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DOCTORAL THESIS / TESIS DOCTORAL

BIOTECHNOLOGICAL PRODUCTION AND UTILIZATION OF NEW ANTIFUNGAL PROTEINS FROM FILAMENTOUS FUNGI

PRODUCCIÓN Y UTILIZACIÓN BIOTECNOLÓGICA DE NUEVAS PROTEÍNAS ANTIFÚNGICAS DE HONGOS FILAMENTOSOS

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CERTIFICAN: Que Dña. Sandra Mª Garrigues Cubells, Licenciada en Biotecnología por la Universidad Politécnica de Valencia, ha realizado bajo su dirección el trabajo de tesis titulado: "Producción y utilización biotecnológica de nuevas proteínas antifúngicas de hongos filamentosos" que presenta para optar al grado de Doctor.

Y para que así conste a los efectos oportunos, firman el presente certificado en Paterna, a 4 de Septiembre de 2018.

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“What we learn with pleasure, we never forget”
Alfred Mercier (1816-1894), American poet.

SUMMARY-RESUMEN-RESUM

SUMMARY

Antimicrobial peptides (AMPs) are promising antifungal alternatives to the fungicides used in agriculture. However, the high cost of chemical synthesis and the difficulties of large-scale biotechnological production have limited their application. Antifungal proteins (AFPs) are a group of natural, small, cationic, secreted, cysteine-rich AMPs that offer a great potential to develop new biomolecules for the control of phytopathogenic fungi. AFPs are naturally present in filamentous fungi, are very stable, and can be produced in large amounts. However, the biological role of these AFPs in their producer fungus is still unclear. In this PhD thesis, we first studied the diversity of AFPs in ascomycetous genomes and proposed a new classification in three different classes (A, B and C). *Penicillium digitatum* is the main citrus postharvest pathogen and encodes only one AFP from class B in its genome (AfpB), while *Penicillium expansum* is the main pome postharvest pathogen and encodes one AFP from each class (AfpA, AfpB and AfpC). We report in this work the first time identification, efficient biotechnological production and characterization of these four AFPs.

We characterized the biological role of the *afpB* gene in *P. digitatum* by the study of its gene expression pattern and the generation of null Δ*afpB* and constitutive expression (*afpB*^{C}) mutants. Results indicated that *afpB* is dispensable for the biology and life cycle of this fungus, although expression of the *afpB* gene under the constitutive *Aspergillus nidulans gpdA* promoter is detrimental to growth and virulence to citrus. Surprisingly, neither the wild type nor the constitutive strains produced detectable amounts of AfpB in spite of the high *afpB* gene expression. Molecular modeling and rational design allowed us to predict the AfpB tertiary structure and design synthetic cysteine-containing peptides to map antifungal motifs within the AfpB primary sequence. We confirmed that the cationic exposed loops L2 and L3 showed moderate antifungal activity and that they can act synergistically.

With the objective of the biotechnological production of AfpB, we used an AFP expression cassette based on the promoter and terminator regions of the well-studied *paf* gene from *Penicillium chrysogenum*, which naturally produces high amounts of its own protein PAF. This *paf* cassette

worked efficiently in *P. digitatum* and allowed the homologous production of AfpB. The data also showed that the signal peptide (SP) and pro-peptide sequences of the translated SP-Pro-AfpB do not determine protein production. We also demonstrated the extreme thermal stability and resistance to proteolytic cleavage of the *P. digitatum* AfpB, and provided data that suggest that tertiary structure is not required for antifungal activity.

Similar to *P. digitatum*, none of the three AFPs were detected in supernatants of cultures of *P. expansum* in rich medium. By contrast, the AfpA was produced with very high yields in *P. expansum* cultures in minimal medium at late times. To complete the repertoire of AFPs from *P. expansum* we produced the three AFPs from *P. expansum* (AfpA, AfpB and AfpC) in *P. chrysogenum* with the use of the above *paf* cassette. With this combined approach, the three *P. expansum* proteins were successfully produced, purified and characterized. None of the four AFPs produced in this work were cytotoxic against mammal erythrocytes. The *P. expansum* AfpA followed by the *P. digitatum* AfpB were the most active AFPs against filamentous fungi, including plant and human pathogens, mycotoxin-producer fungi, and their own producers, a feature that had not been previously described for AFPs. Moreover, AfpA from *P. expansum* and AfpB from *P. digitatum* protected against fungal infection caused by *Botrytis cinerea* in tomato plants, and additionally the *P. expansum* AfpA protected against *P. digitatum* in orange fruits. These results confirm our previous hypothesis that AFPs are good candidates for the development of antifungals in plant protection and postharvest conservation, but also in clinic or food preservation.

RESUMEN

Los péptidos antimicrobianos (AMP) son una alternativa muy prometedora para el desarrollo de nuevos antifúngicos que puedan sustituir a los fungicidas utilizados en agricultura. Sin embargo, el alto coste de la síntesis química y las dificultades para su producción biotecnológica a gran escala han limitado su aplicación. Las proteínas antifúngicas (AFP) son un grupo de AMP naturales, de pequeño tamaño, catiónicas, secretadas y ricas en cisteína que ofrecen un gran potencial para desarrollar nuevas biomoléculas para el control de hongos fitopatógenos. Las AFPs están presentes de forma natural en hongos filamentosos, son muy estables y pueden producirse en grandes cantidades. Sin embargo, el papel biológico de estas AFPs en su hongo productor no se conoce en profundidad. En esta tesis doctoral, primero se estudió la diversidad de AFPs en genomas de hongos ascomicetos y se propuso una nueva clasificación en tres clases diferentes (A, B y C). *Penicillium digitatum* es el principal patógeno postcosecha de cítricos y codifica solo una AFP de la clase B en su genoma (AfpB), mientras que *Penicillium expansum*, el principal patógeno postcosecha de manzana, codifica en su genoma una AFP de cada clase (AfpA, AfpB y AfpC). En este trabajo describimos por primera vez la producción biotecnológica eficiente y la caracterización de estas cuatro AFPs.

Hemos caracterizado el papel biológico del gen *afpB* en *P. digitatum* mediante estudios de expresión génica y la generación de mutantes nulos (Δ*afpB*) y de expresión constitutiva (*afpB*^{C}). Los resultados indicaron que *afpB* es prescindible para la biología y el ciclo de vida de este hongo, aunque la expresión del gen *afpB* bajo el promotor constitutivo *gpdA* de *Aspergillus nidulans* es perjudicial para su crecimiento y virulencia sobre frutos cítricos. Sorprendentemente, ni la cepa parental ni las cepas constitutivas produjeron cantidades detectables de AfpB a pesar de la alta expresión de su gen codificante. El modelado molecular y el diseño racional nos permitieron predecir la estructura terciaria de AfpB y diseñar péptidos sintéticos ricos en cisteínas para mapear motivos antifúngicos dentro de la secuencia primaria de la proteína. Confirmamos que los bucles catiónicos expuestos L2 y L3 mostraron una actividad antifúngica moderada y que pueden actuar de forma sinérgica.

Resumen

Con el objetivo de producir AfpB mediante biotecnología, utilizamos un casete de expresión de AFPs basado en las regiones promotora y terminadora del gen *paf* de *Penicillium chrysogenum*, hongo que produce naturalmente grandes cantidades de su propia proteína PAF. Este casete funcionó de forma eficiente en *P. digitatum* y permitió la producción homóloga de AfpB. Los datos también mostraron que las secuencias del péptido señal (SP) y el pro-péptido de la SP-Pro-AfpB no determinan la producción de proteína. También demostramos la extrema estabilidad térmica y la resistencia a la proteólisis de la AfpB de *P. digitatum*, y aportamos datos que sugieren que la estructura secundaria no es necesaria para la actividad antifúngica.

De forma similar a lo descrito para *P. digitatum*, ninguna de las tres AFPs se detectó en los sobrenadantes de cultivo en medio rico de *P. expansum*. Por el contrario, AfpA se produjo en grandes cantidades en cultivos en medio mínimo de *P. expansum* a tiempos tardíos. Para completar el repertorio de AFPs, produjimos las tres AFPs de *P. expansum* (AfpA, AfpB y AfpC) en *P. chrysogenum* con el uso del casete *paf*. De esta forma, las tres proteínas de *P. expansum* se produjeron, purificaron y caracterizaron con éxito. Ninguna de las cuatro AFPs producidas en este trabajo fue citotóxica frente a eritrocitos de mamíferos. AfpA de *P. expansum* seguida de AfpB de *P. digitatum* fueron las AFPs más activas contra hongos filamentosos, incluyendo patógenos de plantas y humanos, hongos productores de micotoxinas y sus propios hongos productores, una característica que no se había descrito previamente para las AFPs. Además, la AfpA de *P. expansum* y la AfpB de *P. digitatum* protegieron frente a la infección causada por el hongo *Botrytis cinerea* en plantas de tomate, y adicionalmente la AfpA de *P. expansum* protegió frente a *P. digitatum* en frutos de naranja. Estos resultados confirman nuestra hipótesis de que las AFPs son buenas candidatas para el desarrollo de nuevos antifúngicos en protección vegetal y conservación postcosecha, pero también con aplicación en medicina o conservación de alimentos.

RESUM

Els pèptids antimicrobians (AMP) són una alternativa molt prometedora per al desenvolupament de nous antifúngics que puguen substituir als fungicides utilitzats en agricultura. No obstant això, l'alt cost de la síntesi química i les dificultats per a la seua producció biotecnològica a gran escala han limitat la seua aplicació. Les proteïnes antifúngiques (AFP) són un grup d'AMPs naturals, xicotetes, catiòniques, secretades i riques en cisteína que oferixen un gran potencial per a desenvolupar noves biomolécules per al control de fongs fitopatogens. Les AFPs estan presents de forma natural en fongs filamentosos, són molt estables i poden produir-se en grans quantitats. No obstant això, el paper biològic d'estes AFPs en el seu fong productor encara no està clar. En esta tesi doctoral, primer es va estudiar la diversitat d'AFPs en genomes de fongs ascomicets i es va proposar una nova classificació en tres classes diferents (A, B i C). *Penicillium digitatum*, el principal patogen postcollita de cítrics, codifica només una AFP de la classe B en el seu genoma (AfpB). *Penicillium expansum,* el principal patogen postcollita de poma, codifica en el seu genoma una AFP de cada classe (AfpA, AfpB i AfpC). En este treball presentem per primera vegada la producció biotecnològica eficient i la caracterització d'estes quatre AFPs.

Hem caracteritzat el paper biològic del gen *afpB* en *P. digitatum* mitjançant estudis d'expressió gènica i la generació de mutants nuls (Δ*afpB*) i d'expressió constitutiva (*afpB*^{C}). Els resultats van indicar que *afpB* és prescindible per a la biologia i el cicle de vida d'este fong, encara que l'expressió del gen *afpB* davall el promotor constitutiu *gpdA* d'*Aspergillus nidulans* és perjudicial per al seu creixement i virulència sobre fruits cítrics. Sorprenentment, ni el cep parental ni els ceps constitutius van produir quantitats detectables d'AfpB malgrat l'alta expressió del gen *afpB*. El modelatge molecular i el disseny racional ens van permetre predir l'estructura terciària d'AfpB i dissenyar pèptids sintètics rics en cisteína per a identificar motius antifúngics dins de la seqüència primària d'AfpB. Confirmarem que les estructures catiòniques L2 i L3 mostren activitat antifúngica moderada i que poden actuar de forma sinèrgica.

Amb l'objectiu de la producció biotecnològica d'AfpB, utilitzarem un cassette d'expressió d'AFPs basat en les regions promotora i terminadora

del gen *paf* de *Penicillium chrysogenum*, el qual produïx naturalment grans quantitats de la seua pròpia proteïna PAF. Este cassette va funcionar eficientment en *P. digitatum* i va permetre la producció homòloga d'AfpB. Les dades també van mostrar que les seqüències del pèptid señal (SP) i el propèptid de la SP-Pro-AfpB no determinaren la producció de proteïna. També demostrarem l'extrema estabilitat tèrmica i la resistència a l'acció proteolítica d'AfpB de *P. digitatum*, i proporcionem dades que suggerixen que l'estructura terciària no és necessària per a l'activitat antifúngica.

Semblant a *P. digitatum*, cap de les tres AFPs es van detectar en els sobrenadants de medi de cultiu ric de *P. expansum*. Al contrari, AfpA es va produir en grans quantitats en cultius de *P. expansum* en medi mínim a temps tardius. Per a completar el repertori d'AFPs, vam produir les tres AFPs de *P. expansum* (AfpA, AfpB i AfpC) en *P. chrysogenum* mitjançant l'ús del cassette *paf* anterior. D'esta manera, les tres proteïnes de *P. expansum* es van produir, purificar i caracteritzar amb èxit. Cap de les quatre AFPs produïdes en este treball va ser citotóxica front eritròcits de mamífers. AfpA de *P. expansum* seguida d'AfpB de *P. digitatum* van ser les AFPs més actives contra fongs filamentosos, incloent patògens de plantes i humans, fongs productors de micotoxines i els seus propis productors, una característica que no s'havia descrit prèviament per a les AFPs. A més, AfpA de *P. expansum* i AfpB de *P. digitatum* van protegir front la infecció causada pel fong *Botrytis cinerea* en plantes de tomaca, i addicionalment l'AfpA de *P. expansum* va protegir front *P. digitatum* en fruits de taronja. Estos resultats confirmen la nostra hipòtesi anterior de que les AFPs són bones candidates per al desenvolupament d'antifúngics en protecció vegetal i conservació postcollita, però també en medicina o conservació d'aliments.

ABBREVIATIONS

AFP: Antifungal Protein
AMP: Antimicrobial Peptide
ANOVA: Analysis Of Variance
ATMT: *Agrobacterium tumefaciens*-Mediated Transformation
BP: Bubble Protein
CFW: Calcofluor White
CPP: Cell-Penetrating Peptide
CRP: Cysteine-Rich Protein
CW: Cell Wall
DLP: Defensin-Like Protein
dpi: days post-infection/inoculation
ECD: Electronic Circular Dichroism
GPY: Glucose, Peptone, Yeast extract
GPYA: Glucose, Peptone, Yeast extract, Agar
GRAS: Generally Regarded As Safe
IMZ: Imazalil
LB: Luria Bertani medium
LTP: Lipid-Transfer Protein
MALDI-TOF: Matrix-Assisted Laser Desorption/ionization Time-of-Flight
MAPK: Mitogen Activated Protein Kinase
MIC: Minimal Inhibitory Concentration
MOPS: 3-(N-morpholino)-propanesulfonic acid
MS: Mass Spectrometry
MW: Molecular Weight
MWCO: Molecular Weight Cut-Off
NCBI: National Center for Biotechnology
ND: Not Determined
nd: not detected
NI: Not Inhibitory
OD: Optical Density
PBS: Phosphate Buffered Saline
PBG: Phosphate Buffer Glucose
PcMM: *Penicillium chrysogenum* Minimal Medium
PDA: Potato Dextrose Agar
PDB ID: Protein Data Bank Identifier
PDB: Potato Dextrose Broth
PdMM: *Penicillium digitatum* Minimal Medium
PFM: Peptide Mass Fingerprinting

Abbreviations

pI: Isoelectic point
PK: Protein Kinase
RBCs: Red Blood Cells
ROS: Reactive Oxygen Species
RP-HPLC: Reversed Phase High-Performance Liquid Chromatography
SD: Standard Deviation
SDS: Sodium Dodecyl Sulphate
SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SE: Standard Error
SP: Signal Peptide
SS: Signal Sequence
TBZ: Thiabendazole
TFA: Trifluoroacetic Acid
TFE: Trifluoroethanol
UTR: Untranslated Region
YPD: Yeast extract, Peptone, Dextrose

GENERAL INTRODUCTION

Fungal infections represent an important risk to human health, food production and security (Fisher et al., 2012). From many decades to now, fungal diseases have not received as much consideration as their bacterial counterparts, probably as a consequence of the higher impact of bacterial infections in human health. However, fungal infections should not be underestimated. The impact of fungal infections on humans varies from superficial infections that affect 25 % of the world population, to invasive infections with mortality rates up to 50 %. The importance of fungal diseases has increased due to the growing number of immunosuppressive therapies and immunocompromised patients, and the appearance of new pathogenic strains and resistances to commonly used antifungal drugs (Denning et al., 2011; Brown et al., 2012). Fungal diseases are also a challenge in agriculture, since fungi are the main pathogens of plant species that are grown for food production. In addition, fungal mycotoxins represent a major threat for food safety, as these compounds can be present in contaminated food and are dangerous to human health when ingested.

It is also important to highlight the role of fungi in agriculture. Phytopathogenic fungi can infect the plants and/or the fruits in the field, the packing house, and during their distribution and commercialization, resulting in large economic losses worldwide (Palou et al., 2016). Chemical fungicides are the main method for the control of plant disease during cultivation and postharvest conservation. Therefore, the global market for fungicides and antifungals is in continuous growth. But despite the enormous impact on human health and food supply, very few antifungal compounds have been introduced into the market in the last thirty years. Only three different classes of antifungal molecules are currently in use, acting mainly on the biosynthesis of ergosterol, cell wall compounds, and the integrity of the plasma membrane (Brown et al., 2012; Campoy and Adrio, 2017). Very few chemical fungicides with a limited spectrum of action are being massively used, favouring the appearance of resistant strains. Furthermore, the emergence of cross-resistance caused by fungicides used in agriculture has been described in medicine, which is a major medium-term problem for the control of diseases in agriculture. For these reasons, there is an urgent need to find new antifungal molecules, and if possible with modes of action different from those already known.

1. Phytopathogenic fungi and their relevance in agriculture

Plant pathogens, mainly phytopathogenic fungi, cause disease and postharvest decay and are responsible for major losses in crop production, and for the contamination of food through the production of toxins that are detrimental to human health. There is a great variety of fungi that may cause pre- and/or post-harvest diseases, but only a few of them have been deeply characterized due to their economic or scientific relevance, or the availability of suitable experimental tools (Dean et al., 2012).

One of the main plant-pathogenic fungi is the ascomycete *Magnaporthe oryzae,* which is the causal agent of the most destructive disease of rice, the blast disease. Rice is the most important food crop of the developing world and the staple food of more than half of the world's population. This fungus kills the plant by infecting the leaves through a specialized penetration cell called appressorium. The economic importance of this fungus led it to occupy the first place among the 10 most important phytopathogenic fungi to date (Dean et al., 2012). Another important plant pathogen is *Botrytis cinerea*, which is one of the most polyphagous fungi and the causal agent of the grey mould disease in more than 200 plant species, including vegetative organs and also many fruits during postharvest storage and commercialization (Williamson et al., 2007). The costs derived from *Botrytis* damage are very difficult to estimate because of its broad host range. *Fusarium oxysporum,* on the other hand*,* is a ubiquitous soil-borne pathogen that usually infects through the root system and causes vascular wilt on a wide range of plants*. F. oxysporum* species comprises different *formae speciales* (f. sp.), which collectively infect more than 100 different hosts, such as melon, tomato, cotton and banana (Michielse and Rep, 2009). *F. oxysporum* is also an emerging human pathogen that can cause invasive infections in immunocompromised patients (O'Donnell et al., 2004; Nucci and Anaissie, 2007).

Other species of phytopathogenic fungi that deserve special mention are the fruit postharvest pathogens *Penicillium italicum, Penicillium digitatum,* and *Penicillium expansum* (see sections below), *Puccinia* spp., *Colletotrichum* spp. *Phytophthora citrophthora, Rhizhopus stolonifer, Geotrichum candidum, Ustilago maydis,* and *Alternaria citri,* among others (Barkai-Golan, 2001; Dean et al., 2012).

1.1 *Penicillium digitatum*

The ascomycete *P. digitatum* is the main postharvest pathogen of citrus, the most important fruit tree in the world. It is a necrotrophic filamentous fungus that produces the green mould disease by penetrating and infecting citrus fruits through pre-existing wounds on the fruit peel, developing white mycelia and green conidia that completely cover and decay the fruit (Tuset, 1987; Palou, 2014). An important aspect of *P. digitatum* is its host specificity, since this fungus can only infect citrus fruits. *P. digitatum* together with *P. italicum,* are the main responsible agents of postharvest citrus losses worldwide, since more than 90 % of citrus rots are produced by these two species. Only in Spain, annual production of citrus fruits is around five to six million tons, from which approximately up to 10 % of the total production can be lost due to infections caused mainly by *P. digitatum* (http://faostat3.fao.org). Currently, imazalil is the most commonly applied postharvest fungicide by the citrus industry worldwide. Other chemical fungicides used for the control of *P. digitatum* are in order of relevance thiabendazole, sodium ortho-phenylphenate, or fludioxonil, among others (Palou, 2014). However, the continuous and sometimes incorrect use of these chemical fungicides often result in the proliferation of resistant strains (Holmes and Eckert, 1999; Kinay et al., 2007; Pérez-Nadales and di Pietro, 2011).

The economic interest associated with *P. digitatum* has driven the development of genetic and molecular tools to characterize the fruit-pathogen interactions and pathogenicity (Ballester et al., 2011; Buron-Moles et al., 2012; Wang et al., 2012), although the mechanisms by which this fungus infects citrus fruits are not completely understood (Zhang et al., 2013a; Zhang et al., 2013b).

The availability of the *P. digitatum* genome opens up new possibilities for the study of its mechanisms of virulence and pathogenicity, and allows the search of new compounds of interest. Three different isolates of *P. digitatum* have been sequenced: the wild-type CECT 20796 strain isolated from an infected orange in Spain, in which our group has collaborated (Marcet-Houben et al., 2012), the thiabendazole- and imazalil-resistant strain Pd1 isolated from grapefruit (Marcet-Houben et al., 2012), and the imazalil-resistant Pd01 strain from China (Sun et al., 2013).

Analyses of synteny among genomes indicated that the two fungicide-resistant strains (the Spanish Pd1 and the Chinese Pd01) showed better genomic synteny between them than with the wild-type Spanish CECT 20796 (Sun et al., 2013). It should be noted that throughout this PhD work, all studies regarding *P. digitatum* have been performed with the wild-type strain CECT 20796 (isolate PHI26).

