a functional enzyme. Furthermore, the differences of chaperones and different post-translational modifications in yeasts (disulfide bridge, and N-terminal and C-terminal

processing) are important hurdles that must be circumvented (Carro et al., 2019) (Gomez de Santos et al., 2020). To extend the repertoire for heterologous expression systems for industrial applications, we established *M. oryzae* as an expression host for the unspecific peroxygenase from the basidiomycete *Agrocybe aegerita* (*Aae*UPO), which is under the most important industrial relevant glycoproteins (Aranda & del Carmen, 2019). The unspecific peroxygenases (UPO, EC 1.11.2.1) belong to peroxide-using enzymes that are of considerable interest due to their wide range of potential biotechnological applications (Burek et al., 2019). The UPOs are amongst the most attractive biocatalysts for the regioselective and stereoselective introduction of oxygen into organic molecules (Molina-Espeja et al., 2015). They are compact proteins with a thiolate axial ligand of the pivotal $Fe^{3+}$ that controls the heme domain. The UPOs secreted by fungi represent an intriguing enzyme type that selectively transfers peroxide-borne oxygen with high efficiency to diverse substrates including unactivated hydrocarbons (Hofrichter & Ullrich, 2014). Also, UPOs have been shown to modify pharmacological compounds by mimicking the role of human liver P450 monooxygenases, including O-dealkylations and hydroxylations, thereby simulating drug metabolism (Gomez de Santos et al., 2019). And, UPOs perform these reactions under mild conditions with only $H_2O_2$ as the oxygen source (Sigmund & Poelarends, 2020). The rapidly expanding field of environmentally friendly and sustainable chemistry bears a great potential to promote biocatalysts for selective oxyfunctionalizations, such as these hydrogen peroxide-utilizing enzymes. Consequently, further optimization of their properties is required for their effective use in industrial applications. Although thousands of genes encoding (hypothetical) UPOs have been identified in the sequences of fungal genomes, only a few UPO enzymes have been experimentally characterized to date (Linde et al., 2020). That's down to the fact that the challenges as mentioned above associated with the functional expression in heterologous hosts like yeasts are extremely difficult to solve (Martínez et al., 2017). Nevertheless, there have been some works that address protein engineering of UPOs. Many of them without fully satisfactory yields and activities (Molina-Espeja et al., 2016; Mate et al., 2017; Gomez de Santos et al., 2018; Knorrscheidt et al., 2021), although a few also delivered good results (Kinner et al., 2021) (Hofrichter et al., 2020).

Therefore, the expression of functional full-length *Aae*UPO in *M. oryzae* can be regarded as highly technical and industrially relevant. Even more important is that such an expression system can potentially be broadly applied for the expression of further eukaryotic (heme)-enzymes and glycoproteins.

## 2 | MATERIALS AND METHODS

### 2.1 | Strains andculture/growth conditions

The fungal strain used in this study was *Magnaporthe oryzae* (Fungal Genetics Stock Center, Kansas City, USA) (Jacob et al., 2015). The strain was grown at 26°C on complete medium (CM) at pH 6.5, agar (2%) containing per liter: glucose (10 g), yeast extract (1 g), peptone (2 g), casamino acids (1 g), nitrate salt solution (50 mL; containing per liter: $NaNO_3$ (120 g), KCl (10.4 g), $KH_2PO_4$ (30.4 g), $MgSO_4$ (10.4 g) × 7 $H_2O$) and a trace element solution (1 ml; containing per liter: $ZnSO_4$ (22 g) × 7 $H_2O$, $H_3BO_3$ (11 g), $MnCl_2$ (5 g) × 4 $H_2O$, $FeSO_4$ (5 g) × 7 $H_2O$, $CoCl_2$ (1.7 g) × 6 $H_2O$, $CuSO_4$ (1.6 g) × 5 $H_2O$, $Na_2MoO_4$ (1.5 g) × 2 $H_2O$ and $Na_2EDTA$ (50 g), at pH 6.5 adjusted by 1 M KOH). Minimal medium (pH 6.5) contains per liter: glucose (10 g), a 0.01% biotin solution (0.25 ml), nitrate salt solution (50 ml), a trace element solution (1 ml) and a 1% thiamine dichloride solution (1 ml). All chemicals used were pa quality unless otherwise stated.

