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Characterization of three multicopper oxidases in the filamentous fungus *Podospora anserina*: A new role of an ABR1-like protein in fungal development?

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ABSTRACT

The *Podospora anserina* genome contains a large family of 15 multicopper oxidases (MCOs), including three genes encoding a FET3-like protein, an ABR1-like protein and an ascorbate oxidase (AO)-like protein. FET3, ABR1 and AO1 are involved in global laccase-like activity since deletion of the relevant genes led to a decrease of activity when laccase substrate (ABTS) was used as substrate. However, contrary to the *P. anserina* MCO proteins previously characterized, none of these three MCOs seemed to be involved in lignocellulose degradation and in resistance to phenolic compounds and oxidative stress. We showed that the bulk of ferroxidase activity was clearly due to ABR1, and only in minor part to FET3, although ABR1 does not contain all the residues typical of FET3 proteins. Moreover, we showed that ABR1, related to the *Aspergillus fumigatus* ABR1 protein, was clearly and specifically involved in pigmentation of ascospores. Surprisingly, phenotypes were more severe in mutants lacking both *abr1* and *ao1*. Deletion of the *ao1* gene led to an almost total loss of AO activity. No direct involvement of AO1 in fungal developmental process in *P. anserina* was evidenced, except in a *abr1*$^{\Delta}$ background. Overall, unlike other previously characterized MCOs, we thus evidence a clear involvement of ABR1 protein in fungal development.

1. Introduction

Multicopper oxidases (MCOs) belong to a superfamily of enzymes that contains copper atoms in their catalytic center and are able of coupling the oxidation of various substrates, including phenolic compounds, with a four-electron reduction of molecular oxygen to water (Baldrian, 2006). Members of the MCO family have been previously classified as true laccases (EC 1.10.3.2), bilirubin oxidases (EC.1.3.3.5), some nitrite reductases (EC. 1.7.2.1), mammalian ceruloplasmin enzymes (EC 1.16.3.1), ferroxidases (FET3; EC 1.16.3.1) and ascorbate oxidases (AO; EC 1.10.3.3) (Reiss et al., 2013). In some previous phylogenetic analysis including a large set of MCOs from ascomycetes, the FET3 family was subdivided in canonical FET3 and in a group of ABR1 proteins that intermingle within the canonical FET3 that however act in the melanin biosynthesis pathway (Hoegger et al, 2006; Pöggeler, 2011; Kües and Rühl, 2011).

FET3 proteins are essential components of the high-affinity reductive iron assimilation (RIA) pathway in fungi. In *Saccharomyces cerevisiae*, the iron metabolism is well understood for a long time (Shi et al., 2003). It depends on the proper functioning of Fre1p, Fet3p and Ftr1p. Fre1p is a metalloreductase in the yeast plasma membrane that is essential to the uptake of environmental Cu^{2+} and Fe^{3+}. *S. cerevisiae* Fet3p is also located in the plasma membrane and is essential for high affinity iron uptake by catalyzing the oxidization of Fe^{2+} to Fe^{3+}. During the uptake of Fe^{3+}, Fre1p produces Fe^{2+} that is a substrate for Fet3p; the Fe^{3+} produced by Fet3p is the ligand for the iron permease, Ftr1p. Fet3p may also play a role in cellular defense against copper toxicity (Shi et al., 2003). Deletion of FET3 leads to iron deficiency and copper sensitivity. Such phenotype has been previously found in the yeast *S. cerevisiae* by (Askwith et al., 1994) who demonstrated that inactivation of *FET3* gene led to an iron-deficient phenotype. The ability to acquire iron *in vivo* is essential for most microbial pathogens. Albarouki and Deising (2013) have shown that FET3-1 of the hemibiotroph *Colletotrichum graminicola* is specifically expressed in and required for development of biotrophic hyphae, and that this gene is a determinant of virulence. The importance of RIA system via an iron