1.2 *Penicillium expansum*

A second filamentous fungus that deserves special mention in this thesis is another postharvest pathogen from the genus *Penicillium. P. expansum* is a ubiquitous fungus and the causal agent of the blue mould disease, which is considered one of the most important postharvest rots of pome fruits (Wayne et al., 2011; Ballester et al., 2015). Like *P. digitatum, P. expansum* also penetrates the fruit through damaged skin, producing tissue maceration and developing blue conidia that completely cover and decay the fruit. In contrast to the strict host specificity of *P. digitatum*, *P. expansum* has a wide range of hosts, including apples, pears, peaches, nectarines and carrots, among others (Errampalli, 2014). Although contamination of *P. expansum* can occur before harvest, it is commonly found during postharvest and in storage. Postharvest losses of pome crops in Spain caused by fungal decay, mainly from *P. expansum* followed by *B. cinerea,* have been estimated to cost eight million dollars per year (Viñas et al., 1998). As occurs with *P. digitatum* and *P. italicum*, chemical fungicides are the main method for controlling fruit decay caused by *P. expansum.* Benzimidazole fungicides, especially thiabendazole with or without the presence of diphenylamine, are the most widespread fungicides used to control blue mould in apples. However, its extensive application has again led to the appearance of resistant isolates (Errampalli et al., 2006; Morales et al., 2010). Other fungicides, like imazalil and benomyl, can also be used to control postharvest blue mould in apples.

P. expansum is reported to produce a wide array of secondary metabolites, including mycotoxins. Therefore*, P. expansum* is also of great concern to fruit processing industries due to its ability to produce citrinin, ochratoxin A, and patulin, which are highly toxic mycotoxins that can contaminate infected fruits and their derived products (Morales et al., 2010; Ballester et al., 2015).

Given its economic importance, three different strains of *P. expansum* have been sequenced: the Israelite strain Pd1, the Spanish CMP-1, and the American MD-8 (Ballester et al., 2015). All sequenced strains were reported to have approximately 32 Mb genome with around 11,000 predicted protein-coding genes. The availability of these genomes will provide information on the genomic basis of the production of secondary metabolites in *P. expansum,* as well as genomic basis of its virulence and pathogenicity. Throughout this work, the Spanish CMP-1 strain of *P. expansum* was chosen, together with *P. digitatum* PHI26, to carry out the objectives proposed in the present doctoral thesis.

2. Antimicrobial peptides as alternatives for the control of fungal pathogens

Antimicrobial peptides (AMPs) are a broad class of peptides and small proteins with direct killing activity. Generally, AMPs can be classified as antibacterial or antifungal, even though some of them show both activities in different degrees and selectivity. AMPs are widespread in nature and are produced by organisms all along the phylogenetic scale from bacteria, fungi, plants, and insects to more complex animal species like amphibians and mammals, including humans (Zasloff, 2002; Brogden et al., 2005; Hancock and Sahl, 2006). AMPs are evolutionary selected from ancient systems of competition for nutrients or protection/attack against invaders. Depending on each AMP, they have different target organisms ranging from viruses to parasites.

Despite the enormous diversity, AMPs share common biophysical properties. AMPs are gene-encoded and most of them derive from precursor peptides through subsequent proteolytic activation steps. They are short (10-60 amino acid residues), usually cationic due to the presence of arginine and lysine residues, and with a substantial proportion of hydrophobic residues, but their sequences and secondary structures vary widely (Brogden, 2005). The most prevalent structural families of AMPs are: (a) linear α-helical peptides, such as human LL-37, amphibian magainins, insect cecropins and mellitin; (b) small cysteine-rich proteins (CRPs) with multiple disulphide bridges and β-sheet structures, such as the defensins from plants, insects and mammals, and defensin-like antifungal proteins of fungal origin; (c) extended structures rich in specific amino acids as glycine, proline, tryptophan, arginine and/or histidine, such as bovine

indolicidin or human histatine; and (d) loop peptides with one disulfide bridge, for example bovine bactenecin and lactoferricin. Examples of the structures of some of these AMPs are shown in Figure 1.

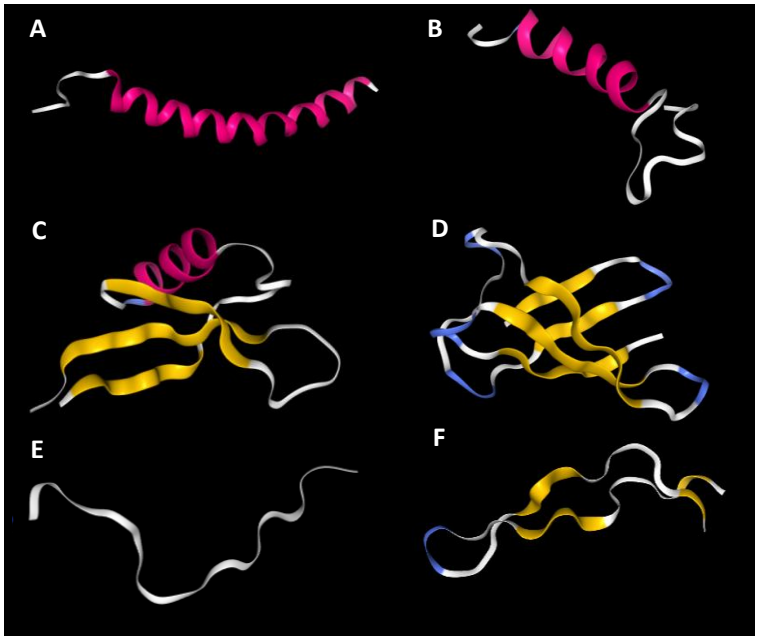

Figure 1: Three-dimensional structures of representative AMPs of natural origin. (A) and (B) represent human LL-37 (Protein Data Bank ID 2K6O) and insect cecropin (PDB ID 2N92), respectively, both belonging to the linear, α-helical peptides family. (C) and (D) represent the cysteine-rich defensin PsDef1 (PDB ID 5NCE) and defensin-like antifungal protein PAF (PDB ID 2MHV), respectively. (E) represents the tryptophan-rich peptide indolicidin (PDB ID 1G89), and (F) the bovine lactoferricin (PDB ID 1LFC).

AMPs have been proposed as promising candidates for the development of novel antimicrobial compounds and therapies due to several factors. In clinical use, they can fight the alarming increase of drug-resistant microbes and help in the treatment of immunocompromised patients (Yeung et al., 2011; Mandal et al., 2014). The use of AMPs in crop protection has been recognized as an alternative to chemical fungicides and antibiotics that have led to an increase of resistant strains, and are hazardous to the environment, harmful to plant photosynthesis, and toxic for human consumers (Rao, 1995; Montesinos, 2007; Marcos et al., 2008; Petit et al., 2012). More importantly, studies show that AMPs have a variety of mechanisms of action that are different from those of existing

antimicrobial compounds, which reinforces the enormous potential that AMPs have as alternative to conventional antibiotics. Currently, there are thousands of AMPs with demonstrated antimicrobial activity (Zhao et al., 2013) and the number is continuously increasing.

Paradoxically, very few peptides have reached the market or are in advanced clinical trial phases (Haney and Hancock, 2013; Rautenbach et al., 2016). An example is the antifungal P113 derived from human histatin 5 (Rothstein et al., 2001), an antifungal tested against oral candidiasis (Table 1). The bacteriocin nisin, a 34-amino acid peptide produced by food-grade lactic acid bacteria, is an example of AMP whose commercial use has been approved for dairy products and canned goods such as vegetables and soups (Cotter et al., 2005). However, despite nearly two decades of serious efforts to exploit the advantageous features of AMPs, there are still important challenges to be solved for their application. Among limitations of natural AMPs are the limited production yield, low bioavailability, unspecific toxicity, and their potential lability to proteases and harsh environmental conditions.

2.1 Antifungal Peptides and Proteins

Over the years, most of the efforts in the study and identification of AMPs have focused on peptides showing antibacterial activity due to their relevance and potential application in human diseases, which are mainly caused by bacteria and viruses (Marcos et al., 2012; van der Weerden et al., 2013). However, the growing importance of fungal diseases in clinic and the need for new antifungals in agriculture has led to an increase in the number of peptides and proteins with demonstrated antifungal activity, which share a common cationic and amphipathic character. Examples of short and linear naturally occurring AMPs with antifungal activity include cecropins and melittin from insects, magainins from amphibians, or indolicidins from mammals (Table 1).

Novel synthetic antifungal peptides with improved properties have been produced through rational design and combinatorial approaches, including fusions of antimicrobial fragments into peptide hybrids, sequence point modifications, *de novo* rational design based on known AMPs, the identification of minimal motifs that retain full activity, or the incorporation of non-natural amino acids or derivatizations (Marcos et al. 2008) (Table 1).

Peptide synthesis also facilitates the design and testing of shorter versions of natural AMPs and proteins in order to define the minimal bioactive motifs, as was demonstrated in the peptides P113 from human histatin 5 (Rothstein et al., 2001), $LfcinB_{17-31}$, and $LfcinB_{20-25}$ from bovine lactoferrin (Muñoz and Marcos, 2006).

Combinatorial peptide chemistry has allowed the preparation of synthetic libraries and led to the identification of the hexapeptide PAF26 as a specific inhibitor of filamentous fungi including the postharvest pathogen *Penicillium digitatum* with a MIC of 8 µM (López-García et al., 2002), and other synthetic peptides with demonstrated antifungal activity (Table 1).

Table 1: Representative AMPs with activity against fungal phytopathogens.

Peptide	Amino acid sequence*	Origin	References
Cecropin A	KWKLFKKIEKVGDNIRDGIIKAGPAVAVVG QATQIAK	Insect	(De Lucca et al., 1997)
Melittin	GIGAVLKVLTTGLPALISSWIKRKRQQ	Insect	(Muñoz et al., 2006)
Magainin 2	GIGKFLHSAKKFGKAFVALKAL	Amphibian	(Gesell et al., 1997)
Indolicidin	Ac-ILPWKWPWWPWRR-NH_2	Bovine	(Muñoz et al., 2007b)
$LfcinB_{17-31}$	Ac-FKCRRWQWRMKKLGA-NH_2	Bovine (fragment of Lactoferrin)	(Muñoz and Marcos, 2006)
P113	AKRHHGYKRKFH	Human (fragment of Histatin 5)	(López-García et al., 2015)
BM0	Ac-RFWWFRRR-NH_2	Combinatorial library	(Monk et al., 2005) (Muñoz et al., 2007b)
PAF26	RKKWFW	Combinatorial library	(Muñoz et al., 2007b)
PAF102	GHRKKWFWAGPARRKKWFWAGPAWRK KWFW	Rational design (based on PAF26)	(López-García et al., 2015)
BP22	Ts-FKLFKKILKVL-NH_2	Rational design	(Badosa et al., 2009)
BP15	KKLFKKILKVL-NH_2	Rational design	(Puig et al., 2016)
D4E1	FKLRAKIKVRLRAKIKL	Rational design	(Cary et al., 2000)

* Ac: acetylated; NH_2: amilated; Ts: Tosylated

2.2 Cysteine-Rich Proteins

A class of peptides that is of particular interest for their biotechnological applications in multiple fields, including agriculture, is the small cysteine-rich proteins (CRPs). These are naturally-produced peptides and small proteins containing multiple cysteine residues that form disulfide bonds and fold into compact structures, conferring a high degree of stability

against adverse biochemical and biophysical conditions. Several groups of CRPs have been reported to play a defense role within their producing organisms and to have specific antimicrobial activity, while others would also have regulatory functions (Ganz, 2003; van der Weerden et al., 2013; Marmiroli and Maestri, 2014). Among cysteine-rich AMPs, we can find the fungal plectasin, which is a 40 amino acid peptide reported to be highly effective against bacteria (Mygind et al., 2005). Other relevant CRPs are the plant thionins, which are around 45 amino acids in length containing 2, 3 or 4 disulphide bonds, and have been reported to show both antibacterial and antifungal activities in different degrees of selectivity (García-Olmedo et al., 1998). Another important cysteine-rich protein family is the plant Lipid-Transfer Proteins (LTPs), which are 9-10 kDa proteins with antimicrobial activity, mainly antifungal (Kader, 1996; Regente and de la Canal, 2000).

Defensins and defensin-like proteins form by far the largest family of CRPs with antimicrobial activity. Plant defensins compose a numerous group of small cationic CRPs (45-54 amino acids in length) that typically include eight cysteines and four intramolecular disulfide bonds (van der Weerden et al., 2013). They are found ubiquitously throughout the plant kingdom as part of the innate immunity against microbial infections, mostly fungal (Carvalho and Gomes, 2009; Sagaram et al., 2011). Plant defensins were first identified in the seeds of wheat, but they can be constitutively expressed in both plant storage and reproductive organs, and also induced locally as well as systemically during plant defense responses. Plant defensins are structurally and functionally related to the previously characterized defensins found in insects and mammals (Carvalho and Gomes, 2009; van der Weerden et al., 2013; Vriens et al., 2014) (Figure 2). Examples of plant defensins with antifungal activity include RsAFP1 and RsAFP2 from *Raphanus sativus*, MsDef1 and MtDef4 from *Medicago* spp., Dm-AMP1 and Dm-AMP2 from *Dahlia merckii*, Psd1 from *Pisum sativum* or NaD1 from *Nicotiana alata*. Plant defensins are very active against phytopathogenic filamentous fungi, with MIC values as low as 1-8 µM (Terras et al., 1993; Sagaram et al., 2011; Bleackley et al., 2016).

The polypeptide precursor of plant defensins contains a signal peptide (SP) that targets the protein to the extracellular space, a pro-peptide that blocks the activity of the mature peptide until necessary, and

the mature protein (Carvalho and Gomes, 2009). The structure of plant defensins typically comprises a cysteine-stabilized αβ-motif (CSαβ) with a prominent α-helix and a triple-stranded antiparallel β-sheet (Figure 2B). Additionally, a three-dimensional peptide signature called the ɣ-core is present in virtually all defensins and other antimicrobial CRPs. This ɣ-core includes two cysteines in the conserved motif Gly-X-Cys-X_{3-9}-Cys, either in dextromeric or levomeric isoform (Yount and Yeaman, 2004). The antimicrobial activity of synthetic peptides comprising this motif has been demonstrated for some plant defensins. This is the case, for example, of the ɣ-core present in MtDef4 from *Medicago truncatula* (Sagaram et al., 2011) or BhDef2 from *Brassica hybrid* (Kaewklom et al., 2016), and is related to its cationic net charge. However, other ɣ-core peptides do not show any antimicrobial activity (Sagaram et al., 2011), suggesting that the ɣ-core serves as a structural scaffold to which other antimicrobial determinants (e.g., β-sheets) are attached (Yount and Yeaman, 2004).

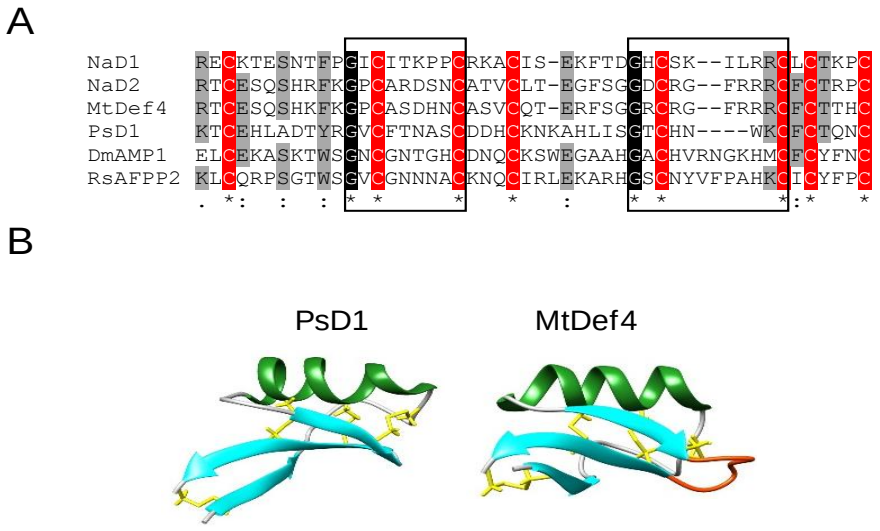

Figure 2: Amino acid sequence and structure of selected plant defensins. (A) Sequence alignment of the plant defensins NaD1 and NaD2 from *N. alata*; MtDef4 from *M. truncatula*; PsD1 from *P. sativum*; DmAMP1 from *D. merckii*; and RsAFP2 from *R. sativus*. The ɣ-core motif is boxed. Highly conserved cysteine and glicine residues are highlighted in red and black, respectively. Other less conserved amino acids are shadowed in grey. (B) Visualization of the protein structure of the plant defensins PsD1 from *P. sativum* (PDB ID 1JKZ,) and MtDef4 from *M. truncatula* (PDB ID 2LR3).

It is interesting to note that distinct but sequence-related plant defensins may have very different antifungal potency and/or specificity for fungi, as shown for the NaD1 and NaD2 from *Nicotiana* (Bleackley et al., 2016), or the *Medicago* MsDef1 and MtDef4 (Sagaram et al., 2011). This indirectly indicates that subtle amino acid changes could alter the antimicrobial properties of the protein and points towards the application of rational design approaches, similar to those used in shorter peptides, to improve the antifungal efficacy of defensins. In fact, point mutations and exchange of motifs between *Medicago* defensins result in modulation of activity and exchange of properties (Sagaram et al., 2011). A very significant improvement in antifungal activity was observed through the exchange of motifs between NaD1 and NaD2, with a resulting inhibitory concentration below the micromolar range, lower than any of the two parental proteins (Bleackley et al., 2016).

2.3 Fungal Antifungal Proteins

Another defensin-like CRP group of interest comprises the antifungal proteins of fungal origin (AFPs). AFPs are small (45-64 amino acid residues) basic proteins that are abundantly produced and secreted to the culture medium by filamentous ascomycetes, initially identified in the genera *Aspergillus* and *Penicillium* (Galgóczy et al., 2010; Tu et al., 2016). They exhibit antifungal activity at low micromolar concentrations (Hegedüs and Marx, 2013; Dos Reis Almeida et al., 2014). The first AFP was identified in *Aspergillus giganteus*. This fungus produces high amounts of two extracellular small proteins when incubated for long periods (>72 h) (Olson and Goerner, 1965; Martínez-Ruiz et al., 1997). First, the larger one (17 kDa) was characterized as α-sarcin, the first member of a class of selective ribotoxin proteins that target the ribosome and have broad biotechnological applications (Olson and Goerner, 1965; Olombrada et al., 2017). The “contaminant” second one (6 kDa) took more than twenty years to be identified as the first fungal AFP, naming a new class of cysteine-rich antifungal proteins (Nakaya et al., 1990; Wnendt et al., 1994; Lacadena et al., 1995).

The second identified AFP was the so-called PAF protein in a screen to select proteins abundantly secreted by the GRAS fungus *Penicillium chrysogenum* (Marx et al., 1995). This protein was initially

identified as a small secreted protein of an apparent molecular mass of 12 kDa in an attempt to find regulatory sequences that would work efficiently for protein production in *P. chrysogenum.* Protein PAF from *P. chrysogenum* together with AFP from *A. giganteus* are the most studied AFPs to date and both are abundantly secreted by their producer fungus with yields above 50 mg/L of culture medium. Additional representative AFPs are AnAFP from *Aspergillus niger* (Gun et al., 1999), AcAFP from *Aspergillus clavatus* (Skouri-Gargouri and Gargouri, 2008), the bubble protein (BP) from *Penicillium brevicompactum* (Seibold et al., 2011), or NFAP from *Neosartorya fischeri* (Kovacs et al., 2011), among others (Table 2). Several of these proteins have activity towards fungal plant pathogens, with inhibitory values in the low micromolar range depending on the fungus (Gun et al., 1999; Vila et al., 2001; Kaiserer et al., 2003; Skouri-Gargouri and Gargouri, 2008), and they were not toxic to plant or mammalian cells (Moreno et al., 2006; Hegedüs and Marx, 2013).

Table 2: Representative AFPs with demonstrated antifungal activity.

AFPs	Mature amino acid sequence	Origin	References
AFP	ATYNGKCYKKDNICKYKAQSGKTAICK CYVKKCPRDGAKCEFDSYKGKCYC	*A. giganteus*	(Lacadena et al., 1995)
PAF	AKYTGKCTKSKNECKYKNDAGKDTFIK CPKFDNKKCTKDNNKCTVDTYNNAVD CD	*P. chrysogenum*	(Marx et al., 1995)
PgAFP	LSKFGGECSLKHNTCTYLKGGKNHVVN CGSAANKKCKSDRHHCEYDEHHKRVD CQTPV	*P. chrysogenum*	(Delgado et al., 2015)
NFAP	LEYKGECFTKDNTCKYKIDGKTYLAK CPSAANTKCEKDGNKCTYDSYNRKVK CDFRH	*N. fischeri*	(Kovacs et al., 2011)
AcAFP	ATYDGKCYKKDNICKYKAQSGKTAICK CYVKVCPRDGAKCEFDSYKGKCYC	*A. clavatus*	(Skouri-Gargouri et al., 2009)
BP	DTCGSGYNVDQRRTNSGCKAGNGDR HFCGCDRTGVVECKGGKWTEVQDCG SSSCKGTSNGGATC	*P. brevicompactum*	(Seibold et al., 2011)
Anisin1	SSCQLGGIFGAGDAACSASCIRAGTYH GGYCNDKQVCICTH	*Aspergillus nidulans*	(Eigentler et al., 2012)
AnAFP	LSKYGGECSLEHNTCTYRKDGKNHVVS CPSAANLRCKTDRHHCEYDDHHRTVD CQTPV	*Aspergillus niger*	(Gun et al., 1999)
Pc-Arctin	DTCGGGYGVDQRRTNSPCQASNGDR HFCGCDRRGIVECKGGKWTEIQDCGG ASCRGVSQGGARC	*P. chrysogenum*	(Chen et al., 2013)

Previously to this thesis work, fungal AFPs had been classified in two separated groups based on phylogenetic analyses: the PAF-cluster (proteins homologous to PAF from *P. chrysogenum*) and the BP-cluster (proteins homologous to the BP from *P. brevicompactum*) (Seibold et al., 2011; Galgóczy et al., 2013b).

Similar to plant defensins, AFPs are secreted proteins, contain the conserved ɣ-core motif, and have to be processed in order to release the mature protein with antifungal activity, even though the functional role of the pro-sequence is not well understood. However, the structure of AFPs differs from the structure of classical defensin-like proteins. AFPs fold into five β-strands forming two packed β-sheets that share a common interface (Figure 3), and have typically six to eight cysteine residues forming three/four disulfide bonds (Campos-Olivas et al., 1995; Batta et al., 2009).

