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Studies on the biological role of the antifungal protein PeAfpA from *Penicillium expansum* by functional gene characterization and transcriptomic profiling

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ABSTRACT

Antifungal proteins (AFPs) from filamentous fungi have enormous potential as novel biomolecules for the control of fungal diseases. However, little is known about the biological roles of AFPs beyond their antifungal action. *Penicillium expansum* encodes three phylogenetically different AFPs (PeAfpA, PeAfpB and PeAfpC) with diverse profiles of antifungal activity. PeAfpA stands out as a highly active AFP that is naturally produced at high yields. Here, we provide new data about the function of PeAfpA in *P. expansum* through phenotypical characterization and transcriptomic studies of null mutants of the corresponding *afpA* gene. Mutation of *afpA* did not affect axenic growth, conidiation, virulence, stress responses or sensitivity towards *P. expansum* AFPs. However, RNA sequencing evidenced a massive transcriptomic change linked to the onset of PeAfpA production. We identified two large gene expression clusters putatively involved in PeAfpA function, which correspond to genes induced or repressed with the production of PeAfpA. Functional enrichment analysis unveiled significant changes in genes related to fungal cell wall remodeling, mobilization of carbohydrates and plasma membrane transporters. This study also shows a putative co-regulation between the three *afp* genes. Overall, our transcriptomic analyses provide valuable insights for further understanding the biological functions of AFPs.

1. Introduction

Filamentous fungi provide great opportunities to humanity through their remarkable diversity, exceptional metabolic capacity and rapid ability to evolve. In contrast, pathogenic fungi represent an increasing risk for human, animal and plant health worldwide [1,2]. Current strategies to control fungal infections mainly rely on chemical fungicides, the application of which is not exempt from criticism due to the growing emergence of resistances. Therefore, there is an urgent need to develop novel antifungal compounds with new mechanisms of action. In this regard, research is oriented towards less toxic, more efficient and environmentally friendly alternatives [1,3].

Antifungal proteins (AFPs) secreted by different fungi from the Ascomycete phylum are small, cationic and highly stable proteins that stand out as a promising option to control deleterious fungi in human health, food, feed and agriculture [4–8]. The *in vitro* antifungal effect of AFPs at micromolar concentrations, their broad antifungal spectra and *in vivo* protection effects together with their lack of toxicity against plant or mammalian cells have been demonstrated [9–13]. Moreover, reliable expression systems in fungal and plant biofactories have been achieved [14–16].

Filamentous fungi genomes harbor between one and three distinct *afp* genes from different phylogenetic classes [17,18], although not all of them result in the production of AFPs. *Penicillium expansum* is the main

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phytopathogenic fungus of pome fruits, causing significant postharvest losses as well as being a producer of health-hazardous mycotoxins [19]. Its genome encodes three phylogenetically differentiated AFPs, PeAfpA, PeAfpB and PeAfpC [11]. Two of them, PeAfpA and PeAfpC, are naturally produced by *P. expansum*, while growth conditions for PeAfpB production have not been identified so far [10]. *P. expansum* produces PeAfpA at yields above 100 mg/mL, and the protein is also successfully produced in safe fungal biofactories [14]. PeAfpA shows the highest antifungal activity among the three PeAFPs against phytopathogenic, dermatophyte and spoilage fungi, including its own producer fungus [11,20]. Regarding its mode of action, we demonstrated that protein O-mannosylation is important for PeAfpA interaction with the cell wall (CW) [21]. Moreover, we have described that the CW integrity (CWI) pathway is a key player in the PeAfpA killing mechanism against *Saccharomyces cerevisiae* [22].

The biological role of AFPs beyond their antifungal action against competitors is still an intriguing question. In this context, available studies based on the characterization of *afp* gene mutants provide different results [17,23,24]. *Penicillium chrysogenum* Δ*paf* mutants showed defects in asexual development and conidia production [23], while *Aspergillus niger* Δ*anafp* and *Penicillium digitatum* Δ*afpB* did not show any phenotypic effects [17,24]. In addition, transcriptomic studies showed that the expression profile of *anafp* coincides with the starvation response and parallels with genes involved in nutrient mobilization and autophagy [24]. Recently, a transcriptomic profile of the *P. digitatum* Δ*afpB* mutant suggested that the *afpB* gene contributes to the overall homeostasis of the cell. Moreover, transcriptomic data showed that PdAfpB repress toxin-encoding genes [25]. All these studies point to different biological roles for distinct AFPs.

To gain further knowledge on the function of PeAfpA in *P. expansum*, we have generated Δ*afpA* mutants. Disruption mutants were phenotypically characterized, and we determined their sensitivity to different treatments including chemical compounds and the three PeAFPs. In addition, we have performed a transcriptomic analysis compared with the parental strain at time points just before and after PeAfpA production is detected.

2. Material and methods

2.1. Strains, media and culture conditions

P. expansum strain CECT 20906 (CMP-1) [26] was used as parental strain. Fungal strains were routinely cultured on Potato Dextrose Agar (PDA; Difco-BD Diagnostics, Sparks, MD, USA) plates at 25 °C. Conidia were collected, filtered and quantified with a hemocytometer. Plasmids were propagated in *Escherichia coli* JM109 grown in Luria Bertani (LB) medium (triptone 1 %, yeast extract 0.5 %, NaCl 0.5 %) supplemented with 25 μg/mL chloramphenicol or 50 μg/mL kanamycin at 37 °C depending on the vector. The *Agrobacterium tumefaciens* AGL-1 strain used for fungal transformation was grown in LB medium plates supplemented with 20 μg/mL rifampicin at 28 °C.

Growth of strains was evaluated by depositing 5 μL of conidial suspension (5×10^4 conidia/mL) on PDA and *P. chrysogenum* Minimal Medium (PcMM) [16] plates. Growth diameter was measured daily. For conidia production, spores were collected, filtered and counted after 4 and 7 days of growth in PDA plates and conidia concentration was normalized to the surface of the fungal colony [27].

For PeAFP production, 100 mL erlenmeyer flasks containing 25 mL of PcMM liquid medium were inoculated with a final concentration of 10^6 conidia/mL and incubated for 5–11 days at 25 °C with shaking.

2.2. Generation of FungalBraid vectors and fungal transformation

The FB modular cloning strategy for gene replacement through homologous recombination and double selection (positive selection in hygromycin and negative selection in 5-fluoro-2′-deoxyuridine, F2dU) was performed to disrupt *afpA* (ID: PEX1_077760) [28]. Flanking 5′ and 3' DNA sequences for gene disruption (around 1 kb size) were domesticated according to Golden Braid (GB) rules and tools (https:// gbcloning.upv.es) and ordered from an external company as synthetic genes (gBlocks™, IDT, Integrated DNA Technologies). Flanking sequences were cloned into the GB/FB entry vector pUPD2 through restriction-ligation reactions as previously described [28,29], obtaining FB110 and FB111 pieces, respectively (Table 1). The FB110 and FB111 elements were assembled with the FB013 (F2dU) and FB012 (*hph*) selection markers into the pDGB3α2 binary vector to obtain the disruption cassette FB113. FB113 was introduced into AGL-1 strain by electroporation and directly used to disrupt *afpA* following the ATMT protocol [29,30]. Disruption transformants were confirmed by PCR amplification of genomic DNA using specific primers at different positions around the target locus (Table S1 and Fig. S1). An ectopic strain (PEME0066) in which the transformed DNA was randomly inserted in a distinct region was used as an additional control.

2.3. Western-blot analysis

PeAFPs were produced and purified as previously described [11]. Strains were grown in PcMM liquid medium [16] and total proteins from the supernatants along with purified AFPs were separated by SDS-16 % polyacrylamide gels and transferred to Amersham Protran 0.20 μm NC nitrocellulose transfer membrane (GE Healthcare Life Sciences, Chicago, IL, USA). Protein detection was accomplished as described previously using anti-PeAfpA, anti-PeAfpB and anti-PeAfpC antibodies [11]. As secondary antibody, a 1:20,000 dilution of ECL NA934 horseradish peroxidase donkey anti-rabbit (GE Healthcare Life Sciences) was used and chemiluminescent detection was performed with ECL™ Select Western blotting detection reagent (GE Healthcare Life Sciences) using the Amersham Imager 680 (GE Healthcare Life Sciences).