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permease (Fer2p) and a FET3 (Fer1p) for biotrophic development has also been shown ten years ago in the *Ustilago maydis*/maize pathosystem (Eichhorn et al., 2006). In this study *fer2*, as well as *fer1* deletion mutants were strongly affected in virulence. In the human pathogen *Aspergillus fumigatus* it has been shown that the high-affinity iron uptake system, *i.e.* siderophore-assisted iron uptake, which are induced upon iron starvation, contributes to pathogenic growth and play a role during infection (Haas et al., 2008; Schrettl et al., 2004, 2007). Close to the canonical FET3, a group of ABR1-like proteins have been described to act in the DHN (1,8-dihydroxynaphthalene)-melanin synthesis pathway, one of the two known fungal pathways leading to formation of dark stained melanins. Such pathway involved the *de novo* synthesis of the phenolic compound DHN by a polyketide synthase (PKS) pathway and the subsequent polymerization producing the DHN melanin (Eisenman and Casadevall, 2012). This last step is mediated by the ABR1 proteins, for example ABR1 in *A. fumigatus* and CHMCO1 in *Cochliobolus heterostrophus* (Saitoh et al., 2010; Tsai et al., 1999). DHN melanin plays different roles in the ascomycete life cycle; for example, it acts as a virulence determinant in *A. fumigatus* (Tsai et al., 1999), and its accumulation in the appressoria of the phytopathogenic fungus *Magnaporthe grisea* is necessary for entering the host tissue (Chumley and Valent, 1990). DHN melanin is also accumulated in reproductive structures, as conidia of *A. fumigatus* or perithecia and ascospores of *Sordaria macrospora*, giving them rigidity and strength (Engh et al., 2007; Tsai et al., 1999). Finally, as reported by Hoegger et al. (2006); Pöggeler (2011) and Kües and Rühl (2011), and despite the fact that they both belong to the same FET3 family, ABR1 proteins clearly differ from canonical FET3 because they do not have a C-terminal TM domain and also because the related gene is not clustered with a permease gene.

Ascorbate oxidases (AOs) are only found in plants and fungi and catalyze oxygen reduction to water preferentially using ascorbate as the electron donor and leading to the production of monodehydroascorbate (Farver et al., 1994; Hoegger et al., 2006). In plants, their function remains controversial and is not fully explained so far. Their expression and activity are induced by auxin and light, suggesting in part a possible role of this enzyme in signal perception/transduction (De Tullio et al., 2004). It has been also shown that AO over-expressing plants are prone to salt stresses, whereas lower expression apparently confers resistance to unfavorable environmental conditions (Yamamoto et al., 2005). More recently, (Balestrini et al., 2012) showed that the expression of a *Lotus japonicus* AO gene is induced in symbiotic interactions both with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi, supporting the hypothesis of a key role of this gene/enzyme in nodule and arbuscular mycorrhizal development and functioning. It has also been shown that AO could play an essential role as a modulator of both ascorbate and oxygen content, with relevant implications related to signaling (De Tullio et al., 2013). Scientific literature is very poor concerning fungal AO. Almost twenty years ago, a gene encoding a thermostable AO was cloned from *Acremonium* sp. and was successfully expressed in *Aspergillus nidulans*. Site-directed mutagenesis at the recombinant enzyme resulted in an increase in azide resistance, as previously found for several AO of plants (Takeda et al., 1998). More recently, it was proven that the oxidative activity of *Acremonium* sp. AO was dominated by the highly selective substrate-binding affinity based on electrostatic interaction beyond the one-electron redox potential difference between AO's type 1 copper site and substrate (Murata et al., 2008).