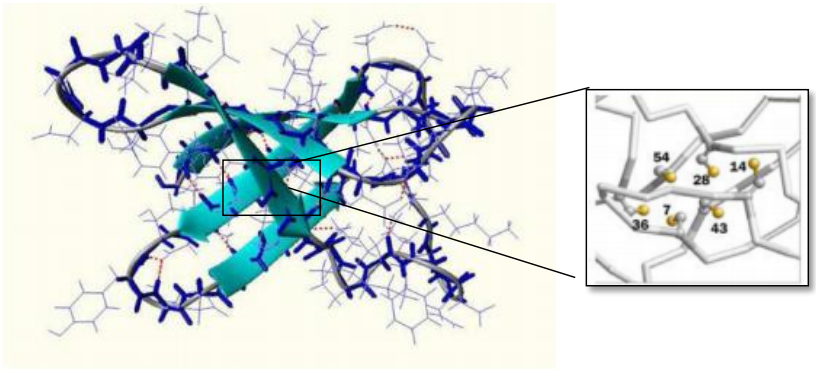

Figure 3: Three-dimensional structure of *P. chrysogenum* PAF. The five conserved β-sheets are shown in light blue. The congested hydrophobic core with the cysteine residues labelled is zoomed. Adapted from (Batta et al., 2009)

The biological role of AFPs in the producer fungus is still unclear. A proposed function related to the competition for nutrients is obvious from their direct killing activity, although no functional experimental evidence is available for this. Null mutation of the *paf* gene in *P. chrysogenum* reduces the production of asexual spores and affects negatively the expression of genes that regulate asexual development, suggesting that AFPs could play a role in conidiogenesis (Hegedüs et al., 2011). A recent *in silico* meta-analysis of the publicly available transcriptomic data in *A*. *niger* explored

the co-expression of the *afp* gene under many different growth conditions and mutants, and suggested novel functions for the AnAFP produced by this fungus in nutrient recycling during starvation, autophagy, and development (Paege et al., 2016). This and other observations led to propose that AFPs might function as cannibal toxins in fungi similar to the phenomenon of cannibalism described for bacteria, in which the sub-population of *Bacillus* cells that enters the sporulation program secretes proteinaceous toxins to kill the non-sporulated cells and recycle their nutrients (Meyer and Jung, 2018). More experimental evidence is needed to elucidate the functional role of AFPs beyond their antifungal activity**.**

As demonstrated in the case of plant defensins, the small size of AFPs make these proteins suitable to rationally design and engineer amino acid substitutions with an impact on their activity, which have been used to study structure-activity relationships (Batta et al., 2009; Sonderegger et al., 2016; Sonderegger et al., 2017). In addition, AFPs can be easily and efficiently produced by biotechnology, which represents a great advantage compared to synthetic peptides in terms of cost and production yields (see below).

3. Modes of Antifungal Action

The study of the mode of action of AMPs is essential for their future development and application. The mode of action of AMPs has been reviewed extensively, especially in the case of antibacterial peptides (Brogden, 2005; Nicolas, 2009; Nguyen et al., 2011) but also in the more specific antifungal peptides (Marcos and Gandía, 2009; Rautenbach et al., 2016). The hexapeptide PAF26 (Table 1) has been proposed by our group as a model to investigate the mode of action and specificity of antifungal peptides by using the fungi *P. digitatum* and the model *Neurospora crassa* as well as the yeast *S. cerevisiae* (Muñoz et al., 2013).

The first step in the AMP mode of action is the physical interaction with the outer structures that surround microbial cells. The cationic properties of many AMPs enable an electrostatic attraction to the negatively charged microbial envelopes (Zasloff, 2002). However, there are examples of AMPs whose interaction and binding with microbes do not rely primarily on electrostatic attraction and is aided by specific components.

The cell wall (CW) and membrane of fungi have specific compositions, different from other eukaryotic cells, and these have been long recognized as targets for antifungal molecules (Munro, 2013). The fungal CW is composed of a highly glycosylated cover of mannoproteins, a thick intermediate layer of β-glucans, and a thin layer of chitin, after which the lipid bilayer of the cell membrane stands as the barrier to the cell interior (Figure 4). The enzymatic degradation of CW reduces the sensitivity of fungal cells to the defensin NaD1 and the synthetic PAF26 (van der Weerden et al., 2010; Harries et al., 2013), demonstrating that an intact CW is required for peptide interaction and activity. Reinforcement of CW is a defensive countermeasure of fungal cells exposed to antifungal peptides and proteins (López-García et al., 2010a; Ouedraogo et al., 2011). Mannosylated proteins and CW composition regulate the sensitivity to the antifungal plant osmotin (Coca et al., 2000; Ibeas et al., 2001). *S. cerevisiae* genes involved in protein glycosylation of CW mannoproteins also determine the interaction and internalization of PAF26 against the yeast (Harries et al., 2013), in a demonstration that the fungal CW is required for internalization of the peptide. These studies were partly extended to *P. digitatum* with the demonstration that mutants in the protein glycosylation gene *pmt2* were selectively more tolerant to PAF26 (Harries et al., 2015).

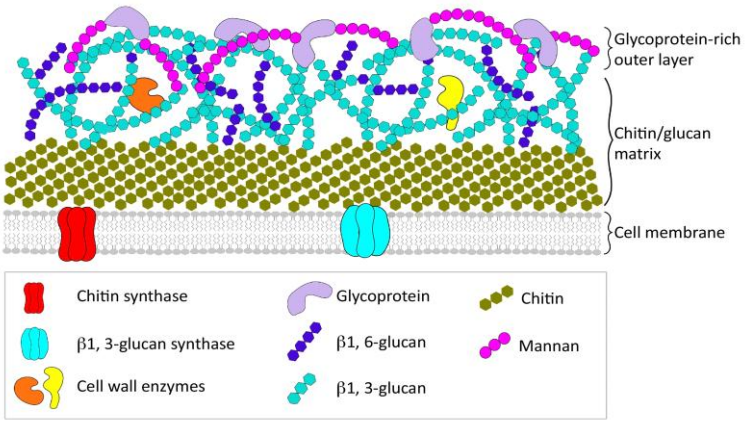

Figure 4: Basic structure and composition of the fungal cell wall (CW). The inner CW is a chitin-glucan-rich interconnected matrix. The outer layer of the wall is rich in mannosylated glycoproteins (Geoghegan et al., 2017).

Once AMPs diffuse through the CW, they find the cell membrane. Different membrane glycosphingolipids and glycerophospholipids from fungi have been shown to determine the interaction and activity of antifungal peptides and defensins (Wilmes et al., 2011; Vriens et al., 2014; Rautenbach et al., 2016).

AMPs can be membrane disruptive or be transported into the cell in a non-disruptive manner. The cationic and amphipathic character of most AMPs would allow the interaction, insertion and disruption of lipid cell membranes causing their rupture, loss of biophysical properties and the cell killing through a lytic mechanism (Brogden, 2005; Nguyen et al., 2011) (Guilhelmelli et al., 2013). However, it must be considered that peptides that mostly act through a membrane disrupting mechanism are usually highly lytic and toxic to different cell types and organisms (Rautenbach et al., 2016). Thus, AMPs with non-lytic mechanisms and specific modes of action and selectivity towards the targeted microorganism are preferred as lead compounds for future development (Marcos and Gandía, 2009). Most AMPs show sequence and biophysical similarities with the so-called cell-penetrating peptides (CPPs), which are able to internalize in a non-disruptive manner (Henriques et al., 2006; Marcos and Gandía, 2009; Nicolas, 2009; Marcos et al., 2012). CPPs are cationic and amphipathic, and have two general pathways to penetrate into the cells: direct translocation/diffusion or endocytosis. Both pathways can act simultaneously for the same peptide, being concentration-dependent (Muñoz et al., 2012; Guidotti et al., 2017). After translocation into the cell interior, these peptides would be able to bind to inner targets and interfere functions inside the cell.

PAF26 is internalized by conidia and mycelium of *P. digitatum* at sub-inhibitory concentrations in a mechanism that preceded any cell lytic effect (Muñoz et al., 2006). In the model fungus *N. crassa*, PAF26 is internalized inside the cells following two different pathways: an energy-dependent endocytic-like mechanism at low peptide concentrations, and an energy-independent translocation at high concentrations (Marcos et al., 2012). PAF26 is localized first at the outer CW; from where it is later observed inside small endocytic vesicles that coalesce and grow with time to fill large central vacuoles, and the intracellular killing correlates with the

active extrusion of the peptide from the vacuoles to the cytosol (Muñoz et al., 2006; Harries et al., 2013; Muñoz et al., 2013).

The plant defensins NaD1 and MtDef4 are also internalized (Lobo et al., 2007; van der Weerden et al., 2008; Sagaram et al., 2013) and accumulated mainly in the cytosol. Two different fungal AFPs were shown to also enter fungal cells as part of their inhibition mechanism (Oberparleiter et al., 2003; Moreno et al., 2006). In the case of the *P. chrysogenum* PAF, the protein accumulated in the cytosol and its internalization was energy-dependent (Oberparleiter et al., 2003). The AFP from *A. giganteus* also entered *M. oryzae* cells, but co-localized within nuclei (Moreno et al., 2006).

Once inside the cell, AMPs may target multiple processes. Common signatures of several antifungal peptides and proteins whose mechanism of action has been studied are the induction of reactive oxygen species (ROS) and the disruption of intracellular Ca^{+2} homeostasis. Examples of the production of ROS include the plant defensins RsAFP2 (Aerts et al., 2007) and NaD1 (van der Weerden et al., 2008), the fungal PAF (Leiter et al., 2005), and the synthetic hexapeptide PAF26 (Carmona et al., 2012). The fungal PAF (Binder et al., 2010) and AFP proteins (Binder et al., 2011) also disrupted cytosolic Ca^{+2} homeostasis in sensitive fungi.

There are intracellular mechanisms that do not necessarily require the internalization of the peptide but rather the activation of signalling cascades led by protein kinase (PK) signalling pathways (Turrà et al., 2014). These pathways could be either hijacked by the antifungal peptides to exert the killing activity or, alternatively, be part of the fungal defense response to counteract the damage produced by the peptides.

A summary of the mode of action of representative AMPs can be seen in Figure 5.

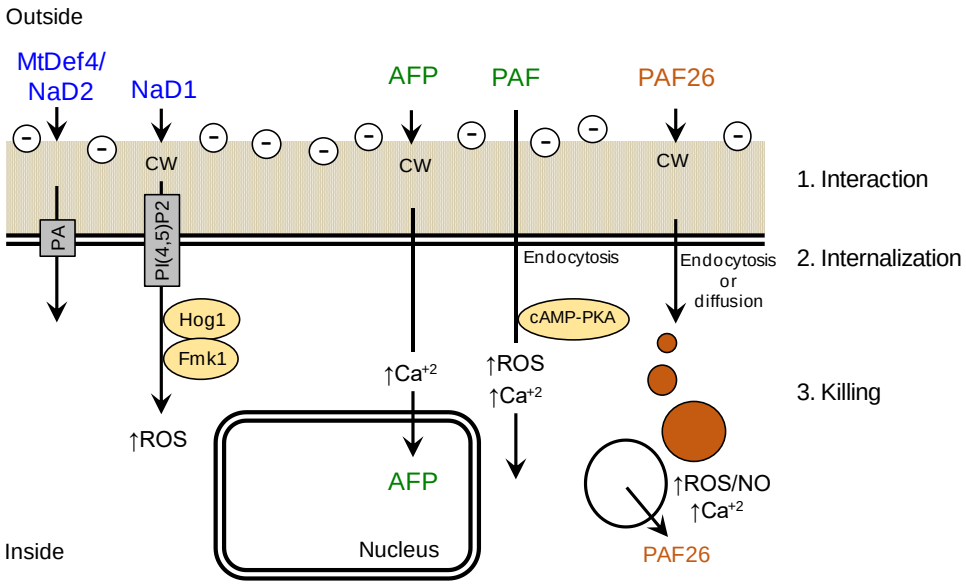

Figure 5: Schematic model of the non-lytic mode of action of antifungal peptides and proteins. The model is divided in three steps: (1) Interaction, (2) Internalization, and (3) Intracellular killing, as indicated at the right. The available data is summarized for the plant defensins MtDef4 from *M. truncatula*, and NaD1 and NaD2 from *N. alata*; the fungal antifungal proteins AFP from *A. giganteus*, and PAF from *P. chrysogenum*; and the synthetic hexapeptide PAF26. PA: phosphatidic acid. PI(4,5)P2: phosphatidylinositol 4,5-bisphosphate. PK pathways determinant of AMPs toxicity are rounded in yellow. See main text for more detail.

4. Use of Antifungal Peptides in Plant Protection

AMPs can be biotechnologically produced through genetic engineering of the host plant to confer disease resistance. Strategies based on the heterologous expression of genes encoding plant AMPs have demonstrated efficacy to enhance resistance in crop and ornamental plants towards fungal pathogens (Terras et al., 1995; Chan et al., 2005; Ghag et al., 2012). A successful example is the AFP from *A. giganteus*, which conferred enhanced resistance to transgenic rice plants against the blast fungus *M. oryzae* (Coca et al., 2004). Other peptides used include linear peptides with broad-spectrum and potent antimicrobial activity as the insect antimicrobial peptides cecropins, attacins, and apidecins, or the frog peptides magainins. (Ko et al., 2002; Coca et al., 2006; Rahnamaeian et al., 2009).

Simultaneous expression of different AMPs in plants, preferably with different properties and modes of action, is an attractive approach to attain resistance against a broad range of pathogens, and to minimize the probability of pathogens to overcome the engineered trait. A successful example is the production in rice plants of a chimeric polyprotein containing two plant defensins DmAMP1 and RsAFP2 fused through the IbAMP linker peptide, which is proteolytically cleaved in plant cells leading to the accumulation of the two independent defensins (Jha et al., 2009). The plants transformed with the polyprotein construct exhibited improved disease resistance to the fungal pathogens of rice *M*. *oryzae* and *Rhizoctonia solani*.

The choice of a suitable promoter to drive transgene expression is relevant to attain AMP-based resistance. In most of the studies, constitutive promoters have been used. However, this strategy presents serious drawbacks, such as the negative impact on fitness of the host plant, or the selection of resistant populations of target pathogens due to the permanent exposure to AMPs. Instead, a controlled production is a more desirable strategy, which can be accomplished by using inducible promoters. As an example, the pathogen-inducible expression of the *afp* gene from *A. giganteus* in rice plants resulted in better protection against rice blast disease compared to that observed in constitutive-expressing plants (Moreno et al., 2005).

Although the benefits of AMPs in enhancing disease resistance in transgenic plants have been repeatedly demonstrated, studies on the transgene-induced changes in the host plants are still scarce. Profiling technologies such as transcriptomics, proteomics, and metabolomics can be used to investigate intended and unintended differences in transgenic AMP plants. Compositional and toxicological analyses are also necessary to assess the substantial equivalence of transgenics compared to their non-transgenic counterparts. The introduction of the AMP gene must be demonstrated not to adversely affect the performance of the crop, and not to introduce unsafe alterations in food or feed products derived from these plants. However, and despite of successful results, the most important challenges currently to the production of AMPs in plants are public concerns about transgenic technologies in general, although AMPs are

natural compounds produced by all living organisms to fight pathogen infections.

4.1. Antifungal peptides in postharvest conservation

The use of AMPs in the control of postharvest decay as alternative to the current use of chemical fungicides has been proposed (López-García et al., 2000; Jones and Prusky, 2002; Palou et al., 2016). There is evidence that antifungal peptides are able to control postharvest decays caused by fungi under controlled experimental conditions as well as fungicides do. In these experiments, synthetic peptides and proteins, which were purified from natural sources or from genetically modified producer microorganisms, were either included in the fungal inoculum or applied before or soon after inoculation. Most of the examples concern citrus and apples, the two most important tree fruits worldwide and which are subject of this doctoral thesis (Table 3).

In the case of the synthetic PAF26 and its derivatives, both the fungicidal activity together with the fungistatic activity is critical to attain a good level of protection (Muñoz et al., 2007a; Badosa et al., 2009). Thus, peptides that have *in vitro* fungistatic activity similar to PAF26 or BM0 but are not fungicidal do not perform well in these experimental assays. This is mostly due to the experimental setup in which peptides are present only at the inoculation site and not in the surrounding fruit regions. Thus, once a few spores are able to germinate and escape from the inoculation site they can infect the fruit. Another parameter to take into account is the peptide susceptibility to degradation on the fruit surface. The introduction of unnatural amino acids or chemical modifications may improve their stability on fruits while maintaining their biological activity (Ng-Choi et al., 2014). For example, the peptide BP22 (Tables 1 and 3) protected apples against *P. expansum* decay as effectively as a commercial formulation of the fungicide imazalil (Badosa et al., 2009).

Table 3: Examples of experimental application of AMPs to control postharvest decay.

Peptide	Origin	Source (Mode of Application)	Fungus	Fruit	Reference
Cecropin A	Insect	Genetically modified *S. cerevisiae* (point co-inoculation)	*Colleotrichum coccodes*	Tomato	(Jones and Prusky, 2002)
PAF26 and derivatives	Peptide library	Synthetic (point co-inoculation)	*P. digitatum* *P. italicum*	Citrus	(López-García et al., 2003) (Muñoz et al., 2007a)
BM0	Peptide library	Synthetic (point co-inoculation)	*P. digitatum*	Citrus	(Muñoz et al., 2007a)
BP22	Peptide library	Synthetic (point pre-application)	*P. expansum*	Apple	(Badosa et al., 2009)
PsD1	Plant defensin (*P. sativum*)	Genetically modified *P. pastoris* (point pre-inoculation)	*P. expansum*	Apple	(Janisiewicz et al., 2008)
Cecropin A	Insect	Genetically modified *P. pastoris* (point pre-inoculation)	*P. expansum*	Apple/ Citrus	(Ren et al., 2012a) (Ren et al., 2012b)
Aq-AMP	Plant (*Amarantus quitensis*)	Recombinantly produced in *Escherichia coli* and purified (point co-inoculation)	*P. digitatum*	Citrus	(Alem et al., 2014)
AFP	Fungal (*A. giganteus*)	Purified from fungal cultures (sprayed on fruit surface)	*A. alternata*	Banana	(Barakat, 2014)
CecA::PsD1	Fusion of modified cecropin A and defensin PsD1	Genetically modified *Pichia pastoris* (point pre-inoculation)	*Geotrichum citri-aurantii*	Citrus	(Kong et al., 2016)

However, synthetic peptides are very difficult to produce in large amounts through biotechnological strategies (see section below), which make these peptides unsuitable for large-scale applications in agriculture. Therefore, further pilot-scale studies that simulate commercial application remain scarce. In these terms, AFPs, which can be easily produced by biotechnology and are very specific against fungi, are the perfect candidates for their large-scale production and application in plant protection and postharvest conservation.

To the best of our knowledge, only one antifungal AMP of fungal origin has been experimentally tested to control plant and postharvest diseases *in vivo*. AFP from *A. giganteus* has been reported to successfully control postharvest decay caused by *M. oryzae* in rice (Vila et al., 2001), *F. oxysporum* in tomato seed (Theis et al., 2005), *A. alternata* when sprayed on banana surfaces (Barakat, 2014) (Table 3) and the infection caused by *B. cinerea* in geranium leaves (Moreno et al., 2003). However, and despite the potential of AFPs to inhibit phytopathogenic fungi at low protein concentrations, these proteins have been poorly studied for their application to control postharvest pathologies to date. In the present thesis, we contribute to the characterization and exploitation of different AFPs for their potential use in plant protection and postharvest conservation *in vivo*.

4.2. Biofactories for AMP production

The development of AMPs for future application requires their production in large amounts. The cost-effective production of peptides of sufficient purity is critical because this will determine whether the practical use of peptides for postharvest conservation is feasible. Even with the increasing number of peptide companies and the declining costs of synthetic procedures, production costs via chemical synthesis and purification are still too high for a cost-effective application in postharvest conservation, even in the case of very short peptides, as it is the case of PAF26. A possible alternative is the purification of AMPs from their natural sources. However, low yields are usually obtained, and this alternative is restricted to natural peptides, and not to those obtained by rational design (Vriens et al., 2014). To overcome this major problem, there are different alternatives to the production of AMPs for the control of postharvest pathogens, and they mostly rely on the biotechnological production of peptides and proteins through genetically modified organisms. One of the primary attractive attributes of AMPs is their peptidic nature, which would theoretically enable their production through biotechnology.

Many different approaches have been used in order to successfully lead AMP expression in different organisms. Engineered plants to produce AMPs can be applied not only to protect host plants against pathogen infection (see section 4), but also as AMP biofactories for further uses (Abdallah et al., 2010). Plants represent a safe, easily scalable, and economic production system of AMPs. Despite the physicochemical

properties of some of these peptides, several plant-based platforms have been reported to be suitable for the large-scale production of AMPs. Among them, there are chloroplasts-based or rice seeds-based production systems (Lee et al., 2001; Montesinos et al., 2016; Montesinos et al., 2017). The success of these strategies is based on limiting the accumulation of AMPs to storage organs in order to avoid interference with plant vegetative growth; or to limit it to specialized subcellular organelles that would both protect AMPs from protease degradation and facilitate their purification (Montesinos et al., 2016; Montesinos et al., 2017).

Another alternative is the expression and *in situ* production of AMP by epiphytic microorganisms growing on the fruit surface, using genetically modified yeast strains that produce the desired AMP. Only a few reports have tested this alternative approach. For instance, the expression of cecropin A or cecropin A-derived peptides (Jones and Prusky, 2002; Ren et al., 2012a; Ren et al., 2012b) or the plant defensin PsD1 (Janisiewicz et al., 2008; Kong et al., 2016), showed promising control of mostly *Colletotrichum* sp. and *Penicillium* spp. in tomato, apple and citrus (Table 3).

