Biotechnology Advances xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Plant biomass degrading ability of the coprophilic ascomycete fungus *Podospora anserina*

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 11 May 2016
Accepted 27 May 2016
Available online xxxx

Keywords:
Lignocellulose
Carbohydrate
Lignin
Enzymes
Metabolism
Appressorium
Colonization
Hyphae

ABSTRACT

The degradation of plant biomass is a major challenge towards the production of bio-based compounds and materials. As key lignocellulolytic enzyme producers, filamentous fungi represent a promising reservoir to tackle this challenge. Among them, the coprophilous ascomycete *Podospora anserina* has been used as a model organism to study various biological mechanisms because its genetics are well understood and controlled. In 2008, the sequencing of its genome revealed a great diversity of enzymes targeting plant carbohydrates and lignin. Since then, a large array of lignocellulose-acting enzymes has been characterized and genetic analyses have enabled the understanding of *P. anserina* metabolism and development on plant biomass. Overall, these research efforts shed light on *P. anserina* strategy to unlock recalcitrant lignocellulose deconstruction.

Contents

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http://dx.doi.org/10.1016/j.biotechadv.2016.05.010
0734-9750/

1. Introduction

Enzymatic conversion of lignocellulosic biomass (i.e. plant material) to bio-products is of great interest for the development of sustainable biorefineries. Plant biomass constitutes the most abundant biological repository of carbon on Earth. It is a complex structure comprising polysaccharides (cellulose, hemicellulose, pectin) and lignin, forming a tight interconnected network. This heterogeneous composition makes it recalcitrant to biotic degradation, which in turn hampers the development of bioprocesses for industrial purposes (Himmel et al., 2007). Because of its complex structure, a number of enzymes with diverse complementary activities are required to perform efficient conversion of plant cell wall to platform molecules or high value compounds, and considerable research efforts have aimed at developing such efficient enzyme systems from microbes.

Filamentous fungi thrive in lignocellulose-rich environments because they are potent biomass degraders thanks to their dedicated enzymatic machinery (Sigoillot et al., 2012) and their ability to penetrate forcibly the biomass. Among them, the filamentous ascomycete fungus *Podospora anserina* has been studied for decades as a model species and numerous studies focused on biological questions such as ageing (Osiewacz et al., 2013), prion mechanisms (Baxa et al., 2004) or fungal reproduction (Silar, 2014; Debuchy et al., 2010). Indeed, genetic analysis is efficient with *P. anserina* thanks to its one-week haplophasic life cycle (Silar, 2013a,b). Moreover, this fungus is easily transformed by exogenous DNA and its genes can be inactivated in one step by replacement with resistance markers (Silar, 2013a,b). One of the outcomes of the genome sequence project of this species (Espagne et al., 2008) was the discovery of a large array of enzymes potentially involved in both cellulose and lignin breakdown, making this fungus a model of choice to better understand the enzymatic deconstruction of plant biomass.

In its natural environment, *P. anserina* is a cosmopolitan coprophilous fungus which frequently develops in the droppings of grass herbivores. As a very late ascomycete degrader that fructifies after the succession of most other coprophilous organisms, *P. anserina* is thought to specifically use the more recalcitrant fraction of lignocellulose (Richardson, 2002). In line with this assumption, this fungus can fructify abundantly using lignocellulosic biomass as sole carbon source (Fig. 1). In addition, it was demonstrated that *P. anserina* differentiates dedicated hyphae to penetrate the biomass, facilitating its digestion (Brun et al., 2009). Development of these hyphae is under the control of signalling pathways that also regulate the transcription of lignocellulolytic genes, connecting differentiation and enzyme production (Bidard et al., 2012).

In this article, we review genomic and post-genomic studies, as well as biochemical and genetic analyses focusing on the exploration of *P. anserina* metabolism and development on biomass.

2. *P. anserina* enzymatic machinery for recalcitrant biomass degradation

Coprophilous fungi hold promise as an enzyme source due to their atypical habitat, as they grow on herbivore dung (Richardson, 2002). The plant biomass that has been digested in the animal digestive track of herbivores is still rich in nutrients. In addition, it is from different origins depending upon the animal diets and ranges from monocotyledons and dicotyledons to ferns and mosses. It has therefore highly variable structures and coprophilous fungus must cope with such variability. The first coprophilous fungi commonly found in dung are saprotrophic zygomycetes that assimilate the most easily utilizable biomass components like hemicelluloses and pectins. For instance, the coprophilous zygomycete *Pilobolus* is often the earliest fungus identified in horse dung (Pointelli et al., 1981; Richardson, 2002). Ascomycetes such as *Chaetomium*, *Sordaria*, and *Podospora* then digest more complex components, and recalcitrant lignin is finally fully degraded by basidiomycetes (e.g., *Coprinopsis cinerea* (Stajich et al., 2010)). As a late grower on the recalcitrant fraction of biomass, *P. anserina* potentially displays a specific and efficient cellulose, hemicellulose and lignin conversion machinery.