To produce *Aae*UPO, the production strain *MoEF1*::*AaeUPO(SP)* was grown at 26°C and 120 rpm in a 5 L glass flask in 2.5 L liquid CM (+30 µM hemin) for 7 days.

### 2.2 | DNA manipulations, construction of the expression vector, and fungal transformation

The DNA of *Magnaporthe oryzae* was isolated from mycelium of three-day-old liquid cultures (grown in CM at 26°C and 120 rpm) using the DNeasy® plant mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions for the purification of DNA from plants and filamentous fungi. The DNA manipulation followed standard procedures (Green & Sambrook, 2012). NEB® 10-ß Competent *Escherichia coli* strains (High Efficiency) were used for the routine bacterial transformations and construction of plasmids. Fungal transformation of *M. oryzae* was conducted using *Agrobacterium tumefaciens*-mediated transformation (ATMT). The detailed procedures followed those described previously (Odenbach et al., 2007). To generate the expression mutant *MoEF1*::*AaeUPO(SP)*, the plasmid used for fungal transformation is based on a backbone of a binary *Agrobacterium*-compatible vector *pSJ + GFP(BAR)* (Bohnert, Antelo, et al., 2019). The mutants were generated by using a glufosinate-ammonium resistance (modified bialaphos resistance gene, BAR). The selection of transformants resistant to glufosinate-ammonium was performed by using 100 µg $ml^{-1}$ of the antibiotic in a minimal medium. The plasmid used for fungal transformation of the *Magnaporthe* wildtype strain (*MoWT*) was generated by the Gibson Assembly® cloning method (Gibson et al., 2009). The 8205 bp expression-plasmid *pSJ + MoEF1*::*AaeUPO(SP)* was generated by using the *Nco*I/*Bsr*GI-restricted *pSJ + GFP(BAR)* as backbone. The cDNA sequence of *AaeUPO* was amplified from the plasmid *pUC57_AaeUPOcDNA* using the primer pair SJ-904/SJ-839. With this primer pair, the signal peptide for protein secretion of the gene MGG_08253 (AAGTTCCTCACCCC GCTCGCCGCCCTTCTCAGCACCGCCTCGGCGGCC) was cloned directly in front of the *Aae*UPO sequence to be expressed. The signal peptide of the gene MGG_08253 was selected because the corresponding protein is present in large quantity in the secretome of *M. oryzae* (Antelo/IBWF, unpublished results). The start codon (ATG) from the *Aae*UPO to be expressed is removed and placed in front of the MGG_08253 signal-peptide (Figure 1).

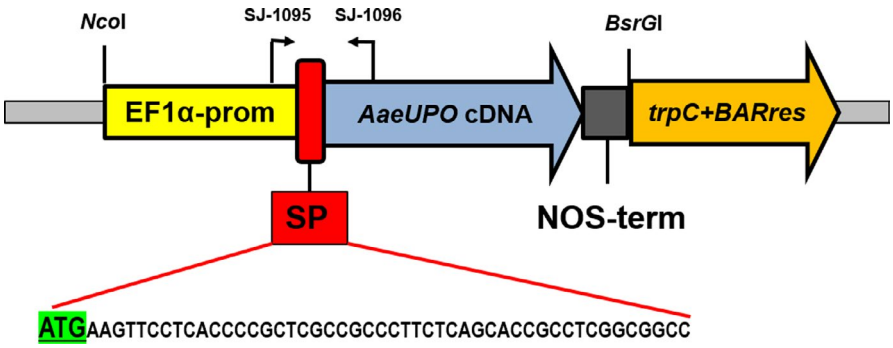


**FIGURE 1** Schematic representation of the expression cassette of the plasmid *pSJ + MoEF1::AaeUPO(SP)*. The coding sequence for the strong *Magnaporthe* own promoter *EF1α* is followed by the ATG-modified secretion signal peptide sequence (from MGG_08253) and subsequently by the cDNA-sequence of *AaeUPO* from *Agrocybe aegerita*. The NOS terminator (terminator of the nopaline synthase gene from *A. tumefaciens*) was introduced at the 3′end to stop translation directly followed by the trpC + BAR glufosinate-ammonium resistance gene from pCB1635 (Sweigard et al., 1997). The primer pair SJ-1095/1096 indicates the location of the DIG-probe for southern blot analysis

Sequence details can be found in the supplementary section and the primers used are listed in Table 1. The accurate assembly of the expression plasmid was confirmed by sequencing. Finally, the plasmid *pSJ + MoEF1::AaeUPO(SP)* was used to transform *MoWT* via ATMT, resulting in the mutant strains *MoEF1::AaeUPO(SP)*. The integration into the genome was confirmed by southern blot analysis (Figure A1).