The most widely explored approach is the use of microorganisms as biofactories for the production of AMPs (Li et al., 2011). Protein production by bacteria offers great advantages for its easy and cost-effective cultivation and high protein yields. However, this expression system presents some disadvantages as incorrect protein/peptide folding, disulphide bonds formation, and codon usage adaptation. In the case of AMPs, the production in microorganisms must also solve the obvious problem of the intrinsic toxicity of the peptide for the producer organism. Strategies such as the fusion to inactivation domains or the strict regulation/induction of gene expression have solved the toxicity issue in some cases. AMP production of functional plant defensins fused to histidine tags that facilitate purification in *Escherichia coli* was reported (Kant et al., 2009). The CRP Ac-AMP2 found in *Amaranthus* (Broekaert et al., 1992) was fused to the protein thioredoxin and a histidine tag, then produced in *E*. *coli* and purified using metal affinity chromatography (Alem et al., 2014). Yields of 38 mg/L of the purified peptide fusion were reported. The purified peptide controlled the green mould decay of citrus under experimental inoculations (Table 3).

Eukaryotic systems are preferred for CRPs with disulphide bonds and complex folding. Some plant defensins and AFPs have been efficiently produced in the yeast *Pichia pastoris*, but sometimes the yields of the well-processed proteins were not generally as high as desired, and usually lower than yields reported in bacteria (Kant et al., 2009; López-García et al., 2010b; Parachin et al., 2012; Viragh et al., 2014). High-throughput and systematic optimization of the expression and production of AMPs is being attempted (Schreiber et al., 2017), but yields reported for bacteria (*E*. *coli*) or yeasts (*P*. *pastoris*) are still too low to support practical use.

4.3. Filamentous fungi as biofactories for the production of AFPs

The biotechnological production and commercial use of proteins and enzymes produced by genetically modified filamentous fungi which are generally regarded as safe (GRAS), as it is the case of *P. chrysogenum* (Rodriguez-Martín et al.) and *A. niger* (Schuster et al., 2002), are well established (Meyer et al., 2016). Thus, it is possible that filamentous fungi could also function as efficient biofactories for the production of AFPs, although it may seem contradictory. However, not so many filamentous fungi have been used as biofactories for the heterologous production of fungal AFPs to date, being *P. pastoris* the preferred biofactory for the heterologous production (Viragh et al., 2014; Patiño et al., 2018). A step forward for the use of fungi as biofactories was recently taken with the development of specific gene promoters (Polli et al., 2016) and expression systems for the efficient production of a variety of heterologous AFPs (Sonderegger et al., 2016), in which our group has also collaborated. Here, a *P. chrysogenum*-based expression system consisting of the *paf* gene promoter, *paf* signal pre-propeptide, and terminator sequences successfully enabled the production of heterologous AFPs in filamentous fungi different from their producer organisms for the first time. Not only *P. chrysogenum,* but also *P. digitatum* were demonstrated to work well for the heterologous production of different AFPs, reaching protein yields up to 80 mg/L. These studies led us to propose that filamentous fungi, as it is the case of *P. chrysogenum* and *P. digitatum,* could be efficiently applied for the homologous/heterologous production of AFPs in high amounts for subsequent applications, and this strategy has been applied throughout this PhD thesis to carry out the objectives proposed.

OBJECTIVES

Antifungal proteins (AFPs) of fungal origin are a promising alternative for the development of new antifungal biomolecules, since they are naturally present in filamentous fungi, can be produced in large amounts, are very stable, and show remarkable antifungal specificity.

This PhD work focuses on the production and characterization of novel AFPs from phytopathogenic fungi for their potential application in plant protection and postharvest conservation. To achieve this goal, four specific objectives have been proposed:

Objective 1: Study of the biological and functional role of the only *afp*-coding gene (*afpB*) present in the citrus postharvest pathogen *Penicillium digitatum*.

Objective 2: Biotechnological production of the antifungal protein AfpB from *P. digitatum*, characterization of its physicochemical properties and antimicrobial activity, and identification of its active motifs.

Objective 3: Production and characterization of the antimicrobial profiles of three AFPs from the postharvest pathogen of pome fruit *Penicillium expansum*.

Objective 4: Evaluation of the most effective AFPs in postharvest conservation and crop protection.

CHAPTER I

CHAPTER I

Occurrence and function of fungal antifungal proteins: A case study of the citrus postharvest pathogen *Penicillium digitatum*

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ABSTRACT

Antifungal proteins (AFPs) of fungal origin have been described in filamentous fungi. AFPs are small, highly stable, cationic cysteine-rich proteins (CRPs) that are usually secreted in high amounts and show potent antifungal activity against non-self fungi. The role of AFPs in the biology of the producer fungus remains unclear. AFPs have been proposed as promising lead compounds for the development of new antifungals. The analyses of available antifungal CRP sequences from fungal origin and their phylogenetic reconstruction led us to propose a new classification of AFPs in three distinct classes: A, B and C. We initiate for the first time the characterization of an AFP in a fungal pathogen, by analysing the functional role of the unique *afpB* gene in the citrus fruit pathogen *Penicillium digitatum*. Null Δ*afpB* mutants revealed that this gene is dispensable for vegetative growth and fruit infection. However, strains that artificially express *afpB* in a constitutive way (*afpB*^{C}) showed a phenotype of restricted growth, distortion of hyphal morphology, and strong reduction in virulence to citrus fruits. These characteristics support an antifungal role for AfpB. Surprisingly, we did not detect the AfpB protein in any of the *P. digitatum* strains and growth conditions that were analysed in this study, regardless of high gene expression. The *afpB*^{C} phenotype is not stable and occasionally reverts to a wild-like phenotype but molecular changes were not detected with this reversion. The reduced virulence of *afpB*^{C} strains correlated with localized fruit necrosis and altered timing of expression of fruit defence genes.

Keywords: Antifungal peptide, Antifungal protein, Antifungals, *Penicillium digitatum*, postharvest pathology, citrus.

Chapter I

INTRODUCTION

Fungal pathogens are a major threat to human health and food security (Fisher et al. 2012). Fungi are currently a major cause of human morbidity and mortality in immunocompromised patients. Fungi also affect major crops globally and contaminate food causing unacceptable food spoilage. Unfortunately, there are few drugs for treating life-threatening fungal infections and resistance against these drugs is increasing alarmingly, as occurs with resistance against the antifungal molecules that are used in agriculture or food preservation. However, very few antifungals have been introduced in recent years. There is thus an urgent need for the development of new antifungal agents. Antimicrobial peptides and proteins (AMPs) have been considered as candidates for the development of novel antimicrobial compounds (Zasloff 2002; Brogden 2005; Fjell et al. 2012) including antifungals (Shah and Read 2013).

A remarkable group of antifungal AMPs are the so called antifungal proteins (AFPs) of fungal origin. The first fungal AFP to be identified and characterized was the one produced by *Aspergillus giganteus* (Nakaya et al. 1990; Wnendt et al. 1994; Campos-Olivas et al. 1995). AFPs are produced by selected species of filamentous ascomycetes, mostly from the genus *Aspergillus* and *Penicillium* (reviewed in Marx et al. 2008; Meyer 2008; Galgóczy et al. 2010). AFPs belong to the wider class of defensin-like AMPs and are small (~50 amino acid residues), cationic and cysteine-rich proteins (CRPs) that fold into compact β-stranded and disulphide-stabilized structures, which confer high stability under adverse biochemical and biophysical conditions. AFPs hold promise as novel antifungal compounds that could efficiently control fungal pathogens. In addition to *A. giganteus* AFP, other AFPs that have been characterized in detail include the PAF from *Penicillium chrysogenum* whose structure has been solved (Batta et al. 2009), the bubble protein (BP) from *Penicillium brevicompactum* (Seibold et al. 2011) or the NFAP from *Neosartorya fischeri* (Kovács et al. 2011). The transgenic expression of *A. giganteus* AFP in plants confirms its potential application in crop protection (Coca et al. 2004).

A detailed understanding of the mode of action of AFP is required for the potential future application of AFPs as antifungal compounds. The most studied AFPs have similarities as well as differences in their mode of action

against sensitive fungi, particularly in relation to whether they induce the cell wall integrity pathway, are internalized as part of their antifungal mechanism, induce disturbances in the intracellular Ca^{+2} concentration, induce the production of reactive oxygen species or affect intracellular signalling (reviewed in Hegedüs and Marx 2013). Particularly obscure is the mechanism by which the producer and related fungi are tolerant to the antifungal action of the self-AFP. The functional roles of the pro-peptide sequence that lies between the signal peptide that determines secretion and the mature AFP are still unknown. The characterization of null mutants of the *paf* gene from *P. chrysogenum* led to the proposal of a role beyond antifungal activity in the regulation of conidiogenesis in this fungus (Hegedüs et al. 2011).

Very few AFPs have been identified in fungal pathogens. The citrus postharvest pathogen *Penicillium digitatum* is a necrotrophic filamentous fungus that is highly specific for citrus and produces very important economic losses in one of the main fruit tree crops worldwide (Macarisin et al. 2007; Marcet-Houben et al. 2012). The main method used to control citrus postharvest decay is the postharvest application of chemical fungicides. However, the massive use of a limited spectrum of commercial fungicides in the citrus industry has boosted the selection of resistant isolates that put at risk worldwide citrus commercialization. These problems have led to the search for new alternatives to the control of this and other fungal postharvest pathogens, as the use of antifungal peptides (Marcos et al. 2008).

The recent sequencing of the genome of *P. digitatum* allowed the identification of several cationic CRP of small size, which at that time were considered potential AFP-like proteins (Marcet-Houben et al. 2012). In this study, phylogenetic analyses confirmed the clustering of one of these hypothetical proteins with known AFPs. Based on these analyses, a new classification and nomenclature for AFPs is proposed. Finally the functional characterization of the new *P. digitatum afpB* gene was initiated through the study of gene expression, and its genetic disruption and constitutive expression.

MATERIALS AND METHODS

Microorganism, media and culture conditions

The fungal strain that was used as the parental isolate was *P. digitatum* CECT 20796 (isolate PHI26) (Marcet-Houben et al. 2012). This strain and all of the transformants that were generated in this work were routinely cultured on potato dextrose agar (PDA) (Difco-BD Diagnostics, Sparks, MD, USA) plates for 7-10 days at 24 ºC. To analyse growth on solid medium, 5 µL of conidial suspension (1×10^3 conidia/mL) of each *P. digitatum* strain were deposited on the centre of PDA plates and the diameter of growth was monitored daily from 3 to 10 days. To determine conidia production, conidia were collected, filtered and counted with a hemacytometer at daily intervals, and normalized to the surface of the fungal colony. To analyse the growth in liquid medium, each *P. digitatum* strain was grown in 100 mL of potato dextrose broth (PDB) (Difco-BD Diagnostics) at 24 ºC with shaking. *Agrobacterium tumefaciens* AGL-1 strain was grown in Luria Bertani medium (LB) that was supplemented with 20 µg/mL rifampicin at 28 ºC.

Protein sequence and phylogenetic analyses

Sequences from different *afp* genes and the corresponding AFP proteins were identified through BLAST searches that were carried out at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov) (Table S1). Multiple sequence alignments were conducted with the ClustalW algorithm (Thompson et al. 1994) (http://www.ebi.ac.uk/Tools/clustalw2/), using the mature protein sequences with previous removal of the signal peptides and the pro-peptides in their N-termini. Sequence alignment was further refined by hand with minor adjustments. The phylogenetic tree was obtained with the Neighbour-Joining method using the MEGA 6.0 program (Tamura et al. 2013), with estimations of nucleotide distances using the p-distance method and computation of bootstrap values using 10,000 replicates.

Fruit Infection assays

The inoculation of *P. digitatum* strains on freshly harvested fruits of mature orange (*Citrus sinensis* L. Osbeck cv Navelina) and sample collection were carried out as previously described (González-Candelas et al. 2010). Briefly, three replicates of five fruits were inoculated with 5 µL of

conidial suspension at four wounds around the equator. Each inoculated wound was scored daily for infection symptoms on consecutive days post-inoculation (dpi). At different dpi depending on the experiments, tissue discs of 5 mm in diameter around the inoculation site were sampled. Tissue samples were crushed and frozen at -80 ºC for use in RNA extraction.

Total RNA extraction and quantitative RT-PCR

Total RNA from (i) time course experiments of *P. digitatum* strains that were grown in PDB or PDA or (ii) infected fruit samples, was obtained following previously described procedures (Rodrigo et al. 2004; Gandía et al. 2012). Total RNA was used to synthesize first strand cDNA as described (Gandía et al. 2014) and cDNA samples were diluted with nuclease-free water. Quantitative PCR was performed and analysed as previously described (Gandía et al. 2012). The gene specific primers that were used for qRT-PCR are shown in Table S2. Relative changes in gene expression between different treatments (times of growth or infection) were normalized using genes coding for *P. digitatum* β-tubulin, ribosomal protein L18a, and 18S rRNA simultaneously as independent housekeeping genes (Table S2). In the case of citrus gene expression, the *Citrus sinensis* defence genes of ethylene biosynthesis ACC synthase 1 and 2 (*acs1* and *acs2*) and ACC oxidase (*aco*), and of the phenylpropanoid pathway phenylalanine-ammonia lyase (*pal*) (Marcos et al. 2005; Alós et al. 2014) were chosen, while genes encoding for glyceraldehyde-3-phosphate-dehydrogenase (*gapdh*) and actin (*act*) were used as reference genes (Romero et al. 2013). The results shown are mean of three technical replicates from each of two biological replicates. The Relative Expression Software Tool (Multiple Condition Solver REST-MCS v2) (Pfaffl et al. 2002) was used to determine the relative quantification of target genes that were normalized to the reference genes and determine the statistical significance of the results.

Generation of deletion and constitutive *P. digitatum* strains

To generate the *PdafpB* gene replacement construct, the hygromycin resistant cassette (*hph*) that was used as a positive selection marker was amplified from pBHt2 (Khang et al. 2006) through PCR with the primers OJM197 and OJM198. All of the PCR procedures were carried out with AccuPrime High-Fidelity polymerase (Invitrogen, Eugene, OR, USA), and the resulting DNA constructs were verified by DNA sequencing. The different primers that were used to generate deletion and constitutive

transformants are listed in Table S2. The *hph* cassette was flanked by fungal DNA fragments of 952 bp (primers OJM375 and OJM376) and 1093 bp (primers OJM365 and OJM377), amplified from *P. digitatum* CECT20796 genomic DNA. This construct was generated by fusion PCR (Szewczyk et al. 2006), and cloned into the binary vector pGKO2 (Khang et al. 2006), whose T-DNA also contains the Herpes Virus thiamine kinase gene (HSV*tk*) that was used as a negative selection marker, to obtain pGKO2_ΔafpB (see the diagram of the construct in Fig. S1). To obtain the constitutive expression construct (pBHt2_PdafpBC), the full-length *PdafpB* coding sequence was cloned under the control of the *A. nidulans* glyceraldehyde triphosphate dehydrogenase (*gpdA*) promoter and the *trpC* terminator into the vector pBHt2 (Mullins et al. 2001) (see Fig. S2). Binary vectors were transformed into *A. tumefaciens* AGL-1, and the fungal transformation of the parental CECT20796 strain was carried out by *Agrobacterium tumefaciens*-mediated transformation (ATMT) essentially as previously described (Khang et al. 2006; Michielse et al. 2008) with minor modifications (Harries et al. 2015).

Homologous recombinants and constitutive transformants were confirmed by PCR amplification of genomic DNA, conducted and analysed as previously described (Gandía et al. 2014). The primers used were located at different positions around the target locus to discriminate and confirm the transformation events (Fig. S1 and S2).

Phenotypic assays on *P. digitatum* transformants

To phenotypically characterize the different transformants, the sensitivity of fungal strains to different temperatures, compounds, osmotic stress and fungicides was assayed. PDA medium was supplemented with 100-150 µg/mL of calcofluor white (CFW, Fluorescent Brightener 28, Sigma-Aldrich, St. Louis, MO, USA), 100-150 µg/mL of sodium dodecyl sulphate (SDS Sigma–Aldrich), H_2O_2 1- 2 M, NaCl 0.5M, sorbitol 1.2 M, or the fungicides thiabendazole (TBZ) 0.5-2 µM and imazalil (IMZ) 0.031-0.125 µM. Suspensions of conidia (5 µL of 5 × 10^4 conidia/mL) were spotted on PDA plates and incubated at 24 ºC. The growth was monitored daily for 7 days.

Fluorescence microscopy

To microscopically visualize the morphology of each fungal strain, sample preparation and fixation were conducted essentially as previously described (Harries et al. 2015). The samples were stained with 50 µg/mL CFW and visualized with fluorescence microscopy (microscope E90i, Nikon Chiyoda, TO, Japan) with excitation and emission wavelengths of 395 nm and 440 nm, respectively. The images obtained in the 40 × objective were captured by the NIS-Elements BR v2.3 software (Nikon).

RESULTS

Phylogenetic tree reconstruction supports the classification of known fungal antifungal proteins in three major classes

The recent sequencing of the *P. digitatum* genome (Marcet-Houben et al. 2012) allowed the identification of a putative antifungal protein gene (PDIG_68840, UniProt K9FGI7) that shows ~33 % amino acid identity with two of the most studied and characterized AFPs to date: the AFP from *A. giganteus* (P17737) (Lacadena et al. 1995) and the PAF from *P. chrysogenum* (B6HWK0) (Batta et al. 2009). The availability of numerous fungal genome sequences provides an opportunity to find additional AFPs and conduct detailed phylogenetic studies. We used these three protein sequences to run BLAST searches and find homologous sequences in the fungal genome databases. Phylogenetic tree reconstruction based on mature protein sequences from the significant BLAST hits (Table S1) supported the grouping of the currently known AFP-like sequences in three major classes A, B, and C (Fig. 1A). Each of the three different classes of AFPs includes at least two proteins for which antifungal activity has been experimentally demonstrated (black dots in Fig. 1A), and class A contains the two most studied AFPs. A unique example is *P. chrysogenum,* which has three AFP with demonstrated antifungal activity; although the two more recent members were characterized in a cured meat (class B) (Rodríguez-Martín et al. 2010) and an Arctic (class C) (Chen et al. 2013) isolates, the corresponding genes are also present in the reference Wisconsin 54-1255 strain that has its genome sequenced, wherein these genes had been overlooked.

Classes A and B are phylogenetically more related, while class C is more divergent. The most representative member of class C is the BP protein from *P. brevicompactum* (P83799) (Seibold et al. 2011). The differences between classes A and B and class C refer not only to protein homology, cysteine pattern and size (Fig. 1B), but also to intron-exon structure or presence of Pfam domains. While the proteins from classes A and B contain two introns in their nucleotide sequence, proteins from class C contain only one (data not shown). Classes A and B also share the presence of the Pfam domain PF11402 (“Antifungal Protein”), whereas the Class C proteins have the non-annotated PF09227 domain (Table S1).

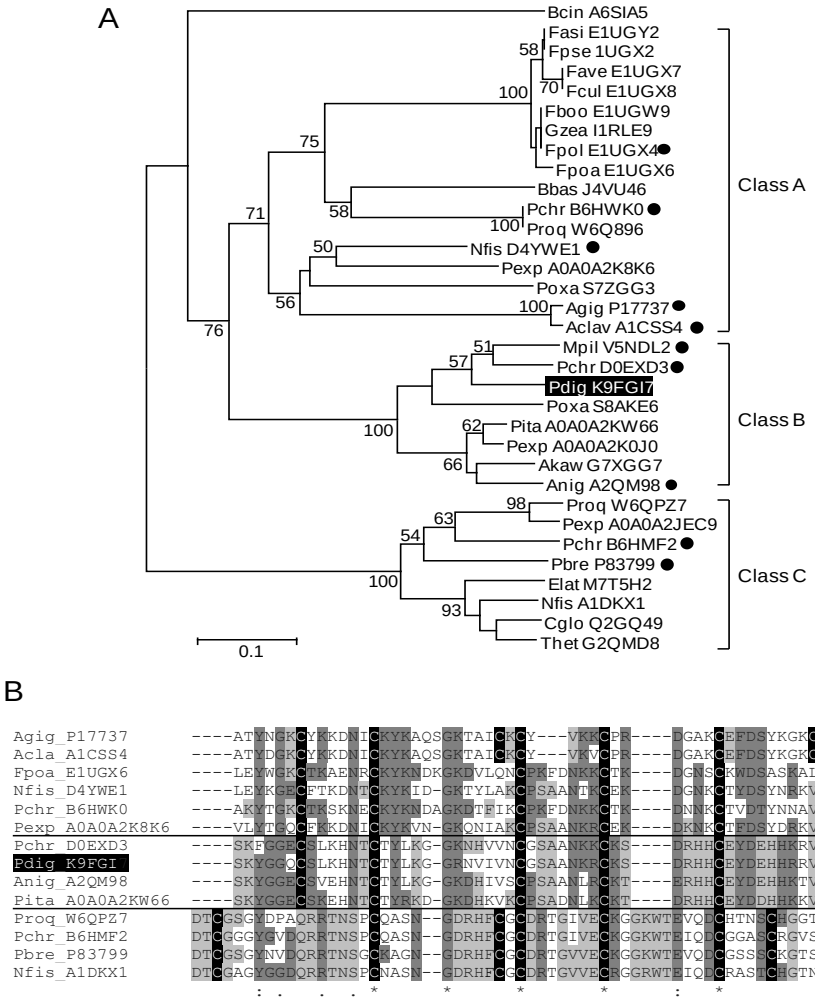

Figure 1: Phylogenetic analysis and sequence alignments of AFP and AFP-like proteins of filamentous fungi. (A) Neighbour-joining phylogenetic tree based on AFP and putative AFP sequences from filamentous fungi. The protein sequences shown in this study are named as follows: the first letter corresponds to the fungal genus; the next three letters correspond to the fungal species; and finally, the alphanumeric code corresponds to the UniProt ID of the protein. The phylogenetic analysis was conducted using MEGA 6.0. The bootstrap values were calculated using 10,000 replicates and are indicated when greater than 40 %. PdAfpB is shadowed in black. Three different classes (A, B and C) are distinguished. Black circles indicate the AFPs with demonstrated antifungal activity. Table S1 includes the accession numbers of all of the sequences used in this analysis. (B) Amino acid sequence alignment of a selection of AFPs and putative AFPs that were used for the phylogenetic analysis (see the alignment of the complete set of proteins in Fig. S3). Proteins belonging to different classes are separated by a line. Cysteine patterns are shadowed in black. Strongly conserved amino acids (or with the same chemical properties) are highlighted in dark grey. Other conserved amino acids are shadowed in light grey.

Class A is the most heterogeneous; it includes sequences with percentage of identity ranging from 36 to 99 % within the class, as well as proteins with two different cysteine patterns of either six or eight cysteines. Classes B and C contain more homogeneous protein sequences with identity percentages ranging from 78 to 88 % and 73 to 92 %, respectively.