2.1. Comparative genomics and post-genomics reveal P. anserina strengths to convert lignocellulose

The sequencing of *P. anserina* genome was carried out in 2008 (Espagne et al., 2008). Annotation of genes encoding carbohydrate active enzymes (CAZymes; www.CAZy.org, Lombard et al., 2014) revealed a large diversity of plant cell wall-targeting activities and one of the highest numbers of Carbohydrate Binding Modules (CBMs) among fungal genomes available at the time. Comparison of closely related fungi such as *Chaetomium globosum* and *Neurospora crassa* and with the industrial workhorse *Trichoderma reesei* confirmed that *P. anserina* encodes a complete machinery for plant cell wall conversion including some auxiliary activity (AA) enzymes potentially targeting lignin (Fig. 2). Enzymes targeting cellulose, i.e., cellobiohydrolases (GH6, GH7) and endoglucanases (GH5, GH12, GH45) are abundant in *P. anserina*. Its genome contains more than 100 CBMs, among which 28 CBM1, specific of cellulose recognition. As a comparison, the cellulose degrader *T. reesei* holds only 36 CBMs. It is generally acknowledged that the presence of CBMs improves the activity of CAZymes by targeting the substrate and binding the catalytic domain to it (for a review see Várnai et al., 2014). Together with *C. globosum*, *P. anserina* displays an impressive set of Lytic Polysaccharide Monooxygenases (LPMOs) with 33 members of the family AA9. This multiplicity of genes raises the question of the functional relevance of LPMOs in fungi, i.e. functional redundancy or functional diversification or fine-tuned regulation of alternative genes and/or adaptations to the degradation of the substrates. Hemicellulolytic enzymes are also abundant with GH10 and GH11 xylanases, GH5 and GH26 mannanases, GH51 and GH62 arabinofuranosidases, and carbohydrate esterases of family CE1 mostly. In contrast to Aspergilli species, only a few pectin-targeting enzymes are found in *P. anserina* (e.g. one member of the GH78 and no member of families GH28 and GH88) (Coutinho et al., 2009). Accordingly, *P.*

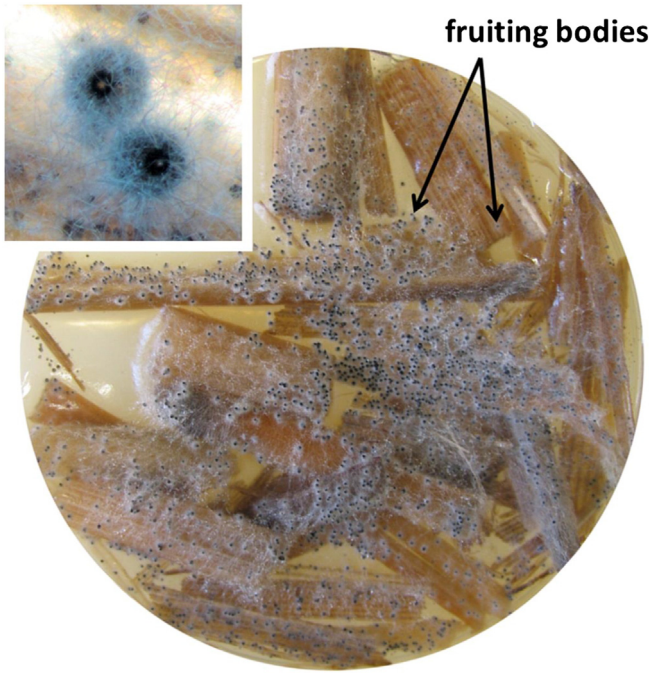

Fig. 1. *P. anserina* fruiting bodies on miscanthus as sole carbon source. Miscanthus supplemented with nitrogen and oligo-elements was inoculated with heterokaryotic self-fertile *P. anserina*. Ten days after inoculation, numerous spore-bearing fruiting bodies are indicative of the ability of the fungus to efficiently scavenge energy from miscanthus.