2.4. Virulence assays on apple fruits

Apple infection assays were conducted as previously described [10]. Briefly, 3 replicates of five fruits were first wounded (4 wounds around the equator) by making punctures with a nail (approximately 5 mm in depth) and 5 μL of conidial suspension (10^4 conidia/mL) were applied to each wound. Apple fruits were stored at 20 °C and 90 % relative humidity. Each wound was scored daily for infection symptoms on consecutive days post-inoculation (dpi). Results were represented as the mean values of the percentage of infected wounds ± standard deviation (SD) of the three biological replicas.

Table 1
FungalBraid plasmids used in this study.

Plasmid	Genetic element/Assembly	Organism	5′/3′ Barcodes	GB Plasmid	Reference
FB012	PtrpC::hph::Ttub	*A. nidulans*; *E. coli*; *N. crassa*	AATG/GCTT	pUPD2	[28]
FB013	P*gpdA*::HSV*tk*::T*tub*[a]	C. heterostrophus; HSV; *N. crassa*	GGAG/TACT	pUPD2	[28]
FB110	*afpA* 5′ fragment	*P. expansum*	TACT/AATG	pUPD2	This study
FB111	*afpA* 3′ fragment	*P. expansum*	GCTT/CGCT	pUPD2	This study
FB113	FB013 + FB110 + FB012 + FB111			pDGB3α2	This study

[a] FB013 is in reverse orientation.

2.5. RNA isolation

Erlenmeyer flasks of 100 mL volume containing 25 mL of PcMM were inoculated with 10^6 conidia/mL. Samples were cultured for 3 or 5 days at 25 °C and 150 rpm. Three replicates were grown for each strain condition. Mycelia were filtered, frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA) following manufacturer's instructions. RNA samples were then subjected to DNAase treatment with the TURBO DNA-free™ Kit (Invitrogen). RNA concentration and quality were assessed using a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis.

2.6. Transcriptomic data analysis

RNA-Seq was performed by the genomic facility of the central service for experimental research (SCSIE) from University of Valencia (UV, Spain) (https://www.uv.es/uvweb/central-service-for-experimental-research/en/central-service-experimental-research-scsie-128586858 2594.html). Integrity of RNA was verified using the 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and samples with RNA Quality Number (RQN) > 7 were subsequently used for high-throughput sequencing. Complementary DNA (cDNA) libraries were prepared for mRNA analysis with the TruSeq stranded mRNA Library prep (Illumina, San Diego, CA, USA) and sequenced on an Illumina NextSeq 500 platform (single-end reads, 75 bp average length).

Analysis was performed using software packages included in OmicsBox software (Version 2.0.36) [31]. Quality (Q) of reads was checked with FastQC version 0.11.8 [32]. Adapters were removed and reads were trimmed (3′ end trimming when Q < 20) and filtered (average Q < 20, read length < 25 bp) using Trimmomatic 0.38 [33]. Filtered reads were mapped onto *P. expansum* CMP-1 genome (NCBI, GenBank assembly accession GCA_000769755.1) with STAR (v.2.7.8a) [34]. The number of reads that overlapped with exon features for each gene was counted based on HT-Seq 0.9.0 [35]. Count data was extracted and used for pairwise differential expression analysis with edgeR [36]. Genes for which Count Per Millions (CPM) < 0.5 in a minimum of three samples were removed. Library sizes were normalized using the Trimmed Mean of M-values (TMM) method and differentially expressed genes (DEGs) were identified using $|\log_2(\text{Fold change})| > 1$ and false discovery rate (FDR) < 0.05.

Gene Ontology (GO) enrichment analysis was performed using the Fisher's exact test (FDR < 0.05) integrated in the FatiGO package [37] included in the OmicsBox software. Combined Pathway Analysis was effectuated using the Fisher's exact test (FDR < 0.05) integrated in OmicsBox by using the KEGG database [38].

Multidimensional scaling (MDS) plots were generated using the plotMDS function from limma (version 3.28.14) Bioconductor package. Gene expression heatmaps were visualized using the heatmap.2 gplots (v3.1.3) package integrated in R (v4.2.2). Hierarchical clustering was effectuated for both rows and columns applying the default "complete method" in the *hclust* function and the "euclidean" distance as the similarity measure. Color scale was applied to the rows (genes).

Raw RNA-Seq read data and count tables are deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under the accession code GSE255791.

2.7. P. expansum CMP-1 genome annotation

Annotation of *P. expansum* CMP-1 genome (assembly accession GCA_000769755.1) was improved with the Functional Annotation package integrated in the OmicsBox suite. BLASTx was used to compare *P. expansum* coding sequences against a pre-generated local database of thirteen fungal proteomes (Table S2). GO terms associated to the BLAST hits were subsequently retrieved in a mapping step. Mapped terms were then assigned to query sequences following the default annotation pipeline [39]. GO annotation was extended by performing a second annotation step with the InterProScan database (InterPro protein annotation).

2.8. Quantitative reverse transcription-PCR analysis (qRT-PCR)

Total RNA was extracted using the Direct-zol RNA Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions, including an in-column DNAase treatment. Synthesis of first-strand cDNA and qRT-PCR assays were conducted as previously described [40]. Gene primers used for qRT-PCR are listed in Table S3. The housekeeping genes coding for *P. digitatum* β-tubulin, ribosomal protein L18a and 18S rRNA were simultaneously employed to normalize gene expression changes. Relative quantification and statistical significance were determined with the relative expression software tool (Multiple Condition Solver REST-MCS v2) [41] (https://www.qiagen.com/es/resources/).

2.9. Sensitivity assays to chemical compounds

Sensitivity of fungal strains to different stressor compounds was tested in 24-well cell culture on PDA and PcMM solid media (1 mL per well). PDA and PcMM were supplemented with 1 M NaCl, 50 to 200 μg/mL sodium dodecyl sulphate (SDS) (Sigma-Aldrich, Burlington, MA, USA), 0.5 to 2 mM H_2O_2 and 125 to 200 μg/mL Congo Red (CR) (Sigma-Aldrich). Five mL of serial 10-fold conidia were applied into each well. Plates were incubated at 24 °C for 3 days. Assays were performed in duplicate.

2.10. Antifungal activity assays

Antifungal activity assays were conducted in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) as previously described [42] with minor modifications. Briefly, 50 μL of 10 % PDB (Sigma-Aldrich) containing 2× conidia solution (5×10^4 conidia/mL) and 0.02 % (*w/v*) chloramphenicol were mixed in each well with 50 μL of 2× protein solutions (PeAfpA, PeAfpB and PeAfpC) from serial 2-fold dilutions (final concentration range from 0.25 to 16 μg/mL for PeAfpA, 1.5 to 96 μg/mL for PeAfpB and 2 to 128 μg/mL for PeAfpC). Plates were statically incubated for 72 h at 25 °C and growth was determined each 24 h by measuring the optical density (OD) at 600 nm (OD_{600}) using a FLUOstar Omega plate spectrophotometer (BMG labtech, Orlenberg, Germany). Dose response curves were generated from measurements after 72 h. The Minimum Inhibitory Concentration (MIC) was defined as the protein concentration that completely inhibited fungal growth.

3. Results

3.1. Generation of P. expansum disruption mutants of the afpA gene

The *afpA* gene from *P. expansum* CMP-1 strain (PEX1_077760) contains an open reading frame (ORF) of 411 bp distributed in three exons and two introns. To disrupt *afpA* (Δ*afpA*) the first exon was replaced by homologous recombination with the *hph* cassette (Fig. S1A). Five disruption strains (PEME0012, PEME0034, PEME0042, PEME0055 and PEME0062) and an ectopic control mutant (PEME0066) from the transformation experiment were confirmed by PCR amplification of genomic DNA (Fig. S1B). Three disruption transformants were randomly selected (PEME0034, PEME0055 and PEME0062) to further validate the non-functionality of *afpA* by SDS-PAGE and Western blot analysis from long-term (11 days) growth culture supernatants. As expected, disruption strains did not produce PeAfpA, in contrast to the parental and ectopic control strains (Fig. 1).