### 2.3 | Protein purification

Culture broth that exhibited activity in the NBD assay (see below) was concentrated (approximately 20-fold) and dialyzed against 10 mM potassium phosphate buffer pH 7 by ultrafiltration over a 10 kDa cutoff membrane (Sartorius Vivaflow 200, PES, 10 kDa). The sample exhibited an NBD activity of 1.3 U $ml^{-1}$. The sample was applied to an FPLC system (Knauer Azura, Berlin, Germany) equipped with a HiPrep DEAE FF 16/10 column (GE Healthcare, Solingen, Germany) and protein was eluted with a NaCl gradient from 0 to 1 M (in 10 mM potassium phosphate pH7) over four column volumes with a flow rate of 5 ml $min^{-1}$. Fractions with significant heme-specific absorption (410 nm) were probed for enzyme activity using the NBD assay and visualized via SDS-PAGE.

### 2.4 | Enzymatic assay

Enzymatic activity was determined using 5-nitro-1,3-benzodioxole (NBD) as a substrate (Poraj-Kobielska et al., 2012). Note, NBD assays were carried out in 1 ml PMMA cuvettes using a Shimadzu UV-1800 photometer. The assay consisted of 550 µl 100 mM potassium phosphate buffer pH 7, 100 µl 5 mM NBD in acetonitrile, 200 µl $H_2O$, 50 µl 20 mM $H_2O_2$, and 100 µl sample. Absorption was measured at 425 nm. Product formation was calculated with an extinction coefficient of 9700 $M^{-1}$ $cm^{-1}$.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Heterologous expression of AaeUPO in *Magnaporthe oryzae*

The cDNA sequence of *Aae*UPO was fused to a *Magnaporthe*-specific signal peptide for secretion and we integrated the modified sequence into the genome of *M. oryzae* under control of a constitutively strong *Magnaporthe*-specific promoter, EF1-α (Figure 1).

The correct integration of multiple *MoEF1::AaeUPO(SP)*-constructs into the fungal genome and consequently, the molecular modification was confirmed by southern blot analysis of the mutant strain *MoEF1::AaeUPO(SP)* (Figure A1). The accurate expression and translation of the recombinant basidiomycetous DNA as well the secretion of the functional enzyme *Aae*UPO was verified in a specific assay using 5-nitro-1,3-benzodioxole (NBD assay, (Poraj-Kobielska et al., 2012) (Figure 2).

The enzyme was purified from the supernatant of a shake flask culture by ion-exchange chromatography. Fractions with increased absorbance at 410 nm, characteristic of heme-containing proteins, corresponded to the fractions with peroxygenase activity when NBD was used as substrate. The culture broth of *MoEF1::AaeUPO(SP)* was concentrated (approximately 20-fold) and the concentrated sample exhibited an NBD activity of 1.3 U/ml. The control samples (i.e. *MoWT*) did not show any enzyme expression at all. No peroxygenase activity was detectable in other fractions (Figure 2). By SDS-PAGE, a predominant protein band with an apparent molecular weight slightly below 55 kDa could be visualized, among others. This corresponds approximately to the molecular weight of the glycosylated enzyme homologously produced in *Agrocybe aegerita* (46 kDa) (Ullrich et al., 2004). The UPO protein engineers have a long and diverse wish list, which has not yet been addressed comprehensively in yeast expression systems: the improvement of the turnover numbers, the modification of selectivity, avoid overoxidation reactivities, the removal of the unwanted