Unlike other filamentous fungi such as *P. chrysogenum*, *Penicillium expansum* or *Neosartorya fisheri*, whose genomes encode more than one AFP from different classes, the *P. digitatum* hypothetical protein that is encoded by PDIG_68840 seems to be the only AFP in the genome of this fungus. The protein has been classified into class B. Thus, we propose to name the corresponding gene *afpB*. PdAfpB has 88 % identity (50 of 57 identical amino acid residues) to the *P. chrysogenum* class B protein (D0EXD3) (Rodríguez-Martín et al. 2010) but only 16 % to the *P. chrysogenum* class C protein (B6HMF2) (Chen et al. 2013).

The *P. digitatum afpB* gene is expressed during fungal growth and the infection of citrus fruit

Expression analyses showed that the expression of *PdafpB* is highly induced during fungal growth, both in liquid and solid rich medium (note the logarithmic scale in Fig. 2), reaching the largest value of this induction after seven days of axenic growth on PDA plates, when the level of sporulation of the fungus is maximum (Gandía et al. 2012). Indeed, the highest amount of *PdafpB* mRNA was found in quiescent conidia. This relatively high *PdafpB* mRNA amount decreased abruptly (by more than three orders of magnitude) in conidia that were incubated for 9 h in rich medium (Fig. 2). At this short time point, most conidia show the typical enlargement that precedes germination and a minor proportion present a breaking germ tube (data not shown).

Additionally, the *PdafpB* gene is expressed during pathogenic interaction with citrus fruit. The mRNA was detected at 3 days post-inoculation, and from this time point the relative level of mRNA remained approximately constant. This expression pattern is considered a late onset of expression because there are fungal genes (including the reference housekeeping genes used in this study) whose expression has been readily observed from earlier times using the same experimental setup (Gandía et

al. 2014; Harries et al. 2015). Therefore, gene expression does not suggest a relevant role of *PdafpB* during fruit infection.

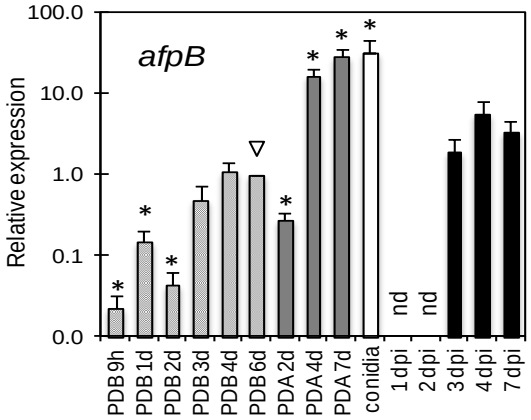

Figure 2: Relative expression of the *P. digitatum afpB* gene in parental strain CECT 20796. The graph shows the relative expression of the *afpB* gene at 9 hours (h), 1, 2, 3, 4 and 6 days (d) of growth in liquid medium PDB (light grey bars); at 2, 4 and 7 days of growth in solid medium PDA (dark grey bars); in purified mature conidia (white bars); and during the infection of citrus fruits at 1, 2, 3, 4 and 7 days post inoculation (dpi) (black bars). Gene expression was normalized to the reference condition: expression at day 6 in liquid PDB (marked by an inverted triangle). In all cases, the bars show the mean ± standard error (SE) of three technical replicates of a representative experiment. Asterisks (*) indicate statistical significance (p<0.05) compared to the reference condition. nd, expression not detected in these samples.

The constitutive expression of *P. digitatum afpB* results in a drastic reduction of axenic growth, abnormal hyphal morphology and delayed conidiogenesis

To study the functional roles of *PdafpB*, null and constitutive expression strains were produced by genetic transformation. We obtained null mutants by homologous recombination and replacement of the entire *PdafpB* ORF with the *hph* cassette from the pGKO2_ΔafpB vector, as a positive selection marker for hygromycin resistance (Fig. S1A). Six independent positive *P*. *digitatum* Δ*afpB* transformants were confirmed by a complete set of PCR amplifications using specific primers (Fig. S1B). Detailed characterization is shown for the two representative null mutants PDMG122 and PDMG135 (Fig. 3); while strain PDMG111 is a control strain that resulted from the ectopic integration of the T-DNA from the vector outside of the *afpB* locus.

We also obtained transformants for *PdafpB* constitutive expression under the strong *gpdA* promoter from *A. nidulans* (Fig. S2). Six of these transformants were confirmed through a combination of PCR amplifications (Fig. S2B). An analysis of the gene copy number by qPCR of genomic DNA indicated that five of these six likely contain one copy of the P*gpdA*:*afpB* (in addition to the native *afpB*), while the sixth (PDMG154) might contain two additional copies (Fig. S2C). PDMG150 and PDMG152 were selected for detailed analysis in this study (Fig. 3). The expression of *afpB* was boosted in these mutants by more than two orders of magnitude (see below).

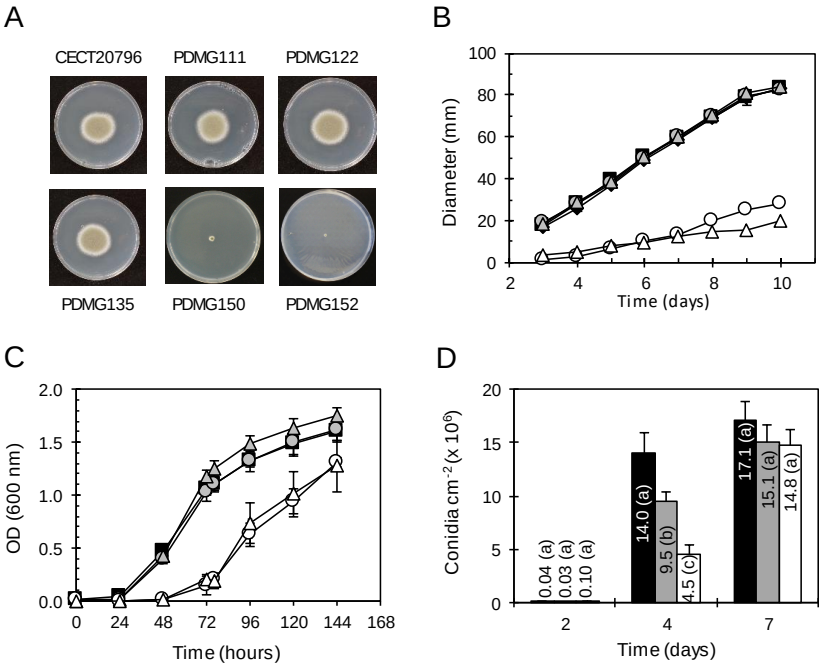

Figure 3: Growth and conidia production of *P. digitatum afpB* strains. (A) Colony morphology after 5 days of growth on PDA plates. (B) Growth in solid PDA as determined by the colony diameter from 3 to 10 days of growth. (C) Growth in liquid PDA as determined by the OD at 600 nm from 0 to 166 h of growth. In (A) to (C) the strains shown are: the parental CECT 20796, an ectopic strain PDMG111, the null Δ*afpB* strains PDMG122 and PDMG135, and the constitutive *afpB*^{C} strains PDMG150 and PDMG152. Parental and ectopic strains are shown in black symbols, null mutants in grey and constitutive strains in white. (D) Conidia production per surface area of colony of parental CECT 20796 (black bars), null Δ*afpB* mutant PDMG122 (light grey bars) and *afpB*^{C} strain PDMG150 (white bars) at 2, 4 and 7 days of growth in PDA plates. Data are mean values ± standard deviation (SD) of three replicate samples. Bars also include the mean value (conidia cm-2 x 106), and letters show significant differences among the strains within each day (ANOVA test, $p < 0.05$).

The growth of the null mutants PDMG122 and PDMG135 in solid PDA plates or liquid PDB rich medium was indistinguishable from that of the CECT 20796 parental strain and the PDMG111 ectopic control under optimal conditions (Fig. 3A-C), or at different temperatures, osmotic or chemical stresses (data not shown). This result demonstrates that the *afpB* gene is dispensable for vegetative growth in *P. digitatum*. In contrast, the constitutive strains PDMG150 and PDMG152 showed a strong reduction of colony diameter and delayed growth on PDA (Fig. 3A and B). The growth of these *afpB*^{C} constitutive mutants in liquid PDB was also slower and delayed compared to that of the wild type and null mutants, although in this case the reduction was not as severe as in solid PDA (Fig. 3C). The restricted growth phenotype of *afpB*^{C} mutants was not recovered in the presence of 1.2 M sorbitol as an osmotic stabilizer nor were these mutants more sensitive to the cell wall (CW)-interfering compounds CFW or SDS (data not shown); indicating that the growth defect is not due to a defective CW.

Null mutants such as PDMG122 showed a slight delay in conidia production that was only statistically significant at intermediate time points (i.e., 4 days of growth in PDA plates, Fig. 3D). This delay was more pronounced in the case of the *afpB*^{C} strains (such as PDMF152, Fig. 3D). In addition, the constitutive strains showed a darker green colour in the conidia-producing area of colonies, compared to the wild type coloration of any of the other strains (see below).

Given the growth limitations of the constitutive strains, the morphology of PDMG152 hyphae was analysed by fluorescence microscopy under CFW staining (Fig. 4). In accordance with the delayed conidia production (Fig. 3D), the micrographs of the mutant showed an absence of conidiophores after growth times at which the parental strain showed conidia producing structures (compare Fig. 4B and 4A, open arrows). Additional morphological differences of PDMG152 compared to the parental, null or ectopic strains were: extensive hyphal branching including tip dichotomous branching very close to the germinating spore (Fig. 4C, solid arrows), and hyphal cells wider than wild type with shorter interseptum distances (Fig. 4B and C, marked by an asterisk). These differences were even more evident after longer incubation times, especially in the case of the greater width and reduced septum distances of the mutant (Fig. 4E and F). These abnormalities indicate an altered pattern of growth. However, the

CFW staining in PDMG152 did not show obvious differences with the parental strain in terms of intensity or distribution, further suggesting that the structure of the CW does not seem to be altered in the mutant.

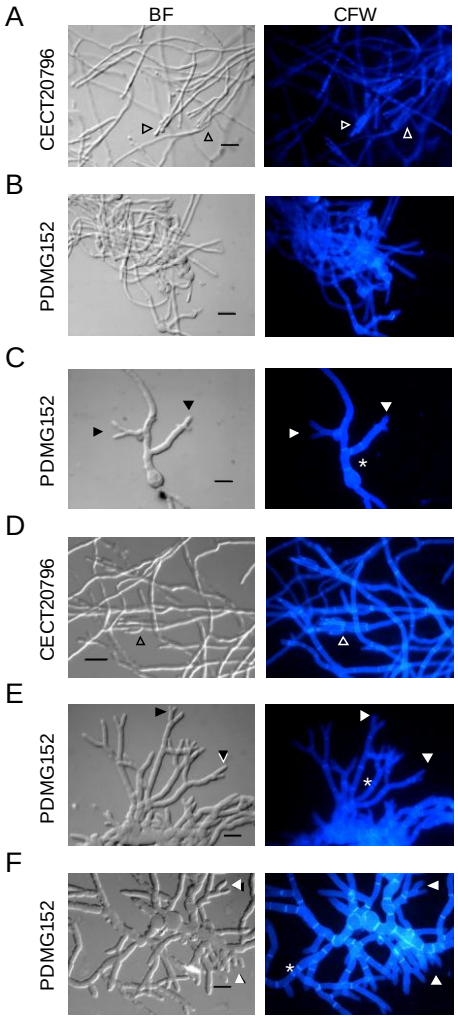

Figure 4: Morphological changes in the *P. digitatum* constitutive *afpB*^{C} strain as determined by fluorescence microscopy**.** Representative bright field (BF, left column) and fluorescence (CFW, right) microscopy images of CFW-stained mycelium of the parental strain CECT 20796 (A and D) and the constitutive strain PDMG152 (B, C, E and F), as indicated. Open arrows, solid arrows and asterisks indicate morphological alterations in the constitutive strain (see main text for details). Samples were grown in liquid PDB for 24 h (A-C) or 48 h (D-F). Scale black bars in BF images are 20 µm.

The reduced growth phenotype of constitutive *afpB*^{C} strains is not stable.

As is standard practice with mutant strains, we always work with monosporic conidia that are preserved as glycerol stocks and are phenotypically stable. However, we observed a rare case of phenotypical instability in the constitutive strains (Fig. 5). We found in all of the strains that were analysed a small population of colonies (<5 %) that reverted to a wild type-like growth phenotype from the restricted growth and dark sporulation described above, and that are easily distinguishable (Fig. 5A compare phenotypes “a” and “b” in a streak on a PDA plate after 6 days of culture). In addition, it was common to see in the isolated colonies of overgrown PDA plates (i.e., grown for more than 14 days) the emergence of this wild type phenotype at the growth limit of the colony (Fig. 5B), giving rise to atypical colonies with lighter colour at the periphery and darker sporulation colour at the centre, from which the mass of conidiophores grows upright in a volcano-like shape (see enlarged picture in Fig. 5B).

Importantly, these wild-like colonies maintained the hygromycin resistance and did not revert back to the mutant phenotype (data not shown). We therefore tested whether there was any difference in their genetic structure or gene expression. The PCR amplification of genomic DNA demonstrated that the *hph* hygromycin cassette and the constitutive *afpB* construct remained unaltered in both “a” and “b” phenotypes (Fig. 5C). The conventional PCR amplification of cDNA showed proper intron processing and no differences in either the “a” or “b” phenotypes compared to their parental PDMG150 or PDMG152 (Fig. 5D). Quantitative qRT-PCR further demonstrated that the more than 100-fold increased mRNA accumulation observed in these constitutive strains remained unchanged (Fig. 5E).

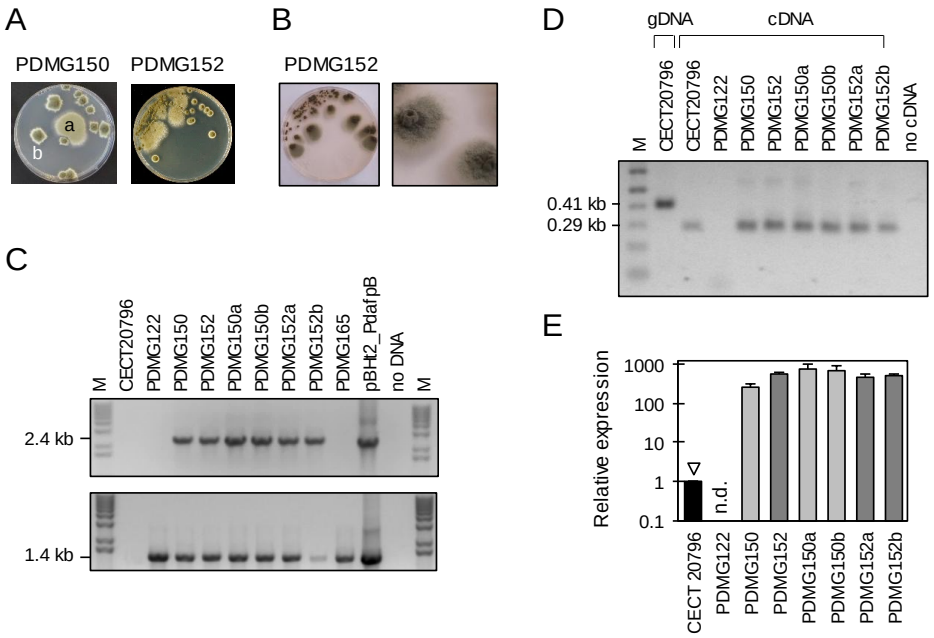

Figure 5: Characterization of the unstable phenotype of the *afpB*^{C} strains**.** (A) Streaks of the *afpB*^{C} PDMG150 and PDMG152 monosporic stocks were grown on PDA plates that were incubated at 24 ºC for 7 days. Occasionally, some colonies reverted to a wild type-like phenotype (a), as opposed to the reduced size and darker sporulation that are typical of the mutants (b). (B) Volcano-like phenotype of the *afpB*^{C} PDMG152 strain that was grown at 24 ºC for 14 days (see main text for a detailed description). (C) Molecular characterization by PCR amplification of genomic DNA (gDNA) of the *afpB*^{C} PDMG150 and PDMG152 strains and their reverted "a" and "b" phenotypes as indicated. Controls are strains CECT 20796 (wild type), PDMG122 (null), and PDMG165 (ectopic), plasmid pBHt2_*PdafpB* and no DNA control. The 2.4 kb bands (upper panel) correspond to the amplification (primers OJM443-446) of the entire constitutive construction only present in the *afpB*^{C} transformants, and the 1.4 kb band (lower panel) corresponds to the hygromycin resistant cassette (*hph*) (primers OJM197-198) that is present in the null, ectopic and constitutive mutants. M: 1 kb DNA ladder. (D) PCR amplification from gDNA or cDNA of the *PdafpB* ORF sequence (primers OJM447-448) from the indicated fungal strains. The 0.41 kb band corresponds to the intron-containing genomic *PdafpB* DNA sequence, while the 0.29 kb bands correspond to the correctly processed cDNA sequence that is present in the parental, constitutive and reverted strains, but absent in the null mutant. (E) Relative expression of the *PdafpB* gene as assayed by qRT-PCR in the parental strain CECT 20796, the null Δ*afpB* PDMG122, the *afpB*^{C} strains PDMG150 and PDMG152 and their reverted phenotypes, as indicated. Gene expression was normalized to the expression of the *afpB* gene in the parental strain (inverted triangle). In all cases bars show the mean ± SE of three replicates. n.d.: expression not detected in the sample. See Fig. S2 for additional details and location of primers.

The *P. digitatum afpB* gene is not required for virulence in citrus fruit

To determine the pathogenicity of the *afpB* strains, infection assays were performed by inoculating citrus fruits with conidial suspensions (Fig. 6). Low inoculum doses (5×10^4 conidia/mL) demonstrated that the null mutants PDMG122 and PDMG135 showed an incidence of infection and symptom development that was very similar to that of the parental strain CECT 20796 or the ectopic control PDMG111 (Fig. 6A and data not shown). At this low inoculum dose, approximately 90-95 % of the wounds that were inoculated with the constitutive mutants PDMG150 and PDMG152 remained as healthy as the wound (water-inoculated) controls (Fig. 6A).

At high inoculum doses (10^6 conidia/mL) the infection with the parental CECT 20796 usually develops so quickly and sharply that at 3 dpi nearly all of the wounds (100%) are infected, and infection develops uniformly and synchronously thereafter (Fig. 6B) to the point that at 7 dpi fruits are covered by a ball of esporulated fungal biomass (Fig. 6C). Under these conditions, the null mutant PDMG122 was indistinguishable from the parental strain (Figs. 6B and C) while the constitutive PDMG152 started to develop symptoms from 6 dpi, showing a clear and significant delay compared to the parental or null strains (Fig. 6B). Infection by PDMG152 was initially identified as a well-defined macerated area of less than 10 mm around the inoculation site; at this site, tiny accumulations of fungal white mycelium were noticeable inside the puncture (Fig. 6D). This growth is not the normal behaviour of *P. digitatum*, as in wild strains mycelium is only distinguishable after 2-3 days of development of the maceration zone, which is then 3-4 cm in diameter (data not show). This observation reflects the restricted growth that was observed in the axenic culture of this strain. At later time points (from 8 dpi) two types of infection were observed in the PDMG152 strain. In some wounds, the maceration area did not increase in size any further and instead became progressively darker, showing signs of necrosis and infection containment (see the enlarged picture of Fig. 6E compared to the wound control, and the fruits at the top in Fig. 6F). This observation mimics the previously described deposition of plant defence metabolites around the inoculation site after the artificial induction of resistance (Ballester et al. 2013a). Other PDMG152-infected fruits developed wild type symptoms, with light olive colour sporulation (Fig. 6F, bottom fruits).

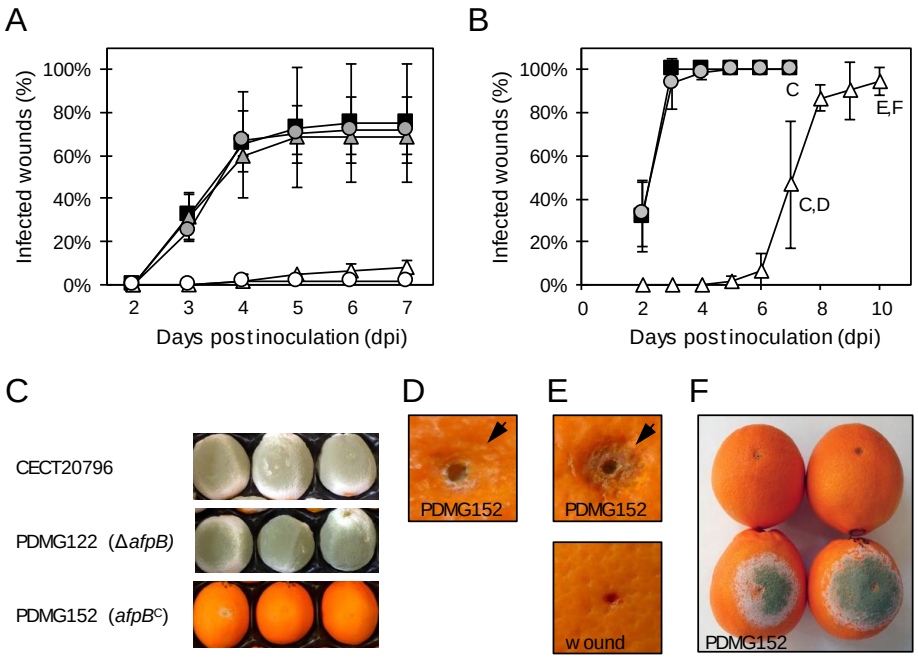

Figure 6: Virulence assays of *P. digitatum* strains on orange fruits. (A) Incidence of infection caused by the parental CECT 20796 and ectopic PDMG111 (black symbols), nulls Δ*afpB* PDMG122 and PDMG135 (gray), and constitutive *afpB*^{C} PDMG150 and PDMG152 (white) at a low inoculum dose (5 × 10^4 conidia/mL). (B) Incidence of infection caused by the parental CECT 20796, the null Δ*afpB* PDMG122 and the constitutive *afpB*^{C} PDMG152, at a high inoculum dose (10^6 conidia/mL). In (A) and (B), the data indicate the percentage of infected wounds (mean value ± SD) at each day post-inoculation (dpi), and the symbols that are used are the same as in Fig. 3. In (B), C-to-F lettering indicates the samples and time points at which pictures (C) to (F) were taken, for clarity. (C) Representative images of orange fruits infected by the indicated strains at 7 dpi. (D) Close-up of an infected wound that was inoculated by PDMG152 after 7 dpi; the arrow indicates the restricted maceration front. Notice the visible mycelium inside the inoculation point. (E) Close-up of a wound that was inoculated by PDMG152 (upper image) after 10 dpi, showing the aborted infection; the arrow indicates the restricted infection front. The bottom image shows a wound from a mock-inoculated control. (F) Representative fruits that were inoculated with PDMG152 after 10 dpi; showing wild type symptoms (bottom) and restricted infections (upper).