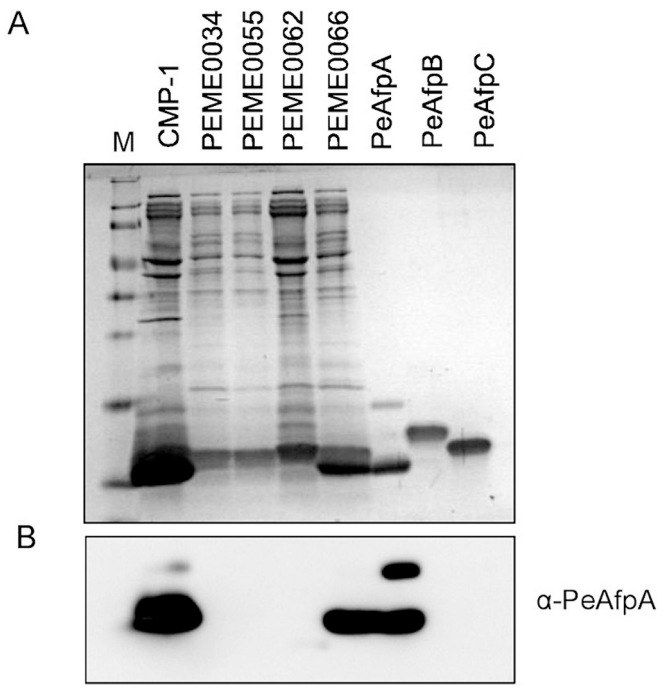

Fig. 1. Western-blot analysis of *P. expansum* growth supernatants. (A) SDS-PAGE and (B) Western blot analyses using the specific anti-PeAfpA antibody of supernatants at 11 days of growth from the wild-type parental strain CMP-1, Δ*afpA* strains (PEME0034, PEME0055 and PEME0062) and the ectopic transformant PEME0066. 2 μg of pure PeAfpA, PeAfpB and PeAfpC are shown as positive controls. M: SeeBlue R® Pre-stained protein standard.

3.2. Characterization of the *P. expansum ΔafpA mutants*

The Δ*afpA* strains (PEME0034, PEME0055 and PEME0062) and the ectopic control PEME0066 were characterized according to their axenic growth, conidia production and virulence (Fig. 2). Morphology of the disruption strains on PDA and PcMM solid media was similar to that of the parental CMP-1 and ectopic strains (Fig. 2A), demonstrating that disruption of *afpA* did not affect vegetative growth of *P. expansum*. Conidiogenesis at 4 and 7 days of growth on PDA plates was not altered (Fig. 2B), nor was the pigmentation of conidia (Fig. 2A).

To determine the role of the *afpA* gene in pathogenicity, the virulence of the mutants was determined by conducting infection assays on apple fruits. Incidence of infection (Fig. 2C) and symptom development (Fig. 2D) of the Δ*afpA* mutants were similar to both the parental CMP-1 and ectopic PEME0066, evidencing that *afpA* is dispensable for the virulence of *P. expansum*.

Sensitivity tests were performed with two Δ*afpA* mutants (PEME0034 and PEME0055) and the parental CMP-1 to identify any involvement of *afpA* in the stress response of *P. expansum*. Osmotic, membrane, CW and oxidative stress conditions were induced by adding NaCl, SDS, Congo Red and H_2O_2 to PDA and PcMM plates, respectively. In PDA, Δ*afpA* mutants did not show altered sensitivity to any of the chemicals compared to the parental CMP-1 (Fig. S2A). In PcMM, NaCl and Congo Red sensitivity was not modified in the Δ*afpA* mutants while a subtle increase in sensitivity in response to H_2O_2 and SDS was evident at the lowest inoculum dose (Fig. S2B).

Finally, we assayed side by side the effect of the three PeAFPs on the two Δ*afpA* mutants and the parental CMP-1 (Fig. S3). Both the mutants and CMP-1 showed a MIC of 2 μg/mL for PeAfpA and 12 μg/mL for PeAfpB, while PeAfpC did not affect fungal growth in the conditions tested (MIC >128 μg/mL). Thus, *afpA* disruption did not alter fungus susceptibility towards any of the AFPs encoded in the *P. expansum* genome.

3.3. Transcriptomic profiling of *P. expansum* parental strain and Δ*afpA* mutant

To further explore the functional role of PeAfpA, we evaluated potential changes produced at gene expression level by its gene disruption. For this purpose, we analysed the transcriptome of one representative Δ*afpA* strain (PEME0055) against the parental CMP-1 at two time points that mark the onset of PeAfpA detection according to our previous data [11]: day 3, at which PeAfpA is not detected yet in the CMP-1 culture supernatant, and day 5, at which the protein is present in the CMP-1 supernatant.

Supernatants from CMP-1 and PEME0055 cultured for 3 or 5 days were subjected to SDS-PAGE and Western blot analysis with anti-PeAfpA, anti-PeAfpB and anti-PeAfpC specific antibodies. As expected, PeAfpA was only identified in CMP-1 supernatants cultured for 5 days while no protein signal was identified in any of the Δ*afpA* samples (Fig. 3). Immunoreaction signal for PeAfpB was not detected in any of the strains, as previously described for CMP-1 [10,11]. Regarding PeAfpC, protein production was also detected at day 5 in the CMP-1 parental strain, while at the same time point PeAfpC was present only in one of the Δ*afpA* replicates, exhibiting a much lower intensity than in the wildtype. A weak PeAfpC-specific signal could also be immunodetected in one of the Δ*afpA* samples at day 3.

Mycelia from these cultures were subjected to transcriptome sequencing (Table S4). Reads alignment against CMP-1 reference genome revealed a total number of uniquely mapped reads per sample between 13.7 and 24.8 million. Specifically, reads that mapped to annotated transcripts ranged between 8 and 17 million, equivalent to 58.52–70.34 %.

Normalized count data were then subjected to multidimensional scaling analysis (MDS). As shown in Fig. 4A, CMP-1 samples cultured for 5 days (C5) were strongly differentiated from all other conditions along the first dimension, which explains 49 % of the variability. The second dimension, which describes 14 % of the variability, separates Δ*afpA* samples according to culture time (M3 and M5, respectively). Hierarchical clustering confirms the separation of PeAfpA-producing CMP-1 samples (C5) from the rest (Fig. 4B). Although there were some clustering discrepancies, all replicates were kept to make the gene expression analysis as reliable as possible for the identification of responses associated to PeAfpA and discard effects from the biological variability caused by filamentous fungi macromorphologies in submerged culture [43].

A total of 89.55 % of the genes annotated in *P. expansum* CMP-1 strain passed the filtering expression threshold for differential gene expression (DGE) analysis. Overall, four pairwise comparisons were effectuated in order to elucidate putative biological role(s) of PeAfpA (Table 2 and Fig. 5A). Table S5 shows the DEG dataset for the four contrasted conditions.