**TABLE 1** List of oligonucleotides used in this study

| Name | Sequence (5′→3′) |
|---|---|
| SJ – 904 (SP + AaeUPO1-for2) | acattatcatcaaaaagagagcaccaaaccgccacatgaagttcctcaccccgctcgccgcccttctcagcaccgcctcggcggccAAATATTTTCC CCTGTTCCCAACCTTG |
| SJ – 839 (AaeUPO1-rev) | acgatctgcagccgggcggccgctttacttTCAATCTCGCCCGTATGGGA |
| SJ – 1095 (DIG-AaeUPO1-for) | CTCCGCGCAAAACTTTG |
| SJ – 1096 (DIG-AaeUPO1-rev) | GAGTGTTGGGATTATAGCG |

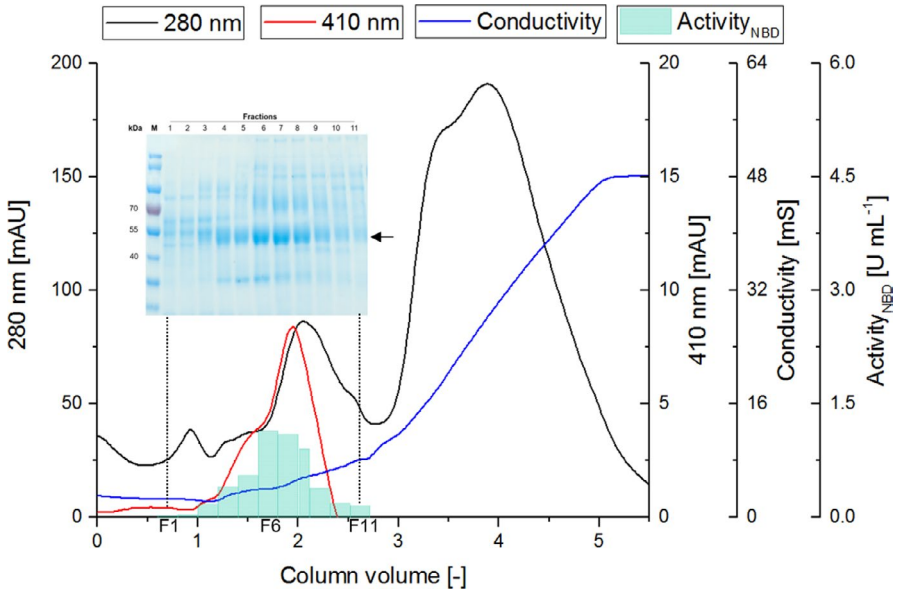


**FIGURE 2** Purification of *Aae*UPO produced in *M. oryzae*. Purification was performed from the culture broth via FPLC on a HiPrep DEAE FF 16/10 column using a NaCl gradient. Enzyme activity (turquoise bars) was determined using NBD as substrate. Corresponding fractions (F1-F11) were analyzed via SDS-PAGE (insert). The lanes of the SDS gel contain (from left to right) molecular markers, fractions 1–11. The arrow indicates the protein bands corresponding to the theoretical molecular weight of *Aae*UPO

peroxidase activity during oxyfunctionalizations, to improve oxidative stability as well as its performance in the presence of organic co-solvents and/or at high temperatures (Hobisch et al., 2020). A major positive aspect of *M. oryzae* is that posttranslational modifications, such as N-glycosylation, are correctly carried out by filamentous fungi (Maras et al., 1999; Punt et al., 2002; Salovuori et al., 1987). Yeasts sometimes perform hyper-glycosylation, and invariably hyper-mannosylated glycoproteins might not be converted into higher fungus type glycoproteins such as *Aae*UPO.

As mentioned above, for all these goals, *M. oryzae* appears to be optimally suited to functionally produce the *Aae*UPO enzyme to sculpture its properties by molecular manipulation. In conclusion, this report is the starting point to improve the use of non-specific peroxygenases in sustainable and resource-efficient chemistry in a much more flexible way than before.

The *Aae*UPO is one of the important enzymes for industrial biotechnology (Molina-Espeja et al., 2015). Nevertheless, *Aae*UPO has not been subjected to successful heterologous production in a filamentous fungus to date (Hobisch et al., 2020). Despite their broad presence in the fungal kingdom, only a handful of UPOs have been characterized so far, and almost nothing substantial is known on their biological function (Bormann et al., 2021). Furthermore, the reliable heterologous expression of UPOs remains challenging, despite all progress in this field (Hofrichter et al., 2020). Missing chaperones along with different post-translational modifications (glycosylation, disulfide bridge, N-terminal and C-terminal processing) among natural and heterologous hosts are high hurdles that must be avoided (Hobisch et al., 2020). Here, we demonstrate that the filamentous fungus *Magnaporthe oryzae* is applicable as a suitable platform organism to functionally produce this specialized eukaryotic glycoprotein or further heme enzymes.