Interestingly, some of these PDMG152-infected fruits showed an area of darker sporulation at the center of the lesion (Fig. 6F, bottom left) that mimics the dual colour colonies that were observed in solid culture (Fig. 5B).The isolation and culture of fungus from both types of infection demonstrated the recovery of hygromycin resistant colonies, confirming that infection is due to the genetically modified strains (data not shown). Therefore, we conclude that the two types of infection reflect the two

distinct phenotypes observed in the axenic culture of the constitutive *afpB*^{C} strains (Fig. 5).

Expression of citrus genes involved in fruit defence in response to infection by *P. digitatum afpB* mutants

The above data with the constitutive strain PDMG152 indicate the induction of localized plant defence around the inoculation site, which could limit fungal colonization. Ethylene and phenylpropanoid biosynthetic pathways are involved in the defence response of citrus fruit to *P. digitatum* infection (Marcos et al. 2005; González-Candelas et al. 2010; Ballester et al. 2011). We therefore determined the expression changes of fruit genes related to these pathways. The genes coding for the ethylene biosynthetic enzymes ACC synthase 1 and 2 (*acs1* and *acs2*) and ACC oxidase (*aco*), and the phenylpropanoid enzyme phenylalanine-ammonia lyase (*pal*) respond to fungal infection (Marcos et al. 2005; Ballester et al. 2011; Ballester et al. 2013b). The expression changes of these four genes were compared during infection by CECT20796, PDMG122 and PDMG152 (Fig. 7). The data revealed an onset of gene induction as early as 1 dpi and a peak of induction at 2-3 dpi for all the four genes in the wild-type phenotypic strains CECT20796 and PDMG122, clearly above the background of the mock-inoculated controls. This peak of induction is coincident with the appearance of maceration symptoms in fruits at 2 dpi (Fig. 6B). This gene induction is consistent with previous observations that were obtained with the parental CECT20796 strain (Marcos et al. 2005; Ballester et al. 2013b).

However, the expression profile was distinct in the case of infection by the constitutive strain PDMG152 (white bars in Fig. 7). While the onset of expression at 1 dpi was similar to that of the other strains and reached similar induction at this time point, the subsequent timing of induction was clearly different. The gene expression was maintained clearly above the background of the controls up to 4 dpi, times at which no noticeable macroscopic infection was observed (Fig. 6B), but it did not peak sharply at 2-3 dpi and did not decrease later.

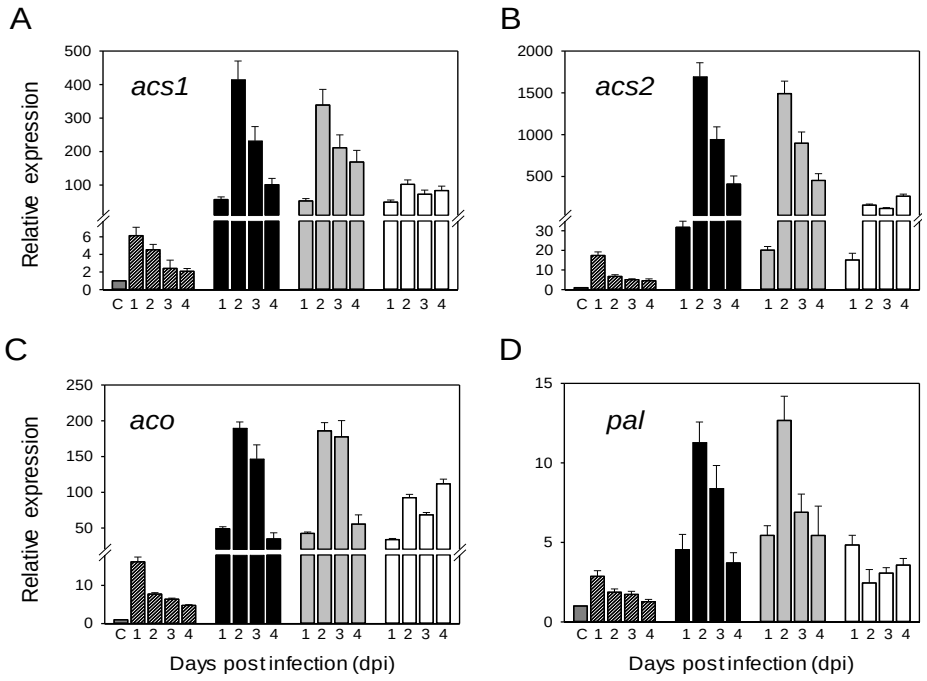

Figure 7: Relative expression of citrus genes involved in fruit defense in response to infection by *afpB* mutants. The bar graphs show the relative expression of (A) citrus ACC synthase 1 (*acs1*), (B) ACC synthase 2 (*acs2*), (C) ACC oxidase (*aco*), and (D) phenylalanine-ammonia lyase (*pal*) 1, 2, 3 and 4 days after inoculation. Hatched bars represent the gene expression in control samples of non-wounded (c) and wounded and mock-inoculated oranges at the same days after wounding. The expression induced by the different strains is indicated as follows: the parental strain CECT 20796 as black bars, the null Δ*afpB* (PDMG122) as grey bars, and the constitutive *afpB*^{C} as white bars. Gene expression was normalized independently for each gene to the control condition (c). In every case the bars show the mean ± standard error (SE) of three technical replicates. The figure shows data from a representative experiment out of two.

DISCUSSION

The phylogenetic analysis of the available AFP and AFP-like sequences that was conducted in this study led to the proposal of their classification in three classes (A, B and C). This proposal is based not only on phylogenetic clustering (Fig. 1A) but also on sequence alignment, cysteine pattern, intron position and Pfam domain identification (Fig. 1B, Table S1, and data not shown). This phylogenetic re-construction expands the two previously reported ones that divided the fungal AFPs into two clusters (Seibold et al. 2011; Galgóczy et al. 2013b): (i) proteins in a PAF-cluster homologous to the *P. chrysogenum* PAF and *A. giganteus* AFP proteins (which included all our class A proteins), and (ii) proteins in a BP-cluster (our class C). The recent discovery of the *P. digitatum afpB* gene and homologous genes from other fungi including *Penicillium* postharvest pathogens resulted in an additional class B cluster. The inclusion in our analyses of the distinct class of the previously identified fungal defensin-like proteins (DLP) (Zhu 2008) did not alter the grouping of the three classes described here (data not shown). We propose the suffix A, B or C to be added to the newly characterized *afp* genes and following this nomenclature we have named *afpB* to the *P. digitatum* gene described in this work.

A remarkable corollary of our study is that it is likely that additional AFP classes are identified following the study of yet uncharacterized genes and proteins. An example of this is the *Botrytis cinerea* A6SIA5, which is a predicted hypothetical protein (NCBI accession XP_001548954). We introduced this sequence because it is mentioned in a previous report (Galgóczy et al. 2013b). Our phylogenetic analyses demonstrate that this sequence diverged before the separation of classes A and B, but after the separation of class C from A and B (Fig. 1A). We cannot confirm that this hypothetical protein has antifungal activity and is the first member of an additional class of antifungal proteins, but this is an attractive hypothesis. In any case, it is increasingly clear that fungi have a complex repertoire of AFP, DLP and maybe other CRPs with antimicrobial properties that offer a remarkable novel source of potential antibiotic drugs (Mygind et al. 2005; Zhu 2008; Galgóczy et al. 2013b; and this study).

Noteworthy, some fungi have three AFP-like proteins, one of each class (for instance, *P. chrysogenum* or *P. expansum*), while others only have two (*N. fischeri* or *Penicillium oxalicum*) or one (*P. digitatum* or

Penicillium italicum). The PAF gene (class A) from *P. chrysogenum* is located in a genome region whose syntenic region is absent in the close relative *P. digitatum* (Marcet-Houben et al. 2012), suggesting that gene loss originated the variations in AFP number between different fungi. Therefore, it is relevant to study whether the distinct AFP-like proteins within a given fungus perform different biological functions. In addition, it is also relevant to initiate the molecular genetic characterization of a member of the class B proteins for the first time, in a fungus such as *P. digitatum* in which the only detected AFP gene belongs to this class. This is also the first AFP-like protein whose biological function is addressed in a pathogenic fungus.

The *afpB* gene is highly expressed at different stages of the biological cycle of *P. digitatum*. The studies described so far in *A*. *giganteus* and *P*. *chrysogenum* have concluded that maximum *afp*-like gene expression and protein recovery occur after reaching the stationary growth phase in submerged cultures (Meyer et al. 2002; Marx 2004). In the *A. giganteus afp*, gene expression was only detectable in the vegetative mycelium and no expression was observed in aerial hyphae or conidia (Meyer et al. 2002). The *P. chrysogenum paf* is expressed during asexual differentiation and no mRNA was detected in mature purified conidia (Hegedüs et al. 2011). These previous observations are qualitatively different from ours (Fig. 2). Although a detailed qRT-PCR quantification of *PdafpB* mRNA demonstrated a strong 10-fold increase during submerged growth, an additional greater than 10-fold increase was observed during aerial culture on solid medium, and purified conidia showed the highest mRNA amount. This high *PdafpB* mRNA concentration rapidly decreased during the incubation of conidia under germinating conditions. This observation suggests a role of *PdafpB* in the quiescent conidial cell that is dispensable after the onset of germination.

In accordance with a potential role in conidiogenesis, the *P. chrysogenum* Δ*paf* mutant showed a 30-70 % reduction in the concentration of conidia, depending on the experimental setup, and the colonies showed a clear phenotype of reduced conidiation (Hegedüs et al. 2011). The appearance of the colonies from the *P. digitatum* null Δ*pafB* mutant was completely normal (Fig. 3A) and a minor delay in conidiation was only noticeable after the quantification of conidia production. The constitutive *afpB*^{C} strain also presented a delay in conidiation. However,

both constitutive and null *P. digitatum* strains reached the amount of conidia that was produced by the wild type at the end of the experiment (Fig. 3). Therefore, although a potential role of *PdafpB* in conidiation cannot be discarded based on its gene expression and the phenotypes of the mutants, it seems that this effect is less drastic than in the case of the class A gene from *P. chrysogenum*.

Considering the previous data from other AFPs that are easily purified and the high induction of expression of *PdafpB*, it is discouraging the failure to detect the corresponding AfpB protein in various experiments conducted up to now (data not shown). Moreover, constitutive *afpB*^{C} mutants, which have more than a 100-fold increase in *PdafpB* mRNA compared to the parental strain (Fig. 5E), did not show a corresponding increase in any positive band, as determined by protein SDS-PAGE electrophoresis followed by mass spectrometry (data not shown). The class B proteins AnAFP (Gun et al. 1999) and PgAFP (Acosta et al. 2009) were purified from culture broth by means of ultrafiltration through MW cut-off membranes and chromatographic columns. Our diverse attempts to concentrate, enrich and/or fractionate the supernatants did not generate positive results and the AfpB protein could not be detected. One possibility is that post-translational modification changes the protein properties in such a way that previously used procedures are not effective. However, post-translational glycosylation has been discarded in other AFP-like proteins (Rodríguez-Martín et al. 2010; Seibold et al. 2011).The heterologous production of the class A protein NFAP from *N. fischeri* (hNFAP) has been reported in *A. nidulans* from the same constitutive promoter that we have used (*gpdA*), in an autonomous replicative plasmid (Galgóczy et al. 2013a). Future deep proteomic analyses of our different strains will try to shed light on the unexpected failure to detect AfpB in *P. digitatum*.

All the data that were obtained in our study on the null mutant supports the conclusion that the *afpB* gene is dispensable for the axenic growth and fruit infection of *P. digitatum*. On the other hand, the constitutive expression of *PdafpB* resulted in a drastic reduction in growth and virulence, and in hyphal morphology distortion (Figs. 3, 4 and 6). Considering that we have not demonstrated the production of the corresponding AfpB protein, we cannot attribute this phenotype to protein over-production. However the reduced growth phenotype is consistent with a potential self-poisoning

effect of AfpB, in a similar way to the hNFAP production in *A. nidulans* (Galgóczy et al. 2013a). *A. nidulans* that produced hNFAP showed abnormal and delayed germination, short germ tubes with extensive branching, and broken hyphal tips. Abnormal hyphae and hyper-branching are also observed in our constitutive *afpB*^{C} mutants (Fig. 4C). Nonetheless, the hyphal damage in the hNFAP *A. nidulans* strains was much more extensive, including destroyed chitin filaments and unstructured CW. All of our tests with CW interfering compounds indicate that the abnormal growth of the *afpB*^{C} strains is not due to a defective CW, in contrast to other *P. digitatum* mutants that have mutations in CW-related genes (Gandía et al. 2014; Harries et al. 2015).

At present, we cannot provide a reasonable explanation for the unstable phenotype of the constitutive *afpB*^{C} mutants (Fig. 5). The molecular or physiological causes remain undetermined but our data indicate that they are not related to gene silencing (as mRNA accumulation does not change), alterations of DNA locus, intron processing, or changes in the accumulation of proteins. Importantly, this finding was observed (with more or less intensity) in all of the independent strains that were analysed (Fig. 5 and data not shown), demonstrating that this finding is not due to differences in the genomic locus where the T-DNA is inserted. This behaviour is unique in all of the genetically modified *P. digitatum* strains that we manage in our laboratory. Possible hypotheses are that this instability is related to the failure to detect the AfpB protein or yet unidentified biological function(s) of the gene or protein. These will be explored in the near future.

The strong reduction of virulence in the constitutive *afpB*^{C} mutants is likely a consequence of their impairment in vegetative growth and thus must be considered a phenotype that is not strictly related to pathogenesis. However, the macroscopic appearance of tissue necrotic areas around the inoculation site (Fig. 6E) and the altered timing of expression of citrus defence genes (Fig. 7) also indicate that the restricted growth of the mutant enables the fruit to mount an effective response and takeover fungal infection, similarly to experimental treatments that elicit citrus fruit resistance to *P. digitatum* (Ballester et al. 2011; Ballester et al. 2013a). In these later studies, defence-related phenolic compounds were locally induced and accumulated around the inoculation site where infection is

restricted. Therefore, the constitutive *afpB*^{C} strains might become a powerful tool to study and characterize the fruit defence response to *P. digitatum* infection.

In conclusion, we have proposed in this study a refined classification for the AFPs of fungal origin, and addressed the characterization of a novel class of *afp* genes in the fungal plant pathogen *P. digitatum*. Our data demonstrate that the *afpB* gene is dispensable for the completion of the life cycle of *P. digitatum* and that its constitutive expression has a negative impact on fungal growth and virulence. Open issues of our work are the inability to detect the corresponding AfpB protein despite the high gene expression and the instability of the phenotype of constitutive *afpB* transformants.

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COMPLIANCE WITH ETHICAL STANDARDS

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CONFLICT OF INTEREST

Authors SG, MG and JFM declare that they have no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

Acosta R, Rodríguez-Martín A, Martín A, Nuñez F, Asensio MA (2009) Selection of antifungal protein-producing molds from dry-cured meat products. Int J Food Microbiol 135**:**39-46

Alós E, Distefano G, Rodrigo MJ, Gentile A, Zacarías L (2014) Altered sensitivity to ethylene in 'Tardivo', a late-ripening mutant of Clementine mandarin. Physiol Plant 151**:**507-521

Ballester AR, Lafuente MT, Forment J, Gadea J, De Vos RCH, Bovy AG, González-Candelas L (2011) Transcriptomic profiling of citrus fruit peel tissues reveals fundamental effects of phenylpropanoids and ethylene on induced resistance. Mol Plant Pathol 12**:**879-897

Ballester AR, Lafuente MT, De Vos RCH, Bovy AG, González-Candelas L (2013a) Citrus phenylpropanoids and defence against pathogens. Part I: Metabolic profiling in elicited fruits. Food Chem 136**:**178-185

Ballester AR, Teresa Lafuente M, González-Candelas L (2013b) Citrus phenylpropanoids and defence against pathogens. Part II: gene expression and metabolite accumulation in the response of fruits to *Penicillium digitatum* infection. Food Chem 136**:**285-291

Batta G, Barna T, Gáspári Z, Sándor S, Kövér KE, Binder U, Sarg B, Kaiserer L, Chhillar AK, Eigentler A, Leiter E, Hegedüs N, Pócsi I, Lindner H, Marx F (2009) Functional aspects of the solution structure and dynamics of PAF - A highly-stable antifungal protein from *Penicillium chrysogenum*. FEBS J 276**:**2875-2890

Brogden KA (2005) Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3**:**238-250

Campos-Olivas R, Bruix M, Santoro J, Lacadena J, Martínez del Pozo A, Gavilanes JG, Rico M (1995) NMR solution structure of the antifungal protein from *Aspergillus giganteus*: evidence for cysteine pairing isomerism. Biochemistry 34**:**3009-3021

Coca M, Bortolotti C, Rufat M, Peñas G, Eritja R, Tharreau D, Martínez del Pozo A, Messeguer J, San Segundo B (2004) Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. Plant Mol Biol 54**:**245-259

Chen Z, Ao J, Yang W, Jiao L, Zheng T, Chen X (2013) Purification and characterization of a novel antifungal protein secreted by *Penicillium chrysogenum* from an Arctic sediment. App Microbiol Biotech 97**:**10381-10390

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484**:**186-94

Fjell CD, Hiss JA, Hancock REW, Schneider G (2012) Designing antimicrobial peptides: Form follows function. Nat Rev Drug Discov 11**:**37-51

Galgóczy L, Kovács L, Vágvölgyi C (2010) Defensin-like antifungal proteins secreted by filamentous fungi. In: Méndez-Vila A (eds) Current Research, Technology and Education Topics in Applied and Microbial Biotechnology. Formatex, pp. 550-559.

Galgóczy L, Kovács L, Karácsony Z, Virágh M, Hamari Z, Vágvölgyi C (2013a) Investigation of the antimicrobial effect of *Neosartorya fischeri* antifungal protein (NFAP) after heterologous expression in *Aspergillus nidulans*. Microbiology (United Kingdom) 159**:**411-419

Galgóczy L, Virágh M, Kovács L, Tóth B, Papp T, Vágvölgyi C (2013b) Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among Fusaria. Peptides 39**:**131-137

Gandía M, Harries E, Marcos JF (2012) Identification and characterization of chitin synthase genes in the postharvest citrus fruit pathogen *Penicillium digitatum*. Fungal Biol 116**:**654-664

Gandía M, Harries E, Marcos JF (2014) The myosin motor domain-containing chitin synthase PdChsVII is required for development, cell wall integrity and virulence in the citrus postharvest pathogen *Penicillium digitatum*. Fungal Genet Biol 67**:**58-70

González-Candelas L, Alamar S, Sánchez-Torres P, Zacarías L, Marcos JF (2010) A transcriptomic approach highlights induction of secondary metabolism in citrus fruit in response to *Penicillium digitatum* infection. BMC Plant Biol 10**:**194

Gun L. D, Yub Shin S, Maeng CY, Zhu Jin Z, Lyong Kim K, Hahm KS (1999) Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. Biochem Biophys Res Commun 263**:**646-651

Harries E, Gandía M, Carmona L, Marcos JF (2015) The *Penicillium digitatum* protein *O*–Mannosyltransferase Pmt2 is required for cell wall integrity, conidiogenesis, virulence and sensitivity to the antifungal peptide PAF26. Mol Plant Pathol

Hegedüs N, Sigl C, Zadra I, Pocsi I, Marx F (2011) The *paf* gene product modulates asexual development in *Penicillium chrysogenum*. J Basic Microbiol 51**:**253-262

Hegedüs N, Marx F (2013) Antifungal proteins: More than antimicrobials? Fungal Biol Rev 26**:**132-145

Khang CH, Park SY, Rho HS, Lee YH, Kang S (2006) Filamentous fungi (*Magnaporthe grisea* and *Fusarium oxysporum*). Meth Mol Biol 344**:**403-420

Kovács L, Virágh M, Takó M, Papp T, Vágvölgyi C, Galgóczy L (2011) Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). Peptides 32**:**1724-1731

Lacadena J, Martínez del Pozo A, Gasset M, Patino B, Campos-Olivas R, Vázquez C, Martínez-Ruiz A, Mancheño JM, Oñaderra M, Gavilanes JG (1995) Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. Arch Biochem Biophys 324**:**273-281

Macarisin D, Cohen L, Eick A, Rafael G, Belausov E, Wisniewski M, Droby S (2007) *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology 97**:**1491-1500

Marcet-Houben M, Ballester A-R, de la Fuente B, Harries E, Marcos JF, Gonzalez-Candelas L, Gabaldon T (2012) Genome sequence of the necrotrophic fungus *Penicillium digitatum*, the main postharvest pathogen of citrus. BMC Genomics 13**:**646

Marcos JF, González-Candelas L, Zacarías L (2005) Involvement of ethylene biosynthesis and perception in the susceptibility of citrus fruits to *Penicillium digitatum* infection and the accumulation of defence-related mRNAs. J Exp Bot 56**:**2183-2193

Marcos JF, Muñoz A, Pérez-Payá E, Misra S, López-García B (2008) Identification and rational design of novel antimicrobial peptides for plant protection. Annu Rev Phytopathol 46**:**273-301

Marx F (2004) Small, basic antifungal proteins secreted from filamentous ascomycetes: A comparative study regarding, expression, structure, function and potential application. App Microbiol Biotech 65**:**133-142

Marx F, Binder U, Leiter É, Pócsi I (2008) The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies. Cell Mol Life Sci 65**:**445-454

Meyer V, Wedde M, Stahl U (2002) Transcriptional regulation of the Antifungal Protein in *Aspergillus giganteus*. Molecular Genetics and Genomics 266**:**747-757

Meyer V (2008) A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. App Microbiol Biotech 78**:**17-28