In the first comparison, transcriptome changes were determined for the CMP-1 strain cultured for 5 (C5, contrast condition) and 3 days (C3, reference condition) (comparison I, Fig. 5A). Therefore, we compared a condition in which PeAfpA is produced and secreted by the parental strain (C5, depicted as a plus symbol in Fig. 5A) against a condition in which the protein is not yet detected (C3, represented as a minus symbol in Fig. 5A). A total of 2347 DEGs corresponding to 22 % of the genes annotated in *P. expansum* were identified. Out of these DEGs, 1069 were up-regulated and 1278 down-regulated (Table 2). Among the five most induced DEGs in comparison I are PEX1_077760 (log_2FC of 11.1), PEX1_010080 (log_2FC of 9.5) and PEX1_023340 (log_2FC of 9.0) that encode for PeAfpA, PeAfpC and PeAfpB, respectively. The other two genes are PEX1_043180 (log_2FC of 9.6), which encode a hypothetical protein of 74 amino acids without any described conserved domain, and PEX1_023220 (log_2FC of 9.1), which codifies a putative cytochrome P450 CYP64-like subfamily monooxygenase. On the contrary, the most repressed genes are PEX1_080220, which encodes an α-amylase type 1–2 (log_2FC of −10.9), and PEX1_059240, annotated as a chitin-binding

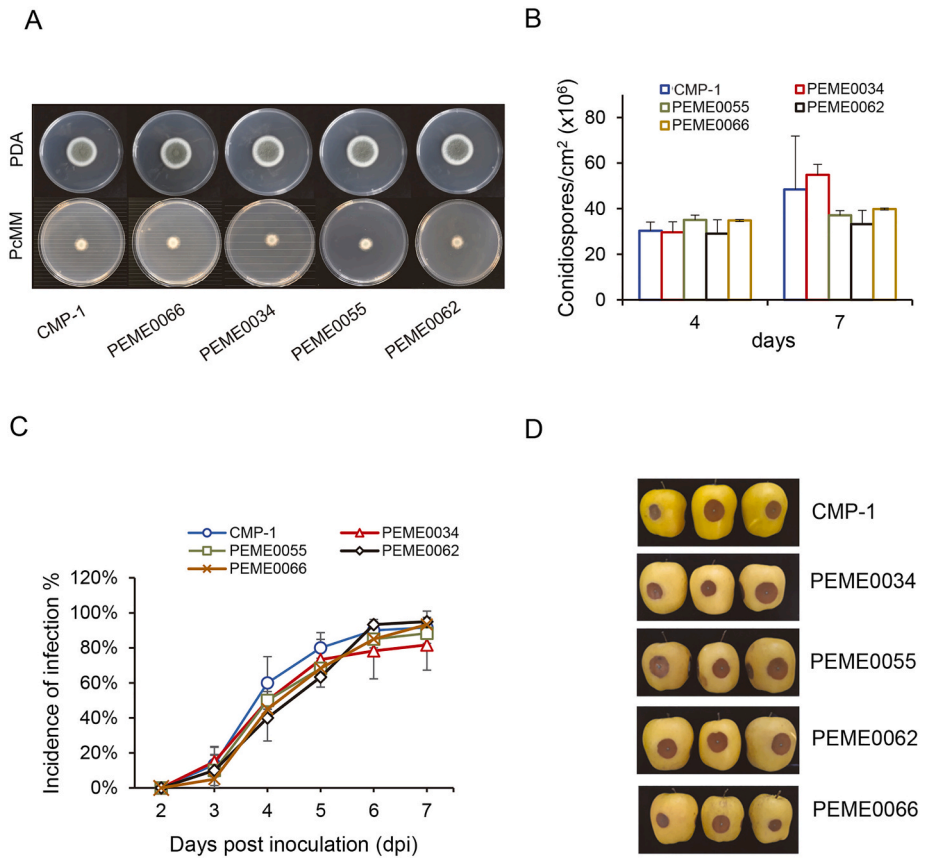

Fig. 2. Characterization of *P. expansum* CMP-1 and transformant strains. (A) Colony morphology of the ectopic transformant PEME0066 and the Δ*afpA* disruption mutants (PEME0034, PEME0055 and PEME0062) compared to the parental strain CMP-1 after 4 days of growth on PDA and PcMM plates. (B) Conidia production per surface area after 4 and 7 days of growth on PDA plates. Data are mean values ± standard deviation (SD) of three replicate samples. (C) Incidence of infection caused by the different strains. Data indicate percentage of infected wounds (mean value ± SD) at each day post inoculation (dpi). (D) Representative images of apple fruits at 7 dpi.

domain 3 protein (log_2FC of −10.8). Importantly, half of the ten most down-regulated genes are described as membrane transporters, including three major facilitator superfamily (MFS) transporters (PEX1_093560, PEX1_072310, PEX1_005120), one sodium/solute symporter (PEX1_000850) and one ammonium transporter (PEX1_087530).

Nevertheless, whether these expression differences in comparison I are caused by PeAfpA production cannot be directly determined by only considering this pairwise comparison, since other variables, such as biomass growth and nutrient consumption over time, can also cause dynamic changes at the gene expression level. Following this rationale, gene expression changes were also determined for the Δ*afpA* disruption mutant PEME0055 cultured for 5 (M5, contrast condition) and 3 days (M3, reference condition) (comparison II, Fig. 5A), as it was previously effectuated for the parental CMP-1 strain in comparison I. Only 145 genes were identified as significant DEGs in PEME0055, being 113 up- and 32 down-regulated (Table 2). These DEGs only represent 1.36 % of total *P. expansum* genome, contrary to the 22 % previously reported for the CMP-1 strain (comparison I). Taken together, this result indirectly supports the hypothesis that PeAfpA production is the variable that plays a major role in the gene expression changes reported for comparison I.

In order to further determine the effect of *afpA* disruption on *P. expansum*, comparison of the null mutant (contrast condition) against the parental strain (reference condition) was carried for samples cultured for 5- (comparison III) and 3-days (comparison IV), respectively (Fig. 5A). Comparison III revealed 1709 genes that were differentially expressed in the null mutant. A total of 980 of these DEGs were up-regulated and 729 were down-regulated (Table 2). The most down-regulated gene was the PeAfpA encoding gene (PEX1_077760) (log_2FC of −13.9), in concordance with the disruption made to this ORF. Apart from this, the four most down-regulated genes were annotated as a dehydrogenase (PEX1_034660, log_2FC of −7.7), monooxygenase (PEX1_007060, log_2FC of −7.3), oxidoreductase (PEX1_023220, log_2FC of - 7.1) and glycoside hydrolases (PEX1_070700, log_2FC of −6.7). Remarkably, the *afpB* and *afpC* genes were also repressed in the *afpA* mutant, exhibiting a log_2FC of −6.1 and − 5, respectively. On the contrary, genes with the highest fold change mostly encoded membrane and transporter proteins, including PEX1_000850 (sodium/solute transporter, log_2FC of 9.1), PEX1_014490 (GPI anchored protein, log_2FC of 8.9), PEX1_036880 (phosphate transporter, log_2FC of 8.8) and PEX1_093560 (MFS monosaccharide transporter, log_2FC 8.6).

Finally, comparison of the Δ*afpA* strain against CMP-1 at 3-days culture (M3 and C3, respectively; comparison IV) only reported 5 up- and 4 down-regulated DEGs (Table 2). This highlights a practically identical transcriptome profile between the null and parental strain when PeAfpA is not yet detected in culture supernatants. It is worth mentioning that the PeAfpC encoding gene (PEX1_010080) is one of the nine significant DEGs, showing an induction (log_2FC of 4.72) in the Δ*afpA* strain at 3 days of culture.