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There is no funding to declare.

## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

**Stefan Jacob:** Conceptualization (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing-original draft (equal). **Sebastian Bormann:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Methodology (equal); Project administration (equal); Writing-original draft (equal). **Michael Becker:** Investigation (equal); Methodology (equal); Visualization (equal). **Luis Antelo:** Conceptualization (equal); Data curation (equal); Investigation (equal); Methodology (equal); Supervision (equal); Writing-review & editing (equal). **Dirk Holtmann:** Conceptualization (equal); Funding acquisition (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing-review & editing (equal). **Eckhard Thines:** Conceptualization (equal); Funding

acquisition (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing-review & editing (equal).

## ETHICS STATEMENT

None required.

## DATA AVAILABILITY STATEMENT

The sequence of the expression plasmid pSJ+SP+AaeUPO1 (8205 bp complete, including the 1116 bp cDNA of AaeUPO1) is available in NCBI GenBank under the accession number MZ711430: https://www.ncbi.nlm.nih.gov/nuccore/MZ711430

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## APPENDIX

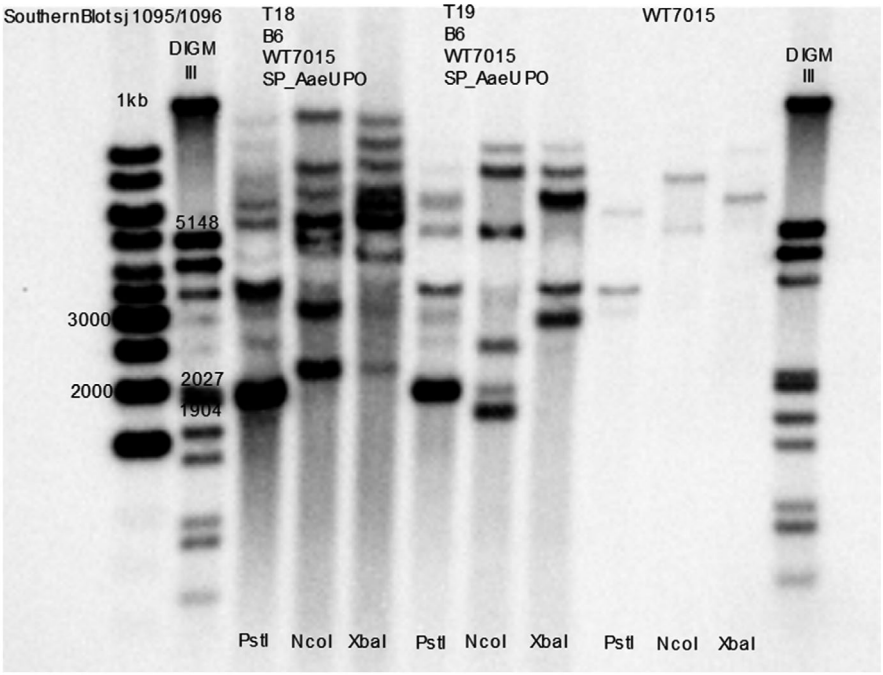


**FIGURE A1** Southern hybridization of the genome from the mutant strains *MoEF1::AaeUPO(SP)*. Southern blot analysis of overexpression mutants *MoEF1::AaeUPO(SP)#18* (T18) *and MoEF1::AaeUPO(SP)#19* (T19) with expression cassette specific probes. Genomic DNA of *M. oryzae* wild-type strain 70-15 and the mutants was isolated and restricted with restriction enzymes *Pst*I, *Nco*I, and *Xba*I. The probes which we used for hybridization with the genomic DNA of the wildtype strain and the corresponding mutant strains were amplified with the primer pair SJ-1095/SJ-1096 and always identical. The DIG marker III (DIGM III, Sigma-Aldrich) was used as control.