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	Pod_ans	Cha_glo	Neu_cra	Tri_ree	Asp_cla	Asp_fum	Asp_ter	Asp_nid	Asp_nig	Asp_fla	Tal_sti	Fus_oxy	Fus_ver	Nec_hae	Fus_gra	Phy_bla	Rhi_ory	Ust_may	
CW (b-glyc)	1	1	2	2	4	5	3	3	3	3	3	6	6	5	3	0	0	0	GH1
CW (b-glyc)	7	7	6	8	3	6	10	10	6	8	5	9	11	11	10	0	0	1	GH2
CW (b-glyc)	11	13	11	13	12	19	21	20	17	24	24	33	29	38	21	3	6	3	GH3
CW (b-glyc)	12	10	6	8	9	14	18	15	10	15	11	21	24	18	14	6	7	12	GH5
PCW (cell)	4	4	3	1	2	1	2	2	2	1	1	1	1	1	1	0	0	0	GH6
PCW (cell)	6	8	5	2	4	4	4	3	2	3	2	3	4	3	2	0	0	0	GH7
CW	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	5	1	GH9
PCW (hemi)	8	7	4	1	2	4	4	3	1	4	2	5	4	3	5	0	0	2	GH10
PCW (hemi)	6	10	2	3	3	3	2	2	4	4	4	4	4	3	3	0	0	1	GH11
PCW (cell)	2	4	2	2	3	4	6	1	4	5	3	4	4	6	4	0	0	0	GH12
PCW (hemi)	1	1	1	0	0	0	0	3	1	1	0	0	0	0	0	0	0	1	GH26
PCW (hemi)	2	3	0	8	3	5	5	3	5	3	4	4	3	2	2	1	2	1	GH27
PCW (pect)	0	1	2	4	3	13	8	9	20	21	8	15	9	11	6	10	18	1	GH28
PCW (hemi)	0	0	0	0	0	0	2	0	1	0	2	2	2	0	1	0	0	0	GH29
PG+ESR+PCW (hemi)	5	6	6	4	5	7	11	10	7	10	7	10	8	9	8	3	3	3	GH31
PCW (hemi)	1	2	2	1	3	5	4	4	5	8	4	7	3	8	3	1	1	1	GH35
PCW (hemi)	1	1	1	2	3	3	4	4	3	3	3	3	5	2	3	3	4	1	GH36
PCW (pect + hemi)	12	17	7	2	13	18	21	15	10	21	11	35	24	32	17	0	2	4	GH43
PCW (cell)	2	2	1	1	0	1	0	1	0	1	2	1	2	1	1	1	5	4	GH45
PCW (pect + hemi)	1	2	1	0	3	2	4	2	3	4	2	3	2	4	2	1	0	2	GH51
PCW (pect + hemi)	1	1	1	0	0	1	1	1	2	2	1	2	1	1	1	0	0	0	GH53
PCW (pect + hemi)	0	0	1	2	1	1	1	1	1	1	3	1	1	1	1	0	0	0	GH54
PCW (hemi)	2	4	0	1	2	2	3	2	1	2	2	1	1	2	1	0	0	1	GH62
PCW (hemi)	1	1	1	1	1	1	2	1	1	1	3	2	3	0	1	0	0	0	GH67
PCW (cell)	1	1	1	1	1	2	1	2	1	0	1	1	1	1	1	0	0	0	GH74
PCW (pect)	1	1	2	1	0	5	4	8	8	12	2	18	10	12	7	0	0	0	GH78
PCW (hemi)	3	4	2	4	0	0	3	1	3	5	1	7	2	2	1	0	0	1	GH79
PCW (pect)	0	0	0	0	0	2	0	2	1	3	0	3	2	5	1	0	0	0	GH88
PCW (hemi)	3	4	2	0	1	3	4	2	0	3	3	5	5	4	2	0	0	0	GH93
PCW (hemi)	0	1	0	4	1	2	3	3	2	3	2	2	1	3	2	0	0	0	GH95
PCW (pect)	0	1	2	1	3	3	2	3	2	4	2	4	3	4	3	0	0	1	GH105
PCW (hemi)	3	2	1	1	1	1	2	1	0	3	1	3	2	2	2	0	0	1	GH115
PCW (cell+b-glyc)	4	4	3	0	1	2	1	1	1	3	0	0	1	1	1	0	0	0	GH131
PCW (hemi)	0	0	0	0	2	1	1	4	0	3	1	1	1	1	0	0	9	0	GH134
PCW (pect)	4	7	1	0	2	6	7	8	6	12	0	10	11	14	9	0	0	1	PL1
PCW (pect)	2	4	1	0	1	3	3	5	0	3	0	7	7	11	7	0	0	0	PL3
PCW (pect)	1	2	1	0	2	3	3	4	2	3	0	3	3	5	2	0	0	0	PL4
PCW (pect)	0	0	0	2	0	0	0	0	0	1	1	0	0	1	0	0	0	0	PL7
PCW (pect)	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	PL8
PCW (pect)	0	1	0	0	0	1	1	1	0	1	0	2	2	1	1	0	0	0	PL9
PCW (pect)	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	PL11
PCW (pect)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	5	0	PL14
PCW (pect)	0	1	1	2	2	1	0	2	0	2	1	0	0	1	1	0	0	0	PL20
PCW (hemi)	13	11	6	2	5	3	4	2	2	4	5	7	5	1	4	0	0	1	CE1
PCW (hemi)	7	7	3	3	0	1	3	5	0	3	0	6	9	11	5	0	0	0	CE3
PCW (hemi)	5	5	5	3	4	4	3	7	5	3	3	10	10	6	7	17	35	7	CE4
PCW (hemi)	7	5	3	4	3	5	8	4	5	6	3	12	12	8	12	0	0	4	CE5
PCW (hemi)	1	2	2	0	7	7	1	3	3	5	2	8	7	8	7	4	6	1	CE8
PCW (hemi)	1	2	1	0	1	3	2	2	2	4	1	4	4	2	3	0	0	0	CE12
PCW (hemi+lign)	3	3	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	CE15
PCW (hemi)	2	2	2	2	3	4	5	3	2	3	4	5	6	5	5	3	3	0	CE16
PCW (lign)	13	8	10	8	5	6	9	2	16	11	13	22	15	13	14	5	5	6	AA1
PCW (lign)	2	1	3	1	2	2	2	0	2	2	2	3	3	2	3	2	3	2	AA2
PCW (cell)	30	24	7	12	17	12	24	16	32	36	18	35	29	33	20	0	1	9	AA3
PCW (lign)	4	0	0	0	0	1	0	0	1	0	2	5	2	7	1	0	0	0	AA4
PCW (hemi)	2	1	2	1	1	1	1	0	0	2	3	5	5	3	5	1	2	4	AA5
PCW	1	1	1	1	1	1	1	0	2	1	2	1	2	1	1	4	5	1	AA6
CW	3	3	2	1	2	1	3	0	4	3	2	7	7	6	2	0	0	2	AA7
PCW	8	7	7	2	4	4	6	2	4	7	2	14	8	5	7	0	0	0	AA8
PCW (cell)	33	44	14	3	7	7	12	9	7	7	1	16	13	12	12	0	0	0	AA9

Fig. 2. Comparative analysis of *P. anserina* CAZymes with other fungi. CAZymes were extracted from the CAZy public database (www.cazy.org). *Asp_cla*, *Aspergillus clavatus*; *Asp_fla*, *Aspergillus flavus*; *Asp_fum*, *Aspergillus fumigatus*; *Asp_nid*, *Aspergillus nidulans* (*Emericella nidulans*); *Asp_nig*, *Aspergillus niger*; *Asp_ter*, *Aspergillus terreus*; *Cha_glo*, *Chaetomium globosum*; *Fus_oxy*, *Fusarium oxysporum f lycopersici*; *Nec_hae*, *Fusarium solani* (*Nectria haematococca*); *Fus_ver*, *Fusarium verticillioides* (*Gibberella moniliformis*); *Neu_cra*, *Neurospora crassa*; *Tal_sti*, *Penicillium emmonsii* (*Talaromyces stipitatus*); *Phy_bla*, *Phycomyces blakesleeanus*; *Pod_ans*, *Podospora anserina*; *Rhi_ory*, *Rhizopus oryzae* (*R. arrhizus*); *T_rees*, *Trichoderma reesei* (*Hypocrea jecorina*); *Ust_may*, *Ustilago maydis*. Enzyme families are represented by their class (GH, PL, CE, AA) and family number according to the carbohydrate-active enzyme database. Known substrates of CAZy families (most common forms in brackets) are indicated to the right: CW, cell wall; ESR, energy storage and recovery; PCW, plant cell wall; PG, protein glycosylation; U, undetermined; b-gluc, β-1,3-glucan; cell, cellulose; hemi, hemicelluloses; lign, lignin; pect, pectin.

anserina can grow on cellulose and hemicellulose such as xylan while growth on pectin is limited (Espagne et al., 2008; Coutinho et al., 2009).