3.4. Identification of differentially expressed genes associated to PeAfpA production

From the four pairwise comparisons effectuated, comparison I and III are especially interesting to unravel the biological function of PeAfpA, since they compare a condition in which the protein is detected in the

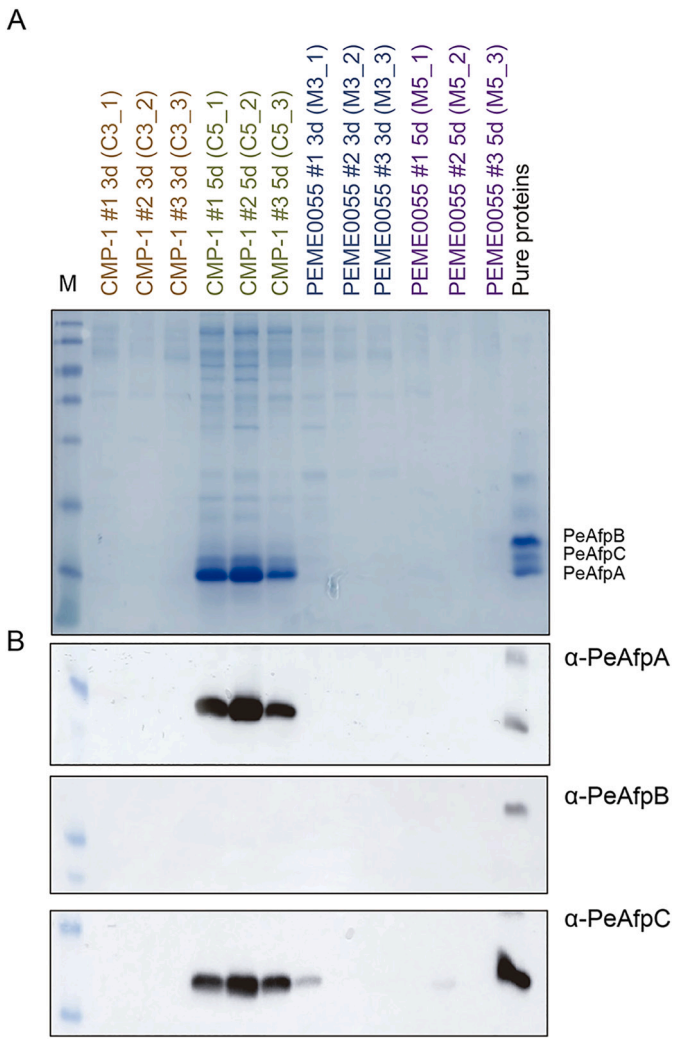

Fig. 3. Western blot analyses of *P. expansum* parental (CMP-1) and Δ*afpA* strain supernatants. (A) SDS page and (B) and Western blot analyses of culture supernatants (10× culture supernatants per lane) after 3 and 5 days of growth in PcMM. One μg of pure PeAfpA and PeAfpC and 2 μg of pure PeAfpB were loaded as positive controls. M: SeeBlue® Pre-stained protein standard.

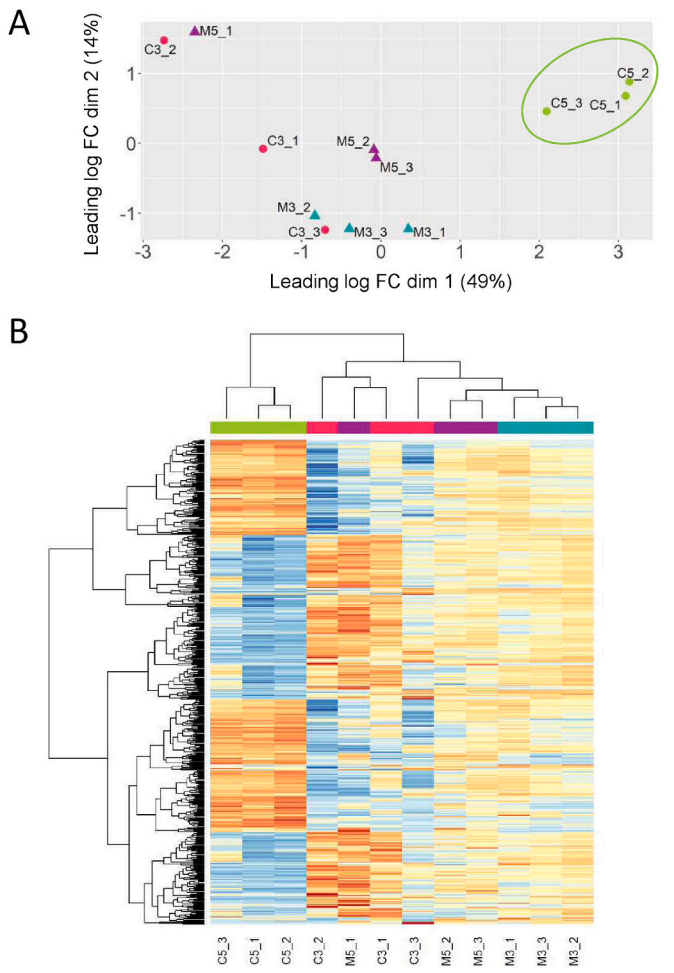

Fig. 4. RNA-Seq profiles of CMP-1 and Δ*afpA* strains after 3 and 5 days of growth in PcMM. (A) Multi-dimensional scaling (MDS) plot of the RNA-seq gene expression profiles. The distances represent the root-mean-square deviation for the genes with the largest standard deviations between samples. FC: fold change. (B) Heatmap of the 500 most variable genes sorted by $\log_2$ counts per million ($\log_2$CPM). Hierarchical clustering is included for the samples (horizontal axis) and genes (vertical axis). Color scaling was applied to the genes (row *Z*-scores).

supernatant (C5) against conditions in which the protein is not yet detected (C3 in comparison I and M5 in comparison III). Following this rationale, DEGs potentially related to PeAfpA putative roles in the host fungus would exhibit a reverse expression pattern between each of these two comparisons (C5 vs. C3 and M5 vs. C5, respectively).

To test this hypothesis, shared DEGs between comparisons I and III were identified. Fig. 5B shows on the four corners of the diagram 1362 shared DEGs between both comparisons, equivalent to 79.9 % of the total DEGs in the *ΔafpA* PEME0055 strain at day 5 (comparison III). Moreover, 99.8 % of these DEGs (1360 out of 1362) showed a reverse expression profile between comparison I and III, supporting them as genes potentially implicated in the functional role of PeAfpA.

Among genes with such a reverse profile, a total of 605 are up-regulated in CMP-1 cultured for 5 days and down-regulated at this time point in the Δ*afpA* mutant (Fig. 5B). We named this group of 605 genes as cluster A, which comprises genes whose induction is positively linked to PeAfpA production and depends on the presence of the *afpA* gene. Cluster A is largely related to catabolic Carbohydrate-Active EnZymes (CAZymes), as it is further explained in next Section 3.5. Importantly, this cluster includes PEX1_077760, PEX1_023340 and PEX1_010080 encoding for PeAfpA, PeAfpB and PeAfpC, which points out to a positive correlation in gene expression of the three *afp* genes.

Oppositely, cluster B is composed of 755 genes that are specifically repressed under PeAfpA production, since they are up-regulated in the Δ*afpA* PEME0055 mutant and down-regulated in the parental CMP-1 strain cultured for 5 days (Fig. 5B). These genes mainly encode transporters, as well as plasma membrane and fungal CW proteins. It also includes two genes involved in chitin biosynthesis, coding for a chitin synthase (PEX1_032770) and the chitin synthase III catalytic subunit (PEX1_001720), and one coding for a chitin binding domain (PEX1_032640). This, together with the overrepresentation of CAZymes in cluster A, suggests an influence of PeAfpA in CW biosynthesis and remodeling.

3.5. Functional enrichment analysis of genes related to PeAfpA production

To further investigate the transcriptome response of *P. expansum* associated to PeAfpA production, Gene Ontology (GO) enrichment analysis was conducted for the cluster A and B data sets previously described in Section 3.4. Since a preliminary analysis indicated that 35.3 % of the genes in CMP-1 were automatically annotated as hypothetical protein, improvement of the *P. expansum* CMP-1 annotation was firstly assessed (see Material and Methods). This annotation pipeline

Table 2
Pairwise comparisons for the determination of differentially expressed genes.