Michielse CB, Hooykaas PJJ, van den Hondel CAMJ, Ram AFJ (2008) *Agrobacterium*-mediated transformation of the filamentous fungus *Aspergillus awamori* Nat Protoc 3**:**1671-1678

Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An efficient tool for insertional mutagenesis and gene transfer. Phytopathology 91**:**173-180

Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sonksen CP, Ludvigsen S, Raventos D, Buskov S, Christensen B, De Maria L, Taboureau O, Yaver D, Elvig-Jorgensen SG, Sorensen MV, Christensen BE, Kjaerulff S, Frimodt-Moller N, Lehrer RI, Zasloff M, Kristensen HH (2005) Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature 437**:**975-980

Nakaya K, Omata K, Okahashi I, Nakamura Y, Kolekenbrock H, Ulbrich N (1990) Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus*. Eur J Biochem 193**:**31-38

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nuc Acids Res 30**:**e36

Rodrigo MJ, Marcos JF, Zacarías L (2004) Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. J Agric Food Chem 52**:**6724-6731

Rodríguez-Martín A, Acosta R, Liddell S, Nuñez F, Benito MJ, Asensio MA (2010) Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. Peptides 31**:**541-547

Romero P, Gandía M, Alférez F (2013) Interplay between ABA and phospholipases A2 and D in the response of citrus fruit to postharvest dehydration. Plant Physiol Biochem 70**:**287-294

Seibold M, Wolschann P, Bodevin S, Olsen O (2011) Properties of the bubble protein, a defensin and an abundant component of a fungal exudate. Peptides 32**:**1989-1995

Shah DM, Read ND (2013) Antifungal peptides come of age. Fungal Biol Rev 26**:**107-108

Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR (2006) Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat Protoc 1**:**3111-3120

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30**:**2725-9

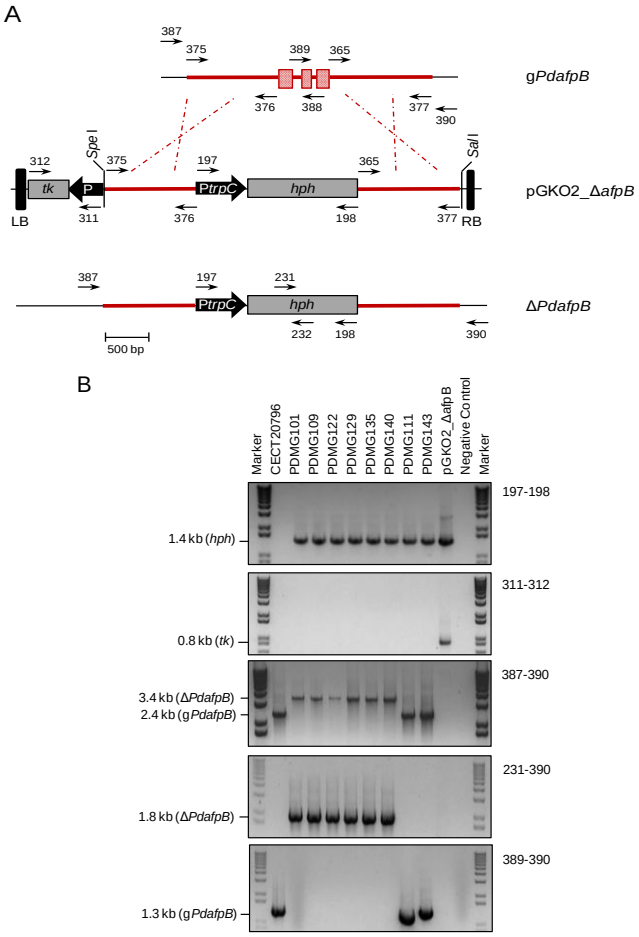
Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuc Acids Res 22**:**4673-4680

Wnendt S, Ulbrich N, Stahl U (1994) Molecular cloning, sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*. Curr Genet 25**:**519-523

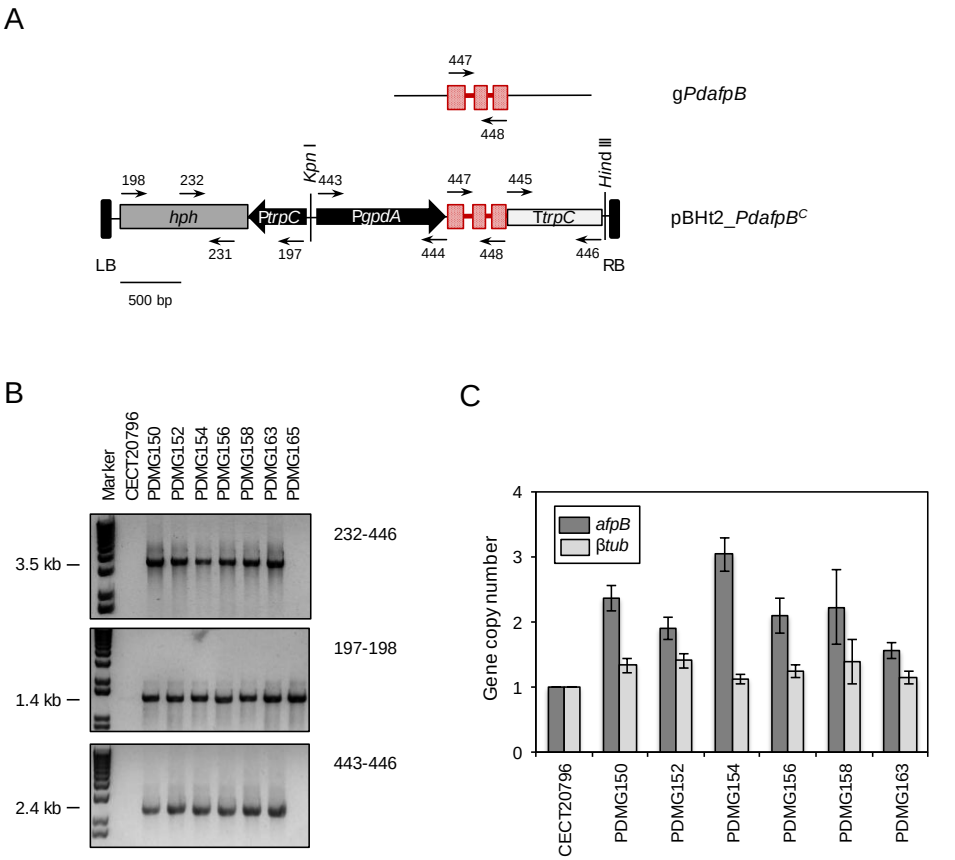
Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415**:**389-395

Zhu S (2008) Discovery of six families of fungal defensin-like peptides provides insights into origin and evolution of the CSabdefensins. Mol Immunol 45**:**828-838

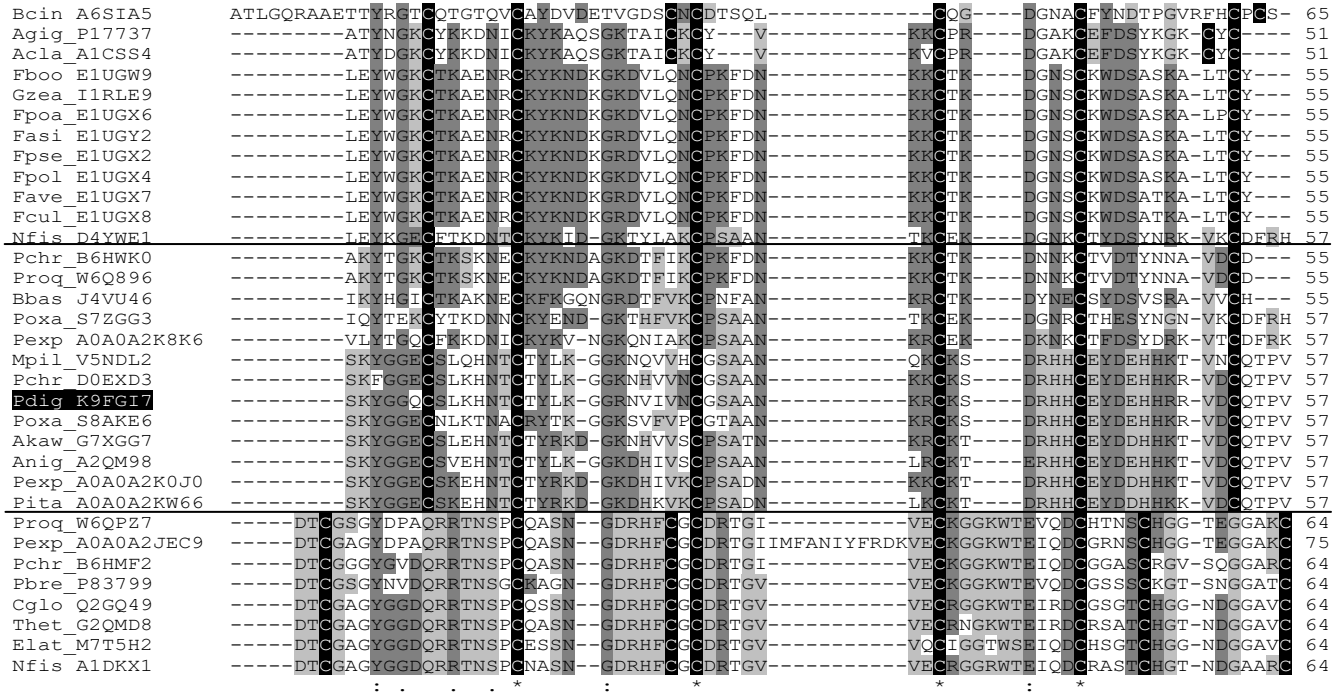
SUPPLEMENTARY MATERIAL



Supp. Fig. 1: Generation of *P. digitatum afpB* deletion strains and confirmation by PCR analysis. (A) Schematic representation of the *PdafpB* gene in the parental strain CECT 20796 (top), the pGKO2_Δ*afpB* vector designed for gene deletion (middle), and the Δ*PdafpB* deleted gene (bottom). All the primers used for PCR analysis are localized in the figure. (B) PCR amplification of genomic DNA of the distinct *P. digitatum* strains with different primer pairs as indicated. All the positive transformants showed an amplicon with primers 197/198 indicating the presence of the hygromycin marker (first panel, *hph*). Only the pGKO2_Δ*afpB* vector showed, as expected, an amplicon of 800 bp using primers 311/312 for HSVtk gene (second panel, *tk*). The deletion (Δ*afpB*) mutants (PDMG101 to PDMG135) showed an amplicon of 3.4 kb with primers 387/390, while the strain CECT 20796 or the ectopic transformants (PDMG111 and PDMG143) showed an amplicon of 2.4 kb (third panel). The Δ*afpB* mutants showed an expected amplification band of 1.8 kb from the 3´region of the deleted *PdafpB* gene amplified with primers 231/390 (fourth panel) or, similarly, a 1.7 kb band from the 5´region amplified with primers 387/232 (data not shown). These set of primers did not produce amplicons either in the wild type strain CECT 20796 or the ectopic transformants PDMG111 and PDMG143 (fourth panel and data not shown). The parental and ectopic strains showed an amplicon of 1.3 kb from the 3´region of the *PdafpB* gene with primers 389/390 (fifth panel) or amplicons of 1.2 kb from the 5´region of the *PdafpB* gene with primers 387/388 (data not shown). These primers did not produce amplicons in the deletion strains (PDMG101 to PDMG135) (fifth panel and data not shown).



Supp. Fig. 2: Generation of *P. digitatum afpB* constitutive transformants, confirmation by PCR analysis and analysis of gene copy number. (A) Schematic representation of the *PdafpB* gene in the parental strain CECT 20796 (top), and of the *PdafpB*^{C} construct for constitutive expression under the *gpdA* promoter in the pBHt2 vector (pBHt2_*PdafpB*^{C}, bottom). All the primers used for PCR analysis are localized in the figure. (B) PCR amplification of genomic DNA of the distinct *P. digitatum* strains with different primer pairs as indicated. The constitutive expression mutants (PDMG150 to PDMG163) showed an amplicon of 3.5 kb with primers 232/446, while the parental strain CECT 20796 or the negative transformant PDMG165 did not show any amplicon (first panel). All the transformants showed a positive amplicon of 1.4 kb with primers 197/198 indicating the presence of the hygromycin marker (second panel). Only the constitutive transformants showed, as expected, a positive amplicon of 2.4 kb with primers 443/446 that amplified the entire constitutive construction (third panel). (C) Evaluation of *afpB* gene copy number in the different constitutive expression transformants (PDMG150 to PDMG163). Genomic DNA was subjected to quantitative PCR with primers specific for *afpB* (466/467) and for the single copy control genes β*tub* (85/86) and *L18a* (151/152). The Ct signal of *afpB* and β*tub* primers was normalized to that of *L18a* used as internal control and to the signal of the parental CECT 20796, and the results are presented as mean values ± SD of three technical replicates. Under this experimental design, the resulting gene copy number is expected to be 1 for β*tub* in all strains, 1 for *afpB* in CECT 20796, and ≥ 2 for *afpB* in all transformants.



Supp. Fig. 3: Sequence alignment of AFP and AFP-like proteins of filamentous fungi. Amino acid sequence alignment of the complete set of mature AFPs and putative AFPs used for phylogenetic analysis in this study (see Fig. 1, main text). Proteins belonging to different classes are separated by a line. Cysteine patterns are shadowed in black. Strongly conserved amino acids (or with the same chemical properties) are highlighted in dark grey. Other conserved amino acids are shadowed in light grey. The PdAfpB is highlighted in black.

Supp. Table 1: AFP and AFP-like sequences used in the phylogenetic analysis.

Organism	Abbreviation	UNIPROT ID	GENBANK ID	UNIPROT annotation	GENBANK annotation	Pfam domains	Antifungal Activity
Aspergillus giganteus	Agig	P17737	CAA37523.1	Experimental evidence at protein level	antifungal protein	PF11402 - Antifungal_prot	+
Aspergillus clavatus	Acla	A1CSS4	XP_001267787.1.	Protein predicted	antifungal protein Afp	PF11402 - Antifungal_prot	+
Fusarium boothi	Fboo	E1UGW9	CAR79010.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Gibberella zeae	Gzea	I1RLE9	ESU10610.1.	Protein predicted	hypothetical protein	PF11402 - Antifungal_prot	
Fusarium poae	Fpoa	E1UGX6	CAR79017.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Fusarium asiaticum	Fasi	E1UGY2	CAR79023.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Fusarium pseudograminearum	Fpse	E1UGX2	CAR79013.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Fusarium polyphialidicum	Fpol	E1UGX4	CAR79015.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	+
Fusarium avenaceum	Fave	E1UGX7	CAR79018.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Fusarium culmorum	Fcul	E1UGX8	CAR79019.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Neosartorya fischeri	Nfis	D4YWE1	CAQ42994.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	+
Penicillium chrysogenum	Pchr	B6HWK0	CAP86946.1.	Protein predicted	Pc24g00380	PF11402 - Antifungal_prot	+
Penicillium roqueforti	Proq	W6Q896	CDM32600.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Beauveria bassiana	Bbas	J4VU46	EJP62050.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Penicillium oxalicum	Poxa	S7ZGG3	EPS29334.1.	Protein predicted	hypothetical protein	PF11402 - Antifungal_prot	
Penicillium expansum	Pexp	A0A0A2K8K6	KGO45316.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Monascus pilosus	Mpil	V5NDL2	AHA86567.1.	Protein predicted	MAFP1	PF11402 - Antifungal_prot	+
Penicillium chrysogenum	Pchr	D0EXD3	ACX54052.1.	Experimental evidence at protein level	antifungal protein preproprotein	PF11402 - Antifungal_prot	+
Penicillium digitatum	Pdig	K9FGI7	EKV08309.1.	Protein predicted	antifungal protein Afp	PF11402 - Antifungal_prot	
Penicillium oxalicum	Poxa	S8AKE6	EPS26293.1.	Protein predicted	hypothetical protein	PF11402 - Antifungal_prot	
Aspergillus kawachii	Akaw	G7XGG7	GAA86026.1.	Protein predicted	hypothetical protein	PF11402 - Antifungal_prot	
Aspergillus niger	Anig	A2QM98	CAK96579.1.	Protein predicted	unnamed protein product	PF11402 - Antifungal_prot	+
Penicillium expansum	Pexp	A0A0A2K0J0	KGO45770.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Penicillium italicum	Pita	A0A0A2KW66	KGO72077.1.	Protein predicted	antifungal protein	PF11402 - Antifungal_prot	
Penicillium roqueforti	Proq	W6QPZ7	CDM36154.1.	Protein predicted	Bubble protein	PF09227	
Penicillium expansum	Pexp	A0A0A2JEC9	KGO48297.1.	Protein predicted	Protein of unknown function	PF09227	
Penicillium chrysogenum	Pchr	B6HMF2	CAP96194.1.	Protein predicted	Pc21g12970	PF09227	+
Penicillium brevicompactum	Pbre	P83799	P83799.1	Experimental evidence at protein level	Bubble protein	PF09227	+
Chaetomium globosum	Cglo	Q2GQ49	EAQ83501.1.	Protein predicted	hypothetical protein	PF09227	
Thielavia heterothallica	Thet	G2QMD8	AEO61118.1.	Protein predicted	hypothetical protein	PF09227	
Eutypa lata	Elat	M7T5H2	EMR61895.1.	Protein predicted	putative bubble protein precursor	PF09227	
Neosartorya fischeri	Nfis	A1DKX1	EAW15442.1.	Protein predicted	conserved hypothetical protein	PF09227	
Botrytis cinerea	Bcin	A6SIA5	XP_001548954.1	Predicted protein	hypothetical protein	none	

Antifungal Activity: "+" denotes protein for which experimental evidence of antifungal activity exists

Supp. Table 2: Primers used in this study.

Name	Use	Sequence 5´- 3´	Tm (ºC)	Restriction sites	Gene	Description
OJM466	F	GACGATCTTGATGCCCGAG	59		*afpB*	qRT-PCR
OJM467	R	AGTCAACCCTCCTGTGGTG	59		*afpB*	qRT-PCR
OJM151	F	TGGGGCAGAGGGAACTTGAG	65		*L18a*	qRT-PCR
OJM152	R	ACCGACGCTGTTGAGGCTCT	65		*L18a*	qRT-PCR
OJM85	F	AGCGGTGACAAGTACGTTCC	65		*βTub*	qRT-PCR
OJM86	R	ACCCTTGGCCCAGTTGTTAC	65		*βTub*	qRT-PCR
OJM334	F	CGACTTCAGGAAGGGGTGTA	56		*rRNA 18S*	qRT-PCR
OJM335	R	CTTGGATGTGGTAGCCGTT	56		*rRNA 18S*	qRT-PCR
OJM199	F	CACAGTGTTCACCAAGGAGTC	60		*acs1*	qRT-PCR
OJM200	R	CGAGTAAATGATACCGACCCTAA	60		*acs1*	qRT-PCR
OJM201	F	TTCGAATCCACTAGGCACAACTT	60		*acs2*	qRT-PCR
OJM202	R	CAACGCTCGTGAACTTAGGAGA	60		*acs2*	qRT-PCR
OJM203	F	TGGAGCACAGAGTGGTTTCTC	60		*aco*	qRT-PCR
OJM204	R	GGATAGATCACAGCATCACTTCC	60		*aco*	qRT-PCR
MJ273F	F	GTTGCATGAGATTGATCCTCTGC	60		*pal1*	qRT-PCR
MJ274R	R	TGTCATTCACCGAGTTGATCTCC	60		*pal1*	qRT-PCR
CsACT	F	TTAACCCCAAGGCCAACAGA	60		*act*	qRT-PCR
CsACT	R	TCCCTCATAGATTGGTACAGTATGAGA	60		*act*	qRT-PCR
CsGAPDH	F	CGTCCCTCTGCAAGATGACTCT	60		*gapdh*	qRT-PCR
CsGAPDH	R	GGAAGGTCAAGATCGGAATCAA	60		*gapdh*	qRT-PCR
OJM375	F	GG**TCTAGA**GGCTTGGTGCATGAATTCCACC	60	*Xba*I	*afpB*	Fungal transformation and transformant verification
OJM377	R	CA**GTCGAC**CTTTGAATCTTAACTTTAATGG	60	*Sal*I	*afpB*	Fungal transformation and transformant verification
OJM376	R	ATGCTCCTTCAATATCAGTTAACGCGAAGATTGTGGTATGGTAGTTG	60		*afpB:hph*	Fungal transformation and transformant verification
OJM365	F	CCGACCGGGAACCAGTTAACACCAATTCCGGGCCATTTGATC	60		*afpB:hph*	Fungal transformation and transformant verification
OJM443	F	C**GAGCTCGGTACC**CTCGAGTACCATTTAATT	60	*Sac*I;*Kpn*I	P*gpdA*	Fungal transformation and transformant verification
OJM444	R	GGTGATGTCTGCTCAAGCGGGG	60		P*gpdA*	Fungal transformation and transformant verification
OJM445	F	GGATCCACTTAACGTTACTGAAATC	60		T*trpC*	Fungal transformation and transformant verification
OJM446	R	G**CTGCAGAAGCTT**TCGAGTGGAGATGTGGAGTG	60	*Pst*I;*Hind*III	T*trpC*	Fungal transformation and transformant verification

OJM447	F	CCCCGCTTGAGCAGACATCACCATGCAGATTACCAGCATTGCC	60		P*gpdA::afpB*	Fungal transformation and transformant verification
OJM448	R	GATTTCAGTAACGTTAAGTGGATCCTCAAACTGGAGTCTGGCAGTC	60		*afpB*::T*trpC*	Fungal transformation and transformant verification
OJM387	F	CGCTGATATTAATCTACCGC	60		*afpB*	Fungal transformation and transformant verification
OJM388	R	CCACCCTTCAGGTACGTGCACG	60		*afpB*	Fungal transformation and transformant verification
OJM389	F	GCGTCGGACGATCTTGATGCCC	60		*afpB*	Fungal transformation and transformant verification
OJM390	R	CGCGACAGTACTCAAACAAGC	60		*afpB*	Fungal transformation and transformant verification
OJM431	F	**CCCGGG**ATGCAGATTACCAGCATTGCC	60	*Sma*I;*Xma*I	*afpB*	Fungal transformation and transformant verification
OJM432	R	**CCCGGG**TCAAACTGGAGTCTGGC	60	*Sma*I;*Xma*I	*afpB*	Fungal transformation and transformant verification
OJM197	F	C**GTTAAC**TGATATTGAAGGAGCAT	60	*Hpa*I	*hph*	Fungal transformation and transformant verification
OJM198	R	T**GTTAAC**TGGTTCCCGGTCGG	60	*Hpa*I	*hph*	Fungal transformation and transformant verification
OJM231	F	GTTGCAAGACCTGCCTGAAACC	60		*hph*	Fungal transformation and transformant verification
OJM232	R	GTTTGCCAGTGATACACATGGG	60		*hph*	Fungal transformation and transformant verification
OJM311	F	CCACGGAAGTCCGCCCGGAGC	60		HSV*tk*	Fungal transformation and transformant verification
OJM312	R	GACGTGCATGGAACGGAGGCG	60		HSV*tk*	Fungal transformation and transformant verification

Restriction sites are in bold.