2.2. *P. anserina* toolbox includes a variety of complementary enzymatic activities

2.2.1. Cellulases

Based on genomic and post-genomic data, several cellulases from *P. anserina* were selected for biochemical characterization in the last few years (Table 1). In contrast with *T. reesei* which contains only one gene encoding a GH6 enzyme, *P. anserina* displays four genes of this family, and three of them were characterized to tackle the question of their redundancy (Poidevin et al., 2013). The enzymes were produced in *Pichia pastoris*, a host of choice for the efficient production of CAZymes from *P. anserina* (Haon et al., 2015). The study revealed complementary activities of the three enzymes, with one endoglucanase (*Pa*Cel6B), one cellobiohydrolase (*Pa*Cel6A) and one processive endoglucanase (*Pa*Cel6C). Another enzyme active on cellulose was identified in *P. anserina* genome that led to the creation of a new GH family, i.e. GH131. Its characterization revealed a surprisingly broad specificity towards β-(1,3)-, β-(1,4)-, and β-(1,6)-glucans (Lafond et al., 2012). Its 3D structure was found to be composed of a β-jelly roll fold (Fig. 3; Jiang et al., 2013) but the catalytic mechanism remains to be elucidated.

P. anserina oxidative enzymes are also involved in cellulose degradation. Notably, two cellobiose dehydrogenases have been biochemically studied (Turbe-Doan et al., 2013; Bennati-Granier et al., 2015). These enzymes are known to be a partner of the newly discovered LPMOs. Several of the LPMOs identified in *P. anserina* secretomes under lignocellulolytic conditions were biochemically studied (Table 1; Bey et al., 2013; Bennati-Granier et al., 2015) and showed activity on cellulose. Among them, LPMOs harbouring a CBM1 module displayed increased efficiency on cellulose as compared to enzymes without CBM. Interestingly, *Pa*LPMO9H displayed a broad specificity as it is able to cleave non-cellulosic substrates containing β-(1–4) linked glucose residues. Because of their different regioselectivity, modularity and specific activity, *P. anserina* AA9 LPMOs are thought to play different roles in plant cell wall deconstruction by targeting not only cellulose but other biomass components, e.g. hemicellulose. Accordingly, association of *P. anserina* CDH with several *P. anserina* LPMOs resulted in redox mediated glycosidic bond cleavage in lignocellulose polysaccharides (Bennati-Granier et al., 2015), assuming a key role of this oxidative system for recalcitrant biomass degradation.

2.2.2. Hemicellulases

As a late degrader of recalcitrant biomass, *P. anserina* harbors a variety of hemicellulose-encoding genes. Reflecting this diversity, a set of two arabinofuranosidases from family GH51 and GH62, one GH11 xylanase and two *endo*-mannanases from families GH5 and GH26 were selected for production in *P. pastoris* and biochemical characterization (Couturier et al., 2011). The five enzymes were able to efficiently act on their predicted substrates. The two mannanases exhibited different end products on mannan substrates, and the two arabinofuranosidases had different activities on arabinose-substituted substrates, highlighting the complementary actions of several enzymes targeting the same substrate. To complete the set of enzymes required for complete degradation of hemicelluloses, two CE members were studied. The methyl glucuronoyl esterase from family CE15 was able to cleave the ester bond between lignin alcohols and xylan-bound 4-*O*-methyl-D-glucuronic acid (Katsimpouras et al., 2014). The acetyl esterase from family CE16 was versatile in terms of activity on xylan. Although it efficiently deacetylated singly- and doubly-acetylated internal xylose residues, the enzyme also displayed *exo*-deacetylase activity (Puchart et al., 2015).

Structural studies were carried out on a few of these enzymes to get further insights into the hemicellulolytic machinery of *P. anserina*. The

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Table 1
List of lignocellulose-acting enzymes from *Podospora anserina* biochemically characterized.

Protein name	Protein ID	CAZy family	Additional module	Activity	Reference
*Pa*Ara51A	CAP62201	GH51	–	Arabinofuranosidase	Couturier et al. (2011)
*Pa*Ara62A	CAP62336	GH62	–		
*Pa*Xyl11A	CAP60973	GH11	CBM1	*Endo*-xylanase	Siguier et al. (2014)
*Pa*Man5A	CAP71606	GH5	–	*Endo*-mannanase	Couturier et al. (2013a,b)
*Pa*Man26A	CAP61906	GH26	CBM35		
*Pa*Cel6A	CAP60942	GH6	–	Cellobiohydrolase	Poidevin et al. (2014)
*Pa*Cel6B	CAP60981	GH6	CBM1	Endoglucanase	
*Pa*Cel6C	CAP61669	GH6	CBM1		
*Pa*Cel131A	CAP61309	GH131	CBM1	Glucanase	Lafond et al. (2012)
*Pa*GE1	CAP60908	CE15	–	Glucuronoyl esterase	Katsimpouras et al. (2014)
*Pa*CE16A	CAP30204	CE16	CBM1	Acetyl xylan esterase	Puchart et al. (2015)
*Pa*LPMO9A	CAP73254	AA9	CBM1	Lytic polysaccharide mono-oxygenase	Bey et al. (2013), Bennati-Granier et al. (2015)
*Pa*LPMO9B	CAP68375	AA9	CBM1		
*Pa*LPMO9C	CAP68173	AA9	–		
*Pa*LPMO9D	CAP66744	AA9	–		
*Pa*LPMO9E	CAP67740	AA9	CBM1		
*Pa*LPMO9F	CAP71839	AA9	–		
*Pa*LPMO9G	CAP73072	AA9	–		
*Pa*LPMO9H	CAP61476	AA9	CBM1		
*Pa*CDHA	CAP68427	AA3_1	AA8	Cellobiose dehydrogenase	Turbe-Doan et al. (2013)
*Pa*CDHB	CAP61651	AA3_1	AA8		Bennati-Granier et al. (2015)
Bilirubin oxidase	CAP65628	–	–	Laccase	Durand et al. (2013)