Comparison	Reference condition	Comparison condition	Pairwise analysis ID	Gene number		
				Total	Up-regulated	Down-regulated
I	CMP-1 3 days	CMP-1 5 days	C5 vs. C3	2347	1069	1278
II	PEME0055 3 days	PEME0055 5 days	M5 vs. M3	145	113	32
III	CMP-1 5 days	PEME0055 5 days	M5 vs. C5	1709	980	729
IV	CMP-1 3 days	PEME0055 3 days	M3 vs. C3	9	5	4

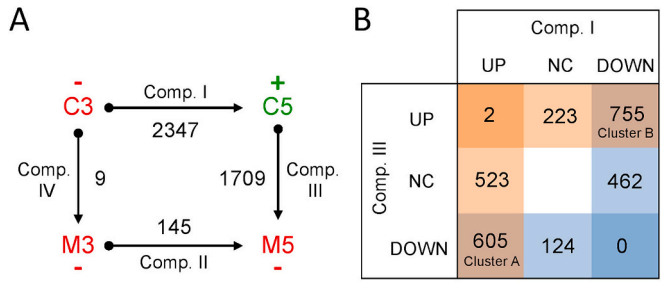

Fig. 5. Pairwise comparisons of CMP-1 and Δ*afpA* RNA-Seq profiles at two time points. (A) Differentially expressed genes (DEGs) associated to each comparison (see Table 2). The plus (+) and minus (−) symbols reflect the detection of PeAfpA in culture supernatants. The dot symbol reflects the reference condition used in each comparison. (B) Shared DEGs between comparison I and III, indicating genes that are up-regulated (UP), down-regulated (DOWN) or not differentially expressed in the other comparison (NC). Genes with an inversed expression pattern are part of cluster A (induced upon PeAfpA production) or cluster B (repressed upon PeAfpA production).

reduced genes described as hypothetical protein to 10.8 % of total genome, and genes without GO annotation were reduced to 19.4 % (2066 genes) (Fig. S4). The improved annotation is available in Table S6 and was used in the following analyses.

As depicted in Fig. 6A, enrichment analysis showed six over-represented GO terms within cluster A that is associated with PeAfpA production (Table S7). Iron ion binding, carbohydrate binding and β-*N*-acetylhexosamidase activity were the significant terms detected for the molecular function category, whilst *N*-acetylglucosamine catabolic process and polysaccharide catabolic process were significantly enriched for the biological processes. The *N*-acetylglucosamine catabolic process is annotated to a glucosamine-6-phosphate isomerase (PEX1_093090), a membrane β-*N*-acetylhexosaminidase subunit alpha/beta (PEX1_045120) and an *N*-acetylglucosamine-6-phosphate deacetylase (PEX1_093060). In concordance, the β-*N*-acetylhexosamidase activity is annotated to three glycoside hydrolases enzymes putatively involved in chitin oligosaccharides cleavage (PEX1_045120, PEX1_002370 and PEX1_074240), highlighting an enrichment in CAZymes with chitinases and chitobiose β-*N*-acetylhexosaminidase activities. Similarly, the carbohydrate binding term shows an induction in genes related to protein-carbohydrate interaction, protein glycosylation and CW remodeling, such as putative mannose-binding lectins, α-1,2-mannosidases and proteins with concanavalin A-like lectin/glucanases superfamily domains, as described in Table S7. On the other hand, genes annotated to the iron ion binding GO term show the overrepresentation of fungal cytochrome P450s monooxygenases, linking *afpA* expression to the regulation of putative secondary metabolism clusters.

Notably, the only significant term for the cellular component category in cluster A was the extracellular region. It showed the highest gene ratio among all significant GO terms, conformed by 35 genes mostly associated to CAZymes of the CW (Table S7). In fact, 8 out of the 9 DEGs from the significant polysaccharide catabolic process shared the extracellular location and included several glycoside hydrolases, one chitinase (PEX1_061900) and one beta-*N*-acetylglucosaminidase (PEX1_064020).

As shown in Fig. 6B, the cluster B that comprises genes negatively correlated with PeAfpA production had an overrepresentation of the mitochondrial respiratory chain complex IV, fungal-type cell wall and plasma membrane GO terms for the cellular component category. The molecular function category evidenced an enrichment in oxidoreductase and transport activities, including cytochrome-c oxidase, nucleobase transmembrane transporter, ammonium transmembrane transporter and sodium inorganic phosphate symporter activities. The biological process category supported these findings, showing a significant enrichment in several cell transport processes GO terms (Fig. 6B and Table S8). Overall, the plasma membrane was the most overrepresented GO term, being annotated to 5.83 % of the cluster B (44 out of 755 genes). Remarkably, 24 out of the 44 genes (57.14 %) encode membrane transporters, including major facilitator superfamily (MFS), ammonium and ATP-binding cassette (ABC) transporters, as further described in Table S8.

KEGG pathway enrichment analysis was also applied to identify specific pathways responsive to PeAfpA production. The analysis revealed significant over-representation only for the amino sugar and nucleotide sugar metabolism pathway (kegg: ko00520) for the cluster A data set. In fact, 20 out of 109 genes (18.3 %) specifically annotated to this pathway in *P. expansum* were part of the cluster. These 20 genes include 5 sequences (PEX1_019210, PEX1_090720, PEX1_018580, PEX1_061900, PEX1_067300) out of a total of 19 genes described as chitinases (EC 3.2.1.14) along with the only 3 genes (PEX1_074240, PEX1_002370, PEX1_045120) assigned to the β-*N*-acetylhexosaminidase enzyme code (EC 3.2.1.52) based on our improved annotation. On the contrary, no specific pathways were significantly overrepresented within the cluster B despite the general down-regulation in transmembrane transporters evidenced through GO analysis, suggesting a multi-target role of PeAfpA inhibiting membrane transport that does not rely on specific pathways.

3.6. *Validation of afpB and afpC gene expression by reverse transcription-quantitative PCR (qRT-PCR)*

Since the transcriptomic data highlighted the down-regulation of *afpB* (PEX1_023340) and *afpC* (PEX1_010080) in the Δ*afpA* mutant, we decided to further validate these results by quantitative reverse transcription-PCR (qRT-PCR). To strengthen this conclusion, we included a second independent Δ*afpA* mutant (PEME0034) along with the PEME0055 strain evaluated in the transcriptomic study. Strains were cultured for 5 days under the same growth conditions applied in the RNA-Seq assay.

RNA-Seq expression data for PEME0055 were firstly validated by comparing RNA-Seq Log_2 fold changes for both *afp* genes with the expression fold changes obtained by means of qRT-PCR, which showed high concordance and confirmed the reliability of the RNA-Seq results (Fig. 7A). Then, both Δ*afpA* mutants revealed an impressive down-regulation in *afpB* and *afpC* gene expression compared to the 5-days cultured parental strain. Remarkably, *afpB* showed 62.5 and 11.1-times expression reduction (equivalent to 0.016 and 0.090 relative fold change, Fig. 7B) in PEME0055 and PEME0034, respectively. Likewise, *afpC* was significantly down-regulated in both mutants, decreasing 52.6 and 6.3-times (equivalent to 0.019 and 0.158 relative fold change, Fig. 7B) compared to the wildtype. Overall, both independent Δ*afpA*