Podospora anserina is a coprophilous filamentous ascomycete that grows on herbivore dungs, a highly competitive habitat where several dozens of species are present and feed on partially degraded plant material (Silar, 2013). *P. anserina* is also used as an efficient laboratory model because of its ease of molecular genetic manipulations, especially in the construction of multiple deletion strains. Moreover, the complete genome sequence of this fungus has been available for several years (Espagne et al., 2008; Grognet et al., 2014). In a previous study, we identified 15 MCO coding sequences from the genomic databases of *P. anserina*, which were then clearly divided in distinct families, *i.e.*, nine canonical laccases (Family A), one related MCO, two bilirubin oxidases-like (BODs-like; Family I), one AO-like (Family F), and two iron transport FET3-like proteins (Family E) (Xie et al., 2014). Characterization of the nine canonical laccases and the related MCO by targeted gene deletion (Xie et al., 2014) showed that laccases are key actors of lignocellulose and plastic degradation, and confirmed that they participated in the detoxification of phenolic substrates. More recently, the functional characterization of the two BODs-like showed that both BODs but also the related MCO were non-canonical thermostable laccases that participated in the degradation of lignocellulose (Xie et al., 2015). Overall, none of previously characterized MCOs was clearly involved in the growth and development of *P. anserina*.

In the present study, single mutants were generated for *Pa_4_3640*, encoding the AO-like protein, as well as for *Pa_2_530* and *Pa_6_4220,* previously thought to encode two ferroxidase FET3-like proteins (Xie et al., 2014), and named here ABR1 and FET3, respectively, according to phylogeny and functional characterization presented in this study. Double and triple mutants were also constructed. All the mutants were characterized for defects in mycelial growth, sexual reproduction (*e.g.*, fruiting body named perithecia, formation and ascospore production), when grown with various substrates including lignocellulose. Our results show that FET3, ABR1 and AO proteins were not involved in lignocellulose degradation. However, the most striking results were that ABR1 was specifically involved in pigmentation of ascospores. Surprisingly ascospores of mutants lacking both *abr1* and *ao1* were not expelled from perithecia and lacked nearly all pigments. We thus evidence a clear involvement of ABR1 in fungal development and a putative interaction between ABR1 and AO1 in this developmental process.

2. Materials and methods

2.1. Strains and growth conditions

All strains of *P. anserina* used in this study were derived from the "S" wild-type strain that was used for sequencing of the *P. anserina* genome (Espagne et al., 2008). The most recent protocols for standard culture conditions, media and genetic methods for *P. anserina* can be found at http://podospora.i2bc.paris-saclay.fr. The Δ*mus51*::*phleoR and* Δ*mus51*::*hygro* mutants strain differed from the S wild-type reference strain by a single deletion of the *mus-51* gene, which has increased frequency of targeted gene replacement (El-Khoury et al., 2008).

2.2. Phylogenetic and multiple sequence analysis

All MCO genes of *P. anserina* were previously identified as reported in Xie et al. (2014). Fifteen MCOs were then described, including two FET3-like (Family E) and one AO-like (Family F). In this new study, the putative FET3 encoded by *Pa_2_530* was named ABR1, the putative FET3 encoded by *Pa_6_4220* was named FET3, and the putative AO encoded by *Pa_4_*3640 was named AO1. As in Xie et al. (2014), 7 putative laccases previously functionally characterized in other filamentous fungi were included in this study: *Botryotinia fuckeliana* (Bflcc1, accession number AF243854 and Bflcc2 accession number AF243855), *Metarhizium anisopliae* (MLAC1, accession number EU769126), *A. fumigatus* (abr2, accession number AF104823) and *Fusarium oxysporum* (Folcc1, accession number EF990894, Folcc3, accession number EF990899 and Folcc5, accession number EF990897). The ABR2-like AnyA from *A. nidulans* (CAA36787.1; Aramayo and Timberlake, 1993) was also included in this study. In order to improve the phylogeny for FET3 and AO, 14 FET3 and one AO previously characterized in other filamentous fungi were added in this study: FET3 from *S. cerevisiae* (ScFet3, accession number NP_013774.1; Askwith et al., 1994), *C. graminicola* (CgFet3-1 and CgFet3-2, accession numbers XP_008097412 and XP_008090819, respectively; Albarouki and