two mannanases, namely *Pa*Man5A and *Pa*Man26A, were further studied and their three dimensional structure was solved, revealing two different catalytic systems (Fig. 3) (Couturier et al., 2013a; Marchetti et al., 2015). The GH62 arabinofuranosidase, *Pa*Man62A, was also selected for further studies, because this family of enzymes is very common among fungi but little is known about their specificity. The three dimensional structure was the first one to be solved in the GH62 family and revealed a five-bladed beta-propeller (Fig. 3) (Siguier et al., 2014). The catalytic apparatus was located in a pocket forming the −1 subsite able to accommodate the arabinose residue to be cleaved, with very probably an inverting mechanism.

2.2.3. P. anserina enzymes in saccharification assays

P. anserina (hemi)cellulolytic enzymes are more diverse than *T. reesei* and therefore enzymes mixtures obtained in different inducing conditions were used to supplement *T. reesei* cellulase cocktails. For instance, the enzymatic diversity of *P. anserina* secretomes induced by a range of carbon sources (i.e. dextrin, glucose, xylose, arabinose, lactose, cellobiose, saccharose, Avicel, Solka-floc, birchwood xylan, wheat straw, maize bran, and sugar beet pulp) was investigated (Poidevin et al., 2014). Compared with the *T. reesei* enzymatic cocktail, *P. anserina* secretomes displayed similar cellulase, xylanase, and pectinase activities and greater arabinofuranosidase, arabinanase, and galactanase activities. When tested for their capacity to supplement a *T. reesei*

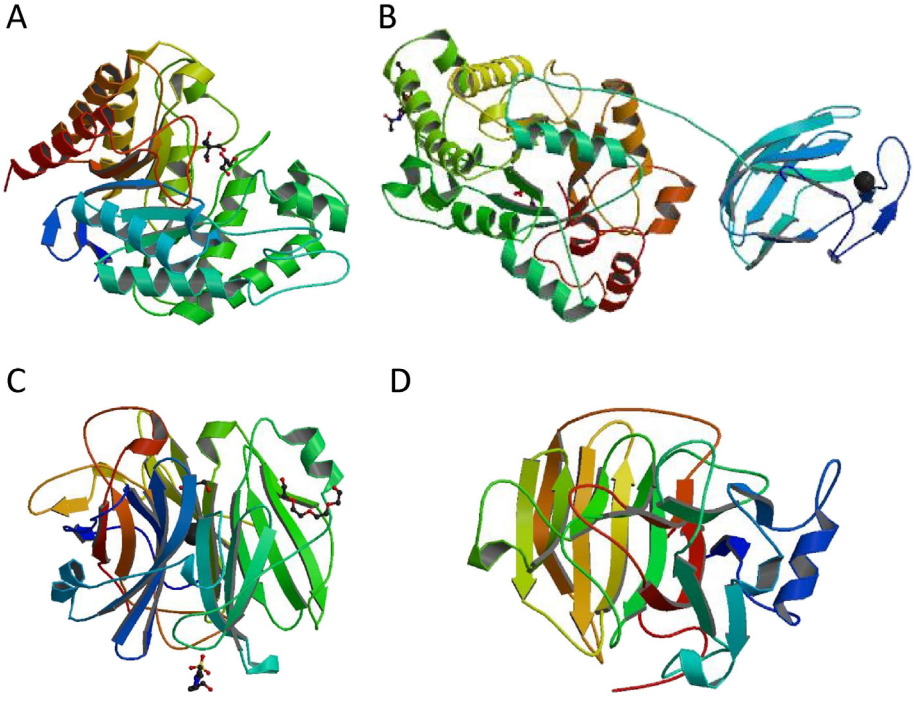

Fig. 3. Three dimensional structures of *P. anserina* hemicellulases. A. GH5 *endo*-mannanase (PDB 3ZIZ); B. GH26-CBM35 *endo*-mannanase (PDB 3Z8M); C. GH62 arabinofuranosidase (PDB 4N4B); D. GH131 beta-glucanase (PDB 4LE3).

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cocktail, several secretomes improved significantly the saccharification yield of steam-exploded wheat straw up to 48%. In this instance, fine analysis of the *P. anserina* secretomes using proteomics revealed a large array of CAZymes with a high number of GH6 and GH7 cellulases, CE1 esterases, GH43 arabinofuranosidases, AA1 laccase-like multicopper oxidases and AA9 LPMOs.