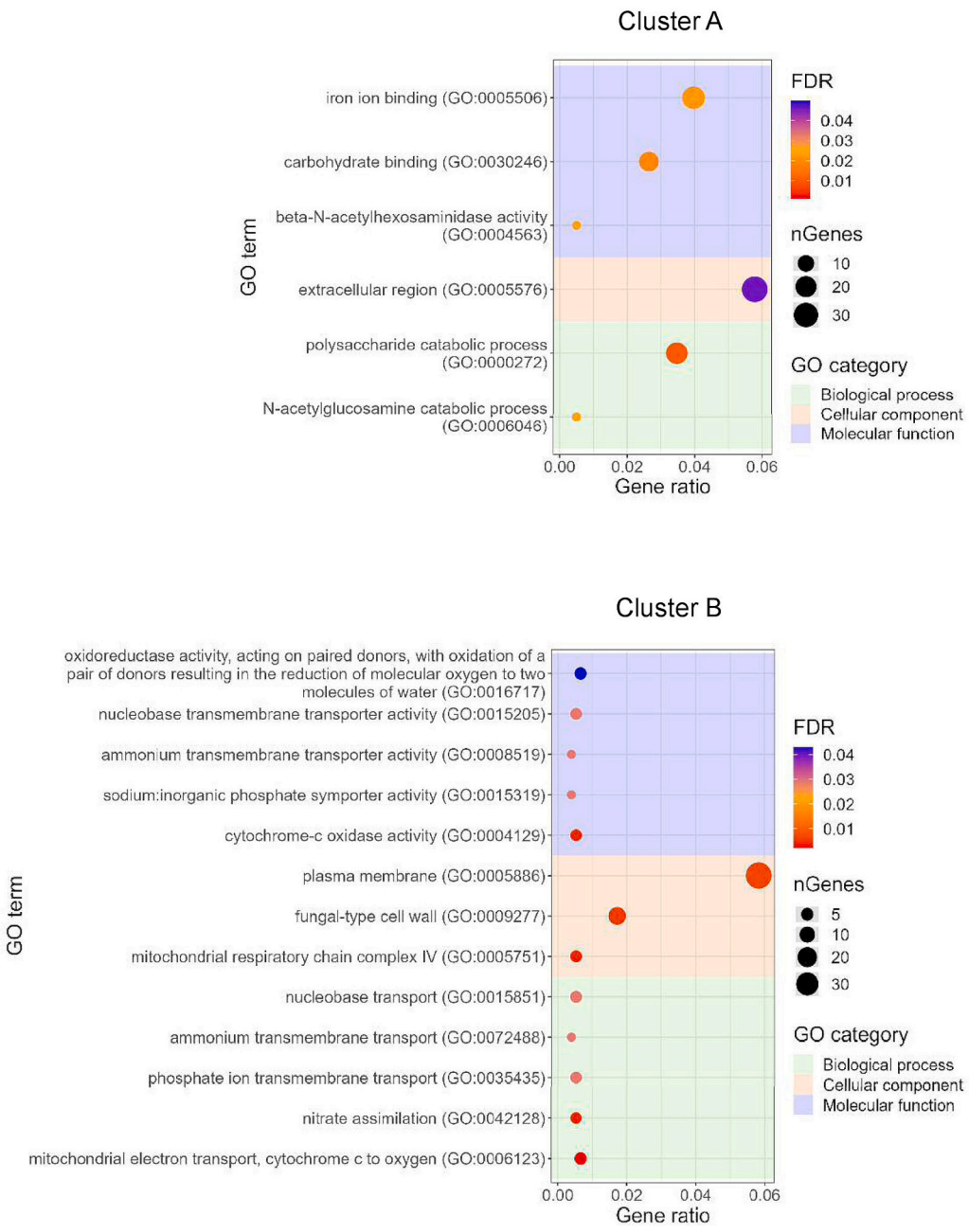

Fig. 6. Gene Ontology (GO) enrichment analysis for the differentially expressed genes (DEGs) of the clusters A and B. Both charts (A and B) show the GO terms significantly enriched (false discovery rate (FDR) < 0.05). The y-axis indicates the GO term and the x-axis shows the gene ratio as the percentage of enriched DEGs associated to each GO term. The bubble size represents the total number of DEGs and the bubble color correlates with the significance level. The color background differentiates each GO category.

strains confirmed the repression of the other two *afp* encoding genes when *afpA* is not expressed.

4. Discussion

AFPs were initially described as highly effective against most filamentous fungi but only moderately active against the producer fungus. Thus, it was generally accepted that their main role was competition with other fungal inhabitants of the same ecological niche. However, the characterization of PdAfpB from *P. digitatum* [42], PAFB from *P. chrysogenum* [44] or PeAfpA from *P. expansum* [11] as highly active against their own fungus, suggested that AFPs might have a biological function that goes beyond antifungal activity. Moreover, several reports emphasize potential distinct roles for different AFPs [17,23,24]. In this study, we aimed to provide new data about the function of PeAfpA in *P. expansum* by studying disrupted mutants in its coding *afpA* gene through phenotypical characterization and transcriptional profiling.

P. expansum is a fungus whose genome encodes three different AFPs, providing an excellent opportunity to address the biological role of its three encoding genes. In previous works, we showed that PeAfpA is a self-inhibitory protein, and that there is not a common pattern for the production of the three PeAFPs or their antimicrobial activity [10,11]. Our previous results also suggested that PeAFPs do not have a relevant role in pathogenesis, since none of them was produced during apple infection [10]. Results presented here confirm that the *afpA* gene is dispensable for pathogenesis to apple fruit, as occurs with *afpB* from *P. digitatum* to citrus fruit [17].

We report here that the three *P. expansum afp* genes might be co-

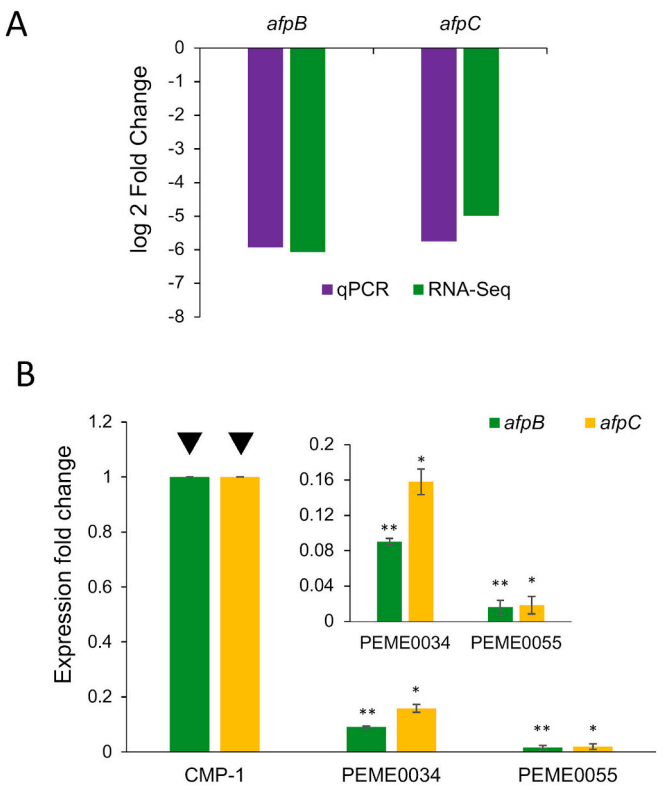

Fig. 7. Expression analysis of the *afpB* and *afpC* genes in Δ*afpA* strains at 5 days of growth in PcMM. (A) Comparison of the $\log_2$ fold change of *afpB* and *afpC* genes between the RNA-Seq (green) and qRT-PCR (blue) assays. (B) Relative gene expression of *afpB* (green) and *afpC* (yellow) determined by qRT-PCR. Reference condition is indicated with an inverted triangle. Bars show the mean ± standard deviation (SD) of three biological replicates. Significant differences are labeled with asterisks (*, $P < 0.05$; **, $P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regulated under AFP-producing conditions (day 5) and that the presence of one of them (*afpA*) is needed for the proper expression of the other two (*afpB* and *afpC*). We demonstrated that the three *afp* genes were among the top five most induced genes of the whole genome after PeAfpA production (comparison I, parental CMP-1 strain at day 5 vs CMP-1 at day 3) and that *afpB* and *afpC* were parallelly repressed in the Δ*afpA* mutant (comparison III, mutant strain vs CMP-1 at day 5). These results are clearly reflected in PeAfpA and PeAfpC production, but not in the case of PeAfpB (Fig. 3) as previously described [10]. Although the induction of *afpA* was predictable given the high yield of PeAfpA produced by this strain, the co-induction with the other two genes and their top level of induction were a completely unexpected finding. In addition, qRT-PCR data of two independent Δ*afpA* strains confirmed the repression of *afpB* and *afpC* genes (Fig. 7), demonstrating that their induction during PeAfpA production was dependent of *afpA*. The repression of *afpC* at day 5 in the Δ*afpA* PEME0055 mutant was additionally confirmed with the extremely low detection of PeAfpC in only one of the three replicates (Fig. 3). We previously reported that the *P. expansum* strain MD-8 does not produce detectable PeAfpA and shows a strong reduction in the amount of PeAfpC [10], resembling the data shown here with the Δ*afpA* mutant and further reinforcing the existence of common mechanisms in the regulation of the production of these two proteins. However, PeAfpC was slightly detected in one of the Δ*afpA* mutant strain replicates at day 3, but in any of the CMP-1 strain at the same time point. This is in accordance with the fact that the PeAfpC encoding gene was one of the five up-regulated DEGs in the Δ*afpA* strain at day 3. Further studies are needed to confirm *afp* co-regulation along culture time.