The hemicellulases described in Section 2.2.2 were also tested to supplement the saccharification of lignocellulosic biomass by *T. reesei* cocktail (Couturier et al., 2011). In particular, the two tested mannanases, *Pa*MAn5A and *Pa*Man26A, were able to improve the saccharification of woody biomass, i.e., spruce. Interestingly, when the mannanases were added to the *T. reesei* enzyme mixture, not only mannose but also glucose release was improved, confirming the great potential of *P. anserina*. It was hypothesized that the mannanases were acting synergistically with *T. reesei* cellulases by degrading the mannan chains that were surrounding cellulose fibrils (Couturier et al., 2011). Hence, a directed evolution strategy was carried out to improve the catalytic efficiencies of *Pa*Man5A and *Pa*Man26A. It allowed the identification of hot-spots within the active site cleft of both mannanases (Couturier et al., 2013b). In particular, a mutant of *Pa*Man26A with an increased activity exhibited two mutations, P140L and D416G. The former was located in the linker between the catalytic domain and the CBM, the latter at the entrance of the catalytic cleft. It was hypothesized that the substitution of a proline by a leucine residue increased the flexibility, and thus resulted in an improved enzymatic activity, while the loss of the D416 side chain could facilitate the entrance of the substrate into the active site (Couturier et al., 2013b).

3. Investigation of *P. anserina* metabolism on biomass

3.1. Genetic engineering and tools developed

One of the interesting properties of *P. anserina* is the efficiency with which genes can be inactivated (Silar, 2013a,b). Moreover, multiple mutants can be easily constructed thanks to the speed of the life cycle, which lasts one week, and the availability of several resistance markers. Different members of gene families can be inactivated with different markers and multiple mutants can be constructed by genetic crossing and obtained within a few weeks. For example, it is possible to construct strains inactivated for up to six genes (Coppin et al., 2012). Genes potentially involved in biomass degradation detected during the genome sequencing were thus tested by targeted gene deletion for their actual involvement in plant biomass degradation. Following mutant constructions, strains can be tested for growth and fruiting body production on various medium. The standard minimal medium for *P. anserina* contains dextrins, as this fungus cannot grow on saccharose, a feature explained by the lack of an invertase gene in its genome, and *P. anserina* is nearly sterile on glucose medium, although it is able to produce an important biomass with this food source. As mentioned in the introduction, these two characteristics are likely associated with the fact that in nature the fungus consumes woody materials, such as remnants of grass, twigs, leaves, etc. and not simple sugars. Dextrin can be replaced with cellulose, either as small crystals or Whatman paper, wood shaving, shredded miscanthus, dried hay, purified lignin, etc. On these, mutant mycelium may or may not grow and may or may not remain sterile. Lack of growth and diminished fertility is accounted for by a lower efficiency in scavenging nutrient and/or a sensitivity to compounds potentially present in the carbon source, such as tannins (Bourdais et al., 2012).

In addition to facilitating reverse genetic engineering, the availability of the genome also permitted to design microarrays (Bidard et al., 2010). These were used to assess the genes regulated during growth on dextrin and those under the regulation of the PaNox1, PaMpk1 and PaMpk2 regulatory pathways. Interestingly genes involved in plant lignocellulose degradation were among those the most regulated during growth and in the regulatory pathways (Bidard et al., 2012). For example, among the 33 genes encoding AA9 LPMOs, one was up-regulated and 15 were down-regulated as growth proceeded. Twenty four out of the 33 genes were under the control or either PaNox1, PaMpk1 or PaMpk2.

3.2. Phenotype: deletion of genes encoding oxidative enzymes associated with biomass conversion

The first genes investigated for their role in plant biomass breakdown in *P. anserina* were those encoding catalases (Bourdais et al., 2012). Catalases are involved in the removal of hydrogen peroxide, a toxic ROS that is suspected to be involved in both the enzymatic degradation through peroxidases and the non-enzymatic degradation through the Fenton reaction. *P. anserina* possesses five such genes. Inactivation of the five genes and analyses of multiple mutants showed that apart from a role in protection against hydrogen peroxide, the catalases had no role when growth is in dextrin medium. The CAT2 gene encoding catalase/peroxidase and in a lesser extent the CATB gene encoding a monofunctional catalase were the most important enzymes for H_2O_2 detoxification. The five genes were also not important for growth and fertility on paper or on crystalline cellulose. Results were different when wood shavings were used as carbon source. The inactivation of CATB alone resulted of a net increase in fertility. A similar phenotype was observed when the three other genes encoding monofunctional catalase, CATA, CATP1 and CATP2 were deleted separately. On the contrary, when CAT2 or multiple mutants were tested, their growth and especially their fertility were much reduced on wood shavings. Once again, CAT2 was the most important enzyme, as its inactivation alone resulted in a drastic decrease in fertility. Moreover, a strain containing only CAT2 was still able to mature a fair number of fruiting bodies. Final proof of the involvement of lignin and the catalase activity was given when the wild type and mutant lacking all five genes were grown on medium were dextrin was replaced by purified lignin. The mutant failed to grow efficiently on this medium. However, addition of external bovine catalase restored a growth similar to the wild-type one. Overall, these data validated a model in which hydrogen peroxide is required for lignin degradation as proposed by many biochemical data (for a review see Ruiz-Dueñas and Martínez, 2009; Dashtban et al., 2010). However, being toxic, its levels must be controlled. *P. anserina* do so in part through catalases, especially CAT2. Intriguingly, *P. anserina* seems to control it too tightly, as removal of minor catalases results in enhanced fertility on wood, hence in better scavenging of nutrients. Since these results were obtained, additional sources of lignocellulose have been tested. While catalases appeared required for growth many woody materials, including dried hay, they seem dispensable for growth and fertility on miscanthus (Tangthirasunun, 2014). This suggests that not all plant materials require high level of H_2O_2 to be degraded. It also suggests that *P. anserina* may tailor its enzymatic machinery to the substrate it wishes to degrade.

The second family of genes tested for their role in lignocellulose breakdown were those encoding multicopper oxidases (MCO), among which the laccases are best known (Xie et al., 2014, 2015) These enzymes were repetitively hypothesized to be involved in lignin breakdown, however, this assumption had never been tested genetically. The genome of *P. anserina* contained nine “true” laccases genes as defined by Kues and Ruhl (2011), one encoding a related MCO, two encoding bilirubin oxidases (BOD), two encoding enzymes related to the iron transport ferric reductases of *Saccharomyces cerevisiae* and one coding a protein related to plant ascorbate oxidases (Xie et al., 2014). Deletion analyses showed once again that all the genes were dispensable for growth and fertility on dextrin containing medium. However, the nine laccases, the related MCO and the two BOD were involved in growth on woody materials. In fact enzyme activity analysis showed that all twelve enzymes had a laccase activity (Xie et al., 2014, 2015; Durand et al., 2013) and that the MCO and BOD enzymes were thermoresistant. The role of the twelve enzymes during biomass

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breakdown was complex. Firstly, when only a single gene was inactivated very little effect was observed while multiple mutants were more severely altered. For example, mutants lacking four genes from laccase subfamily 2 or three genes from subfamily 1 were nearly sterile on wood shavings. Secondly, the mutants presented a large panel of phenotypes: growth on pure lignin was indeed decreased, but also on paper, in the presence of hydrogen peroxide or of various quinones, some of which results from lignin hydrolysis. Poor growth on paper was also correlated with a diminish ability to retrieve glucose from the cellulose fibers, as measured by weight loss of paper pads. Each mutant or multiple mutants presented its own panel of phenotype suggesting a complex array of redundancy and synergism between the twelve enzymes. Therefore, laccases and related enzymes appeared to be very important for lignocellulose breakdown, not only for lignin lysis by also for degrading cellulose, resisting to various by-products of lignin lysis and to the hydrogen peroxide that is used during the breakdown of lignin.

3.3. Biomass colonization mechanisms

Although enzymes are crucial for lignocellulose degradation, they are not the only adaptation evolved by fungi to ensure efficient breakdown. Indeed, many fungi differentiate dedicated structures to penetrate plants, the appressoria or appressorium-like structures. Once inside, they can digest from within, likely resulting in a more efficient breakdown. While a large body of data on living plant penetration exist for phytopathogenic fungi (review in Ryder and Talbot, 2015; Tucker and Talbot, 2001), data are more scarce for saprotrophic ones. Indeed, it was reported in 2009 that *P. anserina* is able to differentiate appressorium-like structures on cellophane (Brun et al., 2009). This was quite surprising at the time as it was thought that the ability to penetrate plants was one of the requirements to be pathogenic. In fact a large array of species, but not all fungi, differentiates such structures on cellophane (Fig. 4), suggesting that the phenomenon is very common. These include both *Basidiomycota* and *Ascomycota*, but apparently not the lower *Mucoromycotina*. *P. anserina* revealed a good model to study the differentiation of these structures.

In *P. anserina*, appressorium-like structures differentiate only in the absence of glucose, as expected for a structure that would enable to scavenge nutrients (Brun et al., 2009). In this fungus, it takes about one day for them to appear, first as reoriented hyphae that grows towards cellophane. These hyphae establish a contact with the layer, and from them small needle-like hyphae are emitted. After 10 μm of growth, the needle-like hyphae expand into palm-like structures, which will generate additional needle-like hyphae that will penetrate further the cellophane. It takes about another day for extensive colonization of the cellophane. Typically, after three days the fungus has crossed a 60 μm-thick cellophane layer. This leads after two days to a

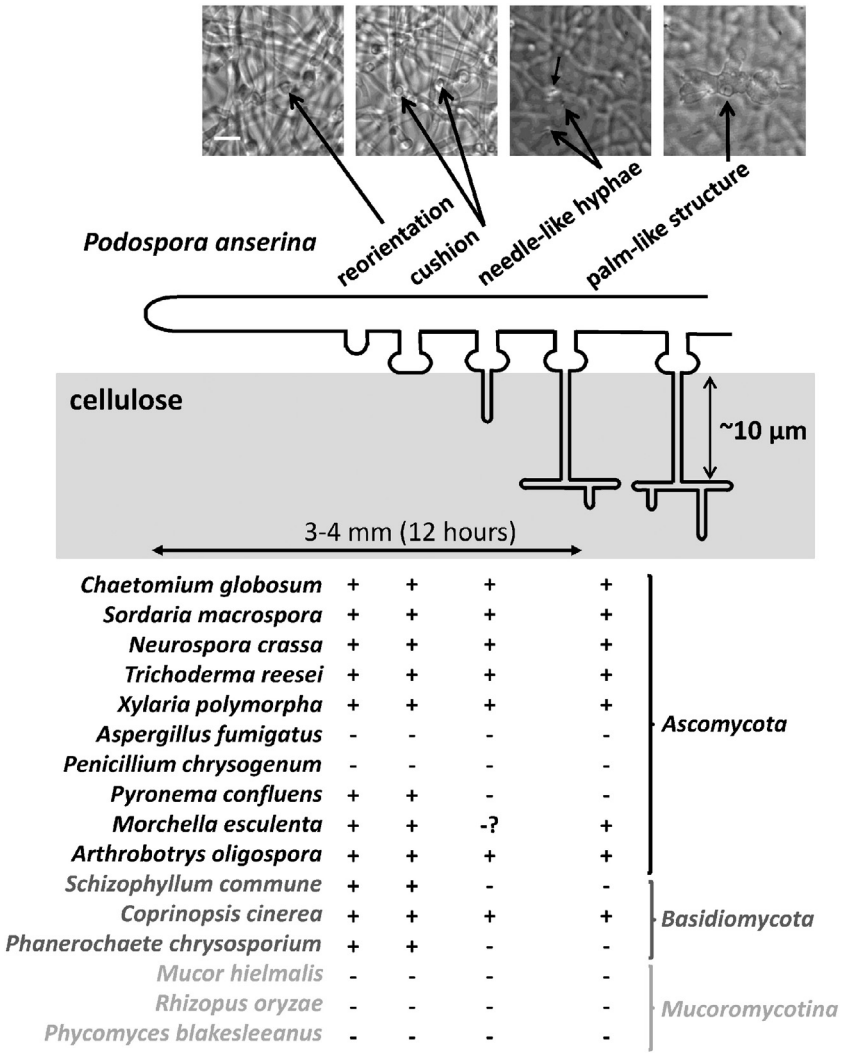

Fig. 4. Appressorium-like structures development in *P. anserina* and other fungi. Their formation in *P. anserina* requires reorientation of hyphae towards cellophane, formation of needle-like hyphae from cushions and finally formation of palm-like structures that enable invasion of the substrate. The same stages are observed in many *Dikarya* fungi.