Unfortunately, disruption of *P. expansum afpA* did not show any phenotype in axenic growth, virulence or response to stress, similarly to *anafp* of *A. niger* or *afpB* of *P. digitatum* [17,24]. The absence of phenotype in our Δ*afpA* mutant PEME0055 is somehow confirmed by its transcriptome nearly identical to the parental CMP-1 under conditions in which no PeAfpA protein is detected in the parental (<0.1 % of DEGs over the total genome, comparison IV). The absence of clearly differential phenotypes could suggest that these AFPs are implicated in processes that have not been assessed through the described assays. Since *P. expansum* encodes more than one AFP, it cannot be discarded an offsetting effect hampering the identification of differential phenotypes in the null mutant. However, disruption of the *paf* gene from *P. chrysogenum*, a fungus that also encodes several AFPs, showed a phenotype of impairment of asexual development and conidia formation [23]. Considering the absence of phenotype of the *afpA* mutant, the production of PeAfpA was surprisingly accompanied by very important changes in the transcriptome. In fact, 12.7 % of *P. expansum* genes (1360 out of 10,663 total genes) changed expression as consequence of PeAfpA production, suggesting an important effect of the protein on the fungal biology, yet to be identified. This was also suggested by the MDS plot (Fig. 4A), since the wild-type samples cultured for 5 days, corresponding to the unique condition in which PeAfpA is detected, were strongly separated from the rest.

The comparison of the transcriptomes of *afp* mutants of different fungi could identify common patterns and potential functions of AFPs. So far, the only previous transcriptomic profiling of an *afp* mutant was our study of the Δ*afpB* from *P. digitatum* [25]. This mutant did not show any phenotype either, but it is important to stress that PdAfpB production in the parental *P. digitatum* has yet to be demonstrated [10,17]. Remarkably, the Δ*afpB* mutant from *P. digitatum* showed approximately 20 times more DEGs (2.3 % of the genome) than the *P. expansum* Δ*afpA* mutant at day 3 (0.1 % of the genome, comparison IV). GO enrichment analysis of Δ*afpB* revealed that the *afpB* gene is required for normal maturation of rRNA, ribosome assembly and RNA transcription, contributing to the overall homeostasis of the cell in *P. digitatum*, and that also represses toxin-encoding genes [25]. Moreover, the Δ*afpB* mutant shows a remarkable induction of a putative inhibitor of apoptosis while the treatment with PdAfpB represses the same gene, suggesting a possible role of PdAfpB in apoptosis [25]. Unfortunately, no homologous to these genes were identified among the few DEGs of PEME0055 and thus our transcriptomic analyses did not reveal any similarity between the *afpA* mutant from *P. expansum* and the *afpB* from *P. digitatum*.

Among the most significant GO terms either positively or negatively regulated with PeAfpA production are those related to the extracellular region, plasma membrane and the fungal CW. In particular, our study highlights the up-regulation of CW polysaccharide and *N*-acetylglucosamine catabolic genes with PeAfpA production, including those directly related to chitin. The fungal CW is primarily composed of interlinked polysaccharides (glucans and chitin) and outer N-linked and O-linked mannosylated glycoproteins [45]. Chitin consists of β-1,4-*N*-acetylglucosamine homopolymers covalently linked to β-1,3-glucans that can be enzymatically degraded by chitinases and *N*-acetylglucosaminidases. Based on our transcriptomic data, the production of PeAfpA would promote chitin disruption mainly through overexpression of chitinases and *N*-acetylglucosaminidases encoding genes and also by a partial repression of chitin biosynthesis at the gene expression level. Down-regulation of chitin synthase genes was also found in *P. digitatum* when the fungus was grown in the continuous presence of exogenous PdAfpB [25,46]. It was also previously shown that AFP from *A. giganteus* inhibited the biosynthesis of chitin in sensitive fungi [47]. The changes in genes encoding essential CW components of *P. expansum* suggest that PeAfpA production has indeed a biological role beyond the elimination of competitors, which could be related to the self-control of the fungal population through CW disruption. The hypothesis that the primary

function of AFPs is to kill genetically identical cells has already been proposed based on functional resemblances between AFPs mode of action and that of bacterial cannibal toxins [48].

Other glycosyl hydrolases encoding genes with annotated catalytic activities towards fungal CW polymers were evidenced upon PeAfpA production, including glucanases, mannosidases and other putative α- and β-glycosidades. In *A. niger*, the induction of hydrolytic enzymes was part of the response to carbon starvation during submerged cultivation [49]. In the meta-transcriptomic study of *A. niger*, the co-regulation of the *anafp* encoding gene with polysaccharide catabolism and glycosyl hydrolase genes was mostly related to nutrient mobilization as response to severe carbon and energy limitation [24]. Incremented transcription of hydrolytic enzymes upon PeAfpA production might also suggest a role of PeAfpA in triggering the mobilization of carbon resources. The disruption of *afpA* also pointed out to transcription changes in genes encoding CAZymes involved in the degradation of plant-derived carbohydrates. These include celullases and xylosidases, whose expression is positively correlated with PeAfpA production. Considering the substrates of these enzymes, their implication in fungal CW disruption seems improbable. In this regard, their overexpression upon PeAfpA production might be part of a fungal active response to synthesize CAZymes that mobilize exogenous carbon sources, as in the scouting response described for *A. niger* under carbon starvation [50]. This hypothesis agrees with the fact that natural PeAfpA production is maximized at long culture times in minimal medium cultures, suggesting a link to nutrient limitation [11].

Despite the expression changes in genes related to CW and CW integrity evidenced here, we did not observe susceptibility changes towards CW stressors in our Δ*afpA* strains cultured on solid media. These results might be related with the improbable production of PeAfpA in solid media, and therefore the absence of the gene would not have any effect under these conditions.

Finally, as much as 44 genes were annotated as plasma membrane in the cell component category among the genes repressed with PeAfpA production, highlighting a notorious representation of transporters and symporters as reflected in the significant GO terms (Fig. 6B) specific for sugars, salts, ion metals, phosphate or nitrogen salts and including ABC and MFS transmembrane transporters. This might somehow indicate a general blockade of the exchange of the fungal cell with the milieu, which presumably would result in a deleterious effect for the cell. This effect is contrary to the active efflux by ABC or MFS transporters that contributes to the fungal resistance towards most toxic compounds, including fungicides [51].

5. Conclusion

In summary, this transcriptomic study comparing both the parental wild-type and the Δ*afpA* strains provides new clues on the potential biological roles of PeAfpA. We show the existence of a putative co-regulation between the three *afp* genes of *P. expansum,* although it remains to be determined whether this result indicates a similar role for the three PeAFPs. In any case, it seems that proper production of PeAfpC requires the presence of PeAfpA. PeAfpA production greatly affects CW-related genes involved in the degradation and mobilization of carbohydrates although it is unclear whether this might be a mechanism to achieve the self-control of fungal population, a response to nutrient limitation or both. PeAfpA might also block the overall exchange of molecules with the environment through a general repression of transporters. However, these results are based on high-throughput gene expression analysis and further studies are still needed. Finally, we showed that PeAfpA does not seem to have a direct role in *P. expansum* asexual development or in pathogenesis. Overall, information provided by this work confirms that PeAfpA has different although similar role(s) to those showed by other AFPs and opens novel questions about AFPs biological function beyond the antifungal activity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.131236.

CRediT authorship contribution statement

Carolina Ropero-Pérez: Writing – original draft, Visualization, Investigation. **Elena Moreno-Giménez:** Investigation. **Jose F. Marcos:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Paloma Manzanares:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Mónica Gandía:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be avalible through the repository DIGITAL CSIC (doi: http://hdl.handle.net/10261/352168)

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