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disruption of the cellophane to a point where it cannot be collected without breaking. The appressorium-like structures differentiated by the other fungi have identical features. They stem for hyphae that reorient their growth towards cellophane. They establish a tight contact from which needle-like hyphae emerges.

Genetic analysis has shown that the production of the *P. anserina* appressorium-like structures is under the control of signalling pathways, which include the PaMpk2/PaMKK2/PaTLK2 MAP kinase module, as well as the PaNox1 and PaNox2 NADPH oxidase enzymes and their regulatory subunits, PaNoxD/PaNoxR and PaPls1/PaNoxR, respectively (Brun et al., 2009; Lalucque et al., 2012; Lacaze et al., 2015). However, it does not depend on the PaMpk1 or PaMpk3 pathways or the PaNox3 NADPH oxidase (Brun et al., 2009; Lalucque et al., 2012). The PaNox2 complex and the PaMpk2 MAP kinase are important for the initial reorientation phase, while the PaNox1 complex is involved in the generation of the needle-like hyphae. The Nox1, Nox2 and Mpk2 orthologues are also involved in the formation of appressoria or appressorium-like structures in phytopathogenic fungi, such as *Magnaporthe oryzae* (Egan et al., 2007) and *Botrytis cinerea* (Segmuller et al., 2008; Siegmund et al., 2015). In these species, the *Nox1*, *Nox2* and *Mpk2* mutants are strikingly blocked at the same stage, suggesting that the machinery used to differentiate the penetration structures is highly conserved, at least in the *Ascomycota*. In *M. grisea*, it has been shown that the Nox1 and Nox2 complexes regulate the remodelling of the actin cytoskeleton and polarized cell growth (Ryder et al., 2013). It is likely that this function is conserved in most fungi, including *P. anserina*. Note that these signalling pathways have pleiotropic roles in addition to controlling appressorium/appressorium-like structure formation, including hyphal anastomoses, fruiting body formation, ascospore germination, etc. Inactivation of these genes leads thus to a large array of defects, some of which are not fully understood.

The role of appressorium-like structures in helping lignocellulose breakdown is very important in *P. anserina*. Indeed, growth and fertility of the *PaNox2* and *PaPls1* mutants are indistinguishable from the wild type ones on medium with glucose or dextrins as sole carbon sources, showing that these mutants do not have any problem to collect glucose even when it is present in the form of dextrins. Yet, they are sterile when grown with cellulose as sole carbon sources (Malagnac et al., 2008). Thus, similarly to industrial processes in which the biomass needs to be pre-treated before the action of the enzymatic cocktails, efficient degradation by *P. anserina* require the action of appressorium-like structures so that enzymes can access the biomass from within. Interestingly, some fungi, known to be very good lignocellulose degraders such as the white rot *Phanerochaete chrysosporium*, do not seem to need appressorium-like structures for degradation (Fig. 4). Possibly, these fungi produce a cocktail of enzymes efficient enough so that attack exclusively from the outside is sufficient for full breakdown. A balance between efficiency of enzymatic degradation versus development of internal degradative structures such as appressorium-like structures may thus govern how the biomaterials are attacked. A good example of the duality has been highlighted by the study of the *PaNox1* mutant of *P. anserina*. This mutant does not produce the needle-like hyphae and thus does not penetrate cellophane (Brun et al., 2009). It has however an up-regulation of many of its CAZymes (Bidard et al., 2012). Accordingly, it degrades paper pads twice as more efficiently as wild-type and is able to cross a 60 μm-thick cellophane layer also in three days despite the lack of appressorium-like structures (Brun et al., 2009). Noteworthy, this mutant exhibits higher ROS production, suggesting a global increase in the redox reactions responsible for lignocellulose breakdown. Interestingly, this mutant is sterile on dextrin medium, yet it is partially fertile on paper medium (Malagnac et al., 2004). This suggests that its better degradation ability may provide energy to this mutant in higher amounts or in a more appropriate manner to complete fruiting body formation when grown on cellulose.

4. Conclusions and future trends

P. anserina is an ideal candidate to better understand the breakdown of plant biomass by fungi. In the last few years, studies have shed light on *P. anserina* enzymatic machinery to process recalcitrant lignocellulose and confirmed its potential as a source of new efficient biocatalysts. Fundamental knowledge has been acquired on the mechanisms and substrate specificities of several glycoside hydrolases, carbohydrate esterases and lytic polysaccharide monooxygenase families. Additional genetic experiments have enabled to better understand how *P. anserina* grows and develops on complex biomass. In the future, the combination of genetic and microbial engineering tools available for *P. anserina* should enable the development of synthetic biology approaches to extend its potential for white-biotechnological applications.

Acknowledgements

The authors would like to thank D. Navarro (INRA BBF) and B. Henrissat (CNRS AFMB) for their kind help to prepare the comparative genomics figure.

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Please cite this article as: Couturier, M., et al., Plant biomass degrading ability of the coprophilic ascomycete fungus *Podospora anserina*, Biotechnol Adv (2016), http://dx.doi.org/10.1016/j.biotechadv.2016.05.010