CHAPTER II

CHAPTER II

Mapping and identification of antifungal peptides in the putative antifungal protein AfpB from the filamentous fungus *Penicillium digitatum*

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ABSTRACT

Antifungal proteins (AFPs) from Ascomycetes are small cysteine-rich proteins that are abundantly secreted and show antifungal activity against non-producer fungi. A gene coding for a class B AFP (AfpB) was previously identified in the genome of the plant pathogen *Penicillium digitatum*. However, previous attempts to detect AfpB were not successful despite high gene expression. In this work, the structure of the putative AfpB was modeled. Based on this model, four synthetic cysteine-containing peptides, PAF109, PAF112, PAF118 and PAF119, were designed and their antimicrobial activity was tested and characterized. PAF109 that corresponds to the γ-core motif present in defensin-like antimicrobial proteins did not show antimicrobial activity. On the contrary, PAF112 and PAF118, which are cationic peptides derived from two surface-exposed loops in AfpB, showed moderate antifungal activity against *P. digitatum* and other filamentous fungi. It was also confirmed that cyclization through a disulfide bridge prevented peptide degradation. PAF116, which is a peptide analogous to PAF112 but derived from the *Penicillium chrysogenum* antifungal protein PAF, showed activity against *P. digitatum* similar to PAF112, but was less active than the native PAF protein. The two AfpB-derived antifungal peptides PAF112 and PAF118 showed positive synergistic interaction when combined against *P. digitatum*. Furthermore, the synthetic hexapeptide PAF26 previously described in our laboratory also exhibited synergistic interaction with the peptides PAF112, PAF118 and PAF116, as well as with the PAF protein. This study is an important contribution to the mapping of antifungal motifs within the AfpB and other AFPs, and opens up new strategies for the rational design and application of antifungal peptides and proteins.

Keywords: Antifungal peptides, antifungal proteins, protein mapping, peptide design, *Penicillium digitatum*, postharvest pathology, *Penicillium chrysogenum*, synergism.

INTRODUCTION

There is an urgent need to develop new antifungal molecules with properties and mechanisms of action different from existing ones (Brown et al., 2012; Fisher et al., 2012). Antimicrobial peptides and proteins (AMPs) have been found in a broad variety of species (Hancock and Sahl, 2006) and are candidates for the development of novel therapeutic compounds (Zasloff, 2002; Brogden, 2005; Fjell et al., 2012) including antifungals (Shah and Read, 2013). A remarkable group of AMPs are the antifungal proteins (AFPs) of fungal origin. AFPs belong to the broad class of defensins and are produced by some species of filamentous Ascomycetes, mostly from the genus *Aspergillus* and *Penicillium* (Marx et al., 2008; Meyer, 2008; Galgóczy et al., 2010; Silva et al., 2014). AFPs are small (~50 amino acid residues), secreted, amphipathic, cationic and cysteine-rich proteins (CRPs) that contain 6 to 8 cysteine residues and fold into compact disulfide-stabilized structures with five β-strands, which confer high stability under adverse biochemical and biophysical conditions (Batta et al., 2009). All the AFPs contain the so-called ɣ-core, a three-dimensional peptide signature present in antimicrobial CRPs spanning biological kingdoms (Yount and Yeaman, 2004). One of the most studied AFPs is the *Penicillium chrysogenum* PAF protein (55 amino acids in length) that can be easily purified from the culture supernatants of the producer fungus *P*. *chrysogenum* (Marx et al., 2008). PAF inhibits the growth of filamentous human and plant pathogenic fungi at µM concentrations and is non-toxic to mammalian cells *in vitro* (Marx et al., 2008) and *in vivo* (Palicz et al., 2013).

A significant diversity of AFP-like genes and proteins are widespread in Ascomycetes, with genomes that encode up to three different sequence-related AFPs (Galgóczy et al., 2013; Garrigues et al., 2016; Tóth et al., 2016), providing a rich source of potentially divergent antifungals. Bioinformatic and phylogenetic analyses suggested the classification of the different AFP-like sequences in at least three different classes: A, B and C (Garrigues et al., 2016). Notably, these fungal genomes code for a different number of AFP-like proteins that belong to diverse classes; *P. chrysogenum* encodes three (classes A, B, and C), *Penicillium roqueforti* two (A and C), and *Penicillium digitatum* just one (B). There is also a significant variation in the amount of AFP produced by each fungus and the growth conditions required to achieve maximum yields. Thus, the above mentioned PAF protein is secreted in large amounts by *P. chrysogenum*

(Marx et al., 2008), while the NFAP and NFAP2 from *Neosartoria fischeri* are produced in modest amounts (Kovács et al., 2011; Virágh et al., 2014). An extreme example of this scenario is that of the previously characterized *afpB* gene identified in the genome of the phytopathogenic fungus *P. digitatum* (Garrigues et al., 2016). Attempts to identify the AfpB protein in the small size protein fraction secreted by the fungus were not successful, even in the constitutive expressing strains that produce up to 1,000 times more *afpB* mRNA than the wild-type (Garrigues et al., 2016). This unexpected finding was one of the main reasons that led us to conduct the study reported here.

In-depth understanding of the structure and mode of action of AFPs is required for their potential future application as antifungal compounds. In addition, AFP and AFP-like sequences are a rich source for the identification and rational design of novel antimicrobial peptides with improved properties (Marcos et al., 2008; Marcos et al., 2012). Finally, the structural domains that account for the antifungal activity of AFPs remain to be fully identified and characterized. In this study, the structure of the putative *P. digitatum* AfpB protein was predicted by *in silico* molecular modeling to aid in the design of peptides based on the AfpB primary sequence and structure, whose antifungal activity was demonstrated and characterized. With this rational approach, peptides that have moderate but specific antifungal activity were identified within the AfpB and PAF amino acid sequences. Importantly, the previously characterized PAF26 hexapeptide (Muñoz et al., 2013a) showed positive synergistic interaction with the AFP-derived peptides and the PAF protein.

MATERIALS AND METHODS

Strains and media

The fungal strains used in this study are *P. digitatum* CECT 20796, *P. chrysogenum* Q176, *Botrytis cinerea* CECT2100, *Fusarium oxysporum* 4287, and *Aspergillus niger* CBS 120.49. Fungi were cultured on potato dextrose agar (PDA) (Difco-BD Diagnostics, Sparks, MD, USA) plates for 7-10 days at 24 ºC with the exception of *F. oxysporum*, which was cultured on potato dextrose broth (PDB) (Difco-BD Diagnostics) at 28 ºC for 4 days with shaking. Conidia (mitotic asexual spores) were collected, filtered and adjusted to the appropriate concentration. For antibacterial assays, the Gram-negative bacterium *E. coli* JM109 was grown in Luria-Bertani (LB) medium at 37 ºC with shaking.

In silico calculations and AfpB structure prediction

The SWISS-MODEL program (http://swissmodel.expasy.org/) (Arnold et al., 2006) was used to predict the 3D structure of the *P. digitatum* antifungal protein AfpB (Garrigues et al., 2016) using the *P. chrysogenum* antifungal protein PAF as template (PDB ID 2MHV) (Fizil et al., 2015). The model obtained was refined using the ModRefiner tool (http://zhanglab.ccmb.med.umich.edu/ModRefiner/) (Xu and Zhang, 2011) and subsequently validated by RAMPAGE to ensure that all the amino acids of the AfpB model were located inside the favored and energetically allowed regions according to the Ramachandran Plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) (Lovell et al., 2003).

The theoretical molecular weight, the pI and the GRAVY of the mature AfpB protein and peptides derived therefrom were examined with the Compute pI/MW and ProtParam tools of the ExPASy Proteomics Server (http://web.expasy.org) (Gasteiger et al., 2005), respectively. The signal peptide and disulfide bridges of the mature protein were predicted using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011) and the DISULFIND software (http://disulfind.dsi.unifi.it/) (Ceroni et al., 2006), respectively. The 3D model of AfpB was visualized by UCSF Chimera software (Pettersen et al., 2004).

PAF protein and synthetic peptides

The PAF protein was produced by *P. chrysogenum* and purified as previously described (Batta et al., 2009; Sonderegger et al., 2016). All the peptides used in this work were synthetic and purchased at >95 % purity from GenScript (Piscataway, NJ, USA) wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. The sequences of the peptides used and their physicochemical properties are listed in Table 1. Peptides PAF109, PAF112, PAF118 and PAF119 derived from the AfpB protein, and peptide PAF116 derived from the PAF protein, were synthetized with a disulfide bond linking the two cysteine residues, respectively. Stock solutions of peptides were prepared at 5.12 mM in 10 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, MO, USA) pH 7 and stored at -20 ºC. The peptide concentrations were prepared by dissolving a given peptide amount (mg) in MOPS buffer (mL), considering the purity of each of the peptides provided by the manufacturer. Additionally, the PAF26 concentration was confirmed spectrophotometrically by measuring the absorbance at 280 nm ($\varepsilon 280 = 5600\ M^{-1}\ cm^{-1}$ for W residue).

ECD spectroscopy

ECD spectroscopic measurements were performed in the 195-260 nm wavelength range (far-UV) to determine the secondary structure of the AfpB-derived peptides. Peptide samples were dissolved in H_2O, and in a 50% Trifuoroethanol (TFE) /H_2O mixture at approximately 0.1 mg/mL concentration and measured in a 0.1 cm path-length quartz cuvette using a Jasco J-815 spectropolarimeter at a scan speed of 100 nm/s at 25 ºC. Solvent spectra were measured similarly and subtracted from the corresponding spectra of peptides. Ellipticity data were given in mdeg units.

Antimicrobial activity assays

Growth inhibition assays were performed in 96-well microtiter plates (Nunc, Roskilde, Denmark) in a total volume of 100 µL as described previously (López-García et al., 2002). A volume of 90 µL of fungal conidia (2.5×10^4 conidia/mL) or bacterial cells (5×10^5 cells/mL) in appropriate growth media (1/20 diluted PDB containing 0.01 % (w/v) chloramphenicol for fungi or 1/10 diluted LB for bacteria) was mixed in each well with 10 µL of 10x concentrated peptide or PAF protein solution from serial 2-fold dilutions (from 1 to 128 µM for PAF109, PAF112, PAF113, PAF116,

PAF118 and PAF119; from 0.25 to 4 µM for PAF26; and from 1 to 16 µM for the protein PAF from *P. chrysogenum*). All samples were prepared in triplicate. Plates were statically incubated for 72 h at the optimal temperature of each microorganism. Growth was determined by measuring the OD at 600 nm (OD_{600}) using a Multiskan Spectrum plate spectrophotometer (Thermo Electron Corporation, Vantaa, Finland), and the OD_{600} mean and SD were calculated. Dose-response curves were generated from measurements after 72 h for fungi, and 48 h for *E. coli*. These experiments were repeated at least twice. The MIC is the peptide concentration that completely inhibited growth in all the experiments performed.

For assays of synergy, different combinations of peptides and concentrations were tested in 96-well microtiter plates. Ten microliters of two different 10x concentrated peptides were mixed in the same well with 80 µL of *P. digitatum* conidia prepared and grown as above (total volume 100 µL). Plates were statically incubated for 72 h at 24 ºC. Data are expressed as OD_{600} mean ± SD of three replicates. Sub-MIC concentrations for each peptide were: 1-32 µM for PAF112, 1-16 µM for PAF118, 1-8 µM for PAF116, and 0.25-2 µM for PAF26. Similarly, the interaction between the PAF protein from *P. chrysogenum* and the PAF26 peptide was tested against *A. niger, P. chrysogenum and P. digitatum*.

Proteolytic digestion assays

The proteolytic digestion assays were performed as described (Ferre et al., 2006; López-García et al., 2015) with minor modifications. Peptides (5 µM) were dissolved in 10 mM MOPS pH 7 and digested with 5 µg/mL of recombinant proteinase K (2.0 U/mg) (Roche, Mannheim, Germany) at 30 ºC. Aliquots were withdrawn from the reaction mixtures at 0, 1, 2, and 24 h of incubation and immediately heated at 80 ºC for 10 min to inactivate the enzyme. Analyses of peptide digests were carried out by RP-HPLC using a Waters system (Waters Corporation, Milford, MA, USA) equipped with a 1525 Binary HPLC pump, a 2996 Photodiode Array Detector and a 717 plus Autosampler. A Symmetry C18 column (4.6 x 150 mm, 5 µm, Waters) kept at 40 ºC was operated at a flow rate of 1 mL/min. Peptides were eluted with a linear gradient of solvent B (acetonitrile with 0.1 % TFA) in solvent A (water with 0.1 % TFA) 0 to 40 % in 20 min and detected at 214 nm. The experiments were repeated at least twice.

Statistical analysis

All the statistical analyses carried out to determine the synergistic interactions were performed using STATGRAPHICS Centurion 16.1.17. (http://www.statgraphics.com/). The significant differences between sets of data were determined by a bi-factorial ANOVA and Tukey's HSD test. Significance was regarded as $p < 0.01$.

RESULTS

AfpB structural modeling

In silico translation of the *afpB* gene (PDIG_68840) shows that the AfpB primary structure contains 92 amino acid residues (Garrigues et al., 2016). A signal peptide and a pro-peptide (35 residues in total) are predicted to be cleaved from the N-terminal end of the protein, liberating the mature AfpB which is a small basic CRP consisting of 57 amino acid residues (Figure 1A). It has a calculated molecular mass of 6.46 kDa, a pI value of 9.06 and a GRAVY of -1.084. The dextromeric isoform of the ɣ-core with the consensus sequence $GXCX_{3-9}C$ (Yount and Yeaman, 2004) is present near the N-terminus (Figure 1A, boxed). The structure of mature AfpB was predicted by homology modeling using the SWISS-MODEL online resource, which selected the protein PAF from *P. chrysogenum* as template for prediction (PDB ID 2MHV) (Fizil et al., 2015), and refined using the ModRefiner tool (Figure 1).

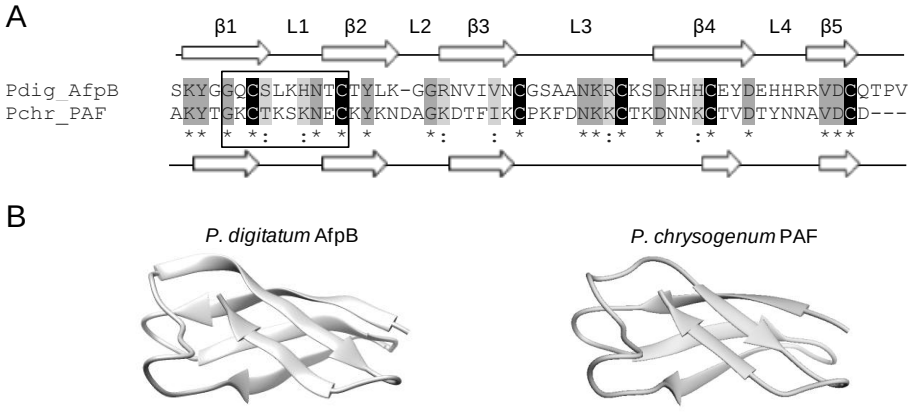

Figure 1: Molecular modeling of the *P. digitatum* class B antifungal protein AfpB. (A) Amino acid sequence alignment of AfpB and *P. chrysogenum* antifungal protein PAF. Cysteines are shadowed in black, other conserved amino acids in dark gray and similar amino acids in light gray. PAF and predicted AfpB β-sheets are labelled as β1 to β5 white arrows; and loops are labeled as L1 to L4. The ɣ-core motif is boxed. (B) Comparison of the tertiary structure of PAF (right) with the 3D molecular model obtained for AfpB (left).

AfpB shows 33 % amino acid identity and 44 % similarity with the *P. chrysogenum* PAF. The refined AfpB model showed 56 of 57 (98 %) of the amino acid residues positioned in energetically favored regions and one

residue in energetically allowed regions according to the Ramachandran plot (Supplementary Figure S1). The structural model of AfpB contains five antiparallel β-strands connected by three small loops and a big surface-exposed loop (Supplementary File S2 and Figure 1). The antiparallel β-strands create two packed β-sheets and the six conserved cysteine residues form three disulfide bridges. The most probable disulfide bond pattern predicted by the DISULFIND software is "abcabc", between cysteines 7 and 35; 14 and 42; and 27 and 53 (see also Figure 2A), which also corresponds to the disulfide bond pattern determined in PAF (Váradi et al., 2013).

Identification of AfpB-derived antifungal peptides with specific antifungal activity

To identify putative antimicrobial motifs in AfpB, the cysteine-containing peptides PAF109, PAF112, PAF118 and PAF119 were derived from the AfpB sequence (Table 1). Peptides (Figure 2A) were rationally designed with intramolecular disulfide bonds to promote folding into a loop or β-hairpin structure corresponding to the regions within the protein (Figure 2B).

Table 1: Amino acid sequences and properties of the peptides used in this study.

ID	Sequence[1]	MM (Da)	Net Charge	pI	GRAVY	Source
PAF26	RKKWFW	950.2	3.0	11.2	-1.883	(López-García et al., 2002)
PAF109	GQCSLKHNTCT	1189.3	1.1	8.1	-0.718	AfpB (*P. digitatum)*
PAF112	NCGSAANKRAKSD RHHCE	1982.1	2.2	8.9	-1.600	AfpB (*P. digitatum)*
PAF113	NAGSAANKRAKSD RHHAE	1920	2.2	10.0	-1.678	AfpB (*P. digitatum)*
PAF118	NTCTYLKGGRNVIV NCG	1810.1	2.0	8.9	-0.065	AfpB (*P. digitatum)*
PAF119	HCEYDEHHRRVDC Q	1824.9	-1.7	5.8	-2.014	AfpB *(P. digitatum)*
PAF116	KCPKFDNKKATKD NNKCT	2081.4	4.0	9.5	-1.906	PAF (*P. chrysogenum)*

[1]Underlined cysteine residues in PAF109, PAF112, PAF116, PAF118, and PAF119 are linked by disulfide bonds.

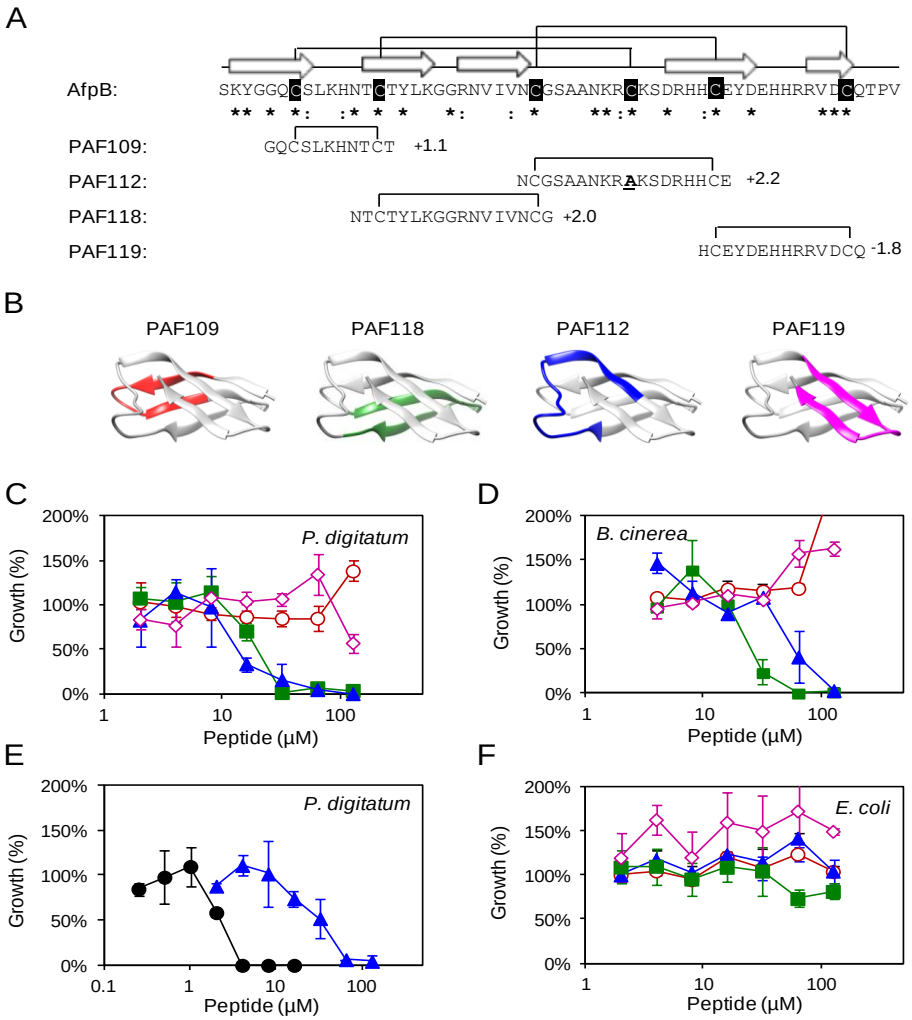

Figure 2: Design and *in vitro* inhibitory activity of the synthetic AfpB-derived peptides. (A) Amino acid sequence and net charge of the AfpB-derived peptides and their location in the protein sequence. Cysteine residues are shadowed in black. Asterisks and colon show conserved residues (see Figure 1). Arrows show the predicted β-sheets in AfpB. Black lines show the predicted disulfide bond pattern for AfpB (top) and the disulfide bonds in the AfpB-derived PAF109, PAF112, PAF118 and PAF119 (bottom). (B) Color-coded location of the AfpB-derived peptides in the modeled tertiary structure of AfpB. (C-F) Color-coded dose-response curves showing the antimicrobial activity of the peptides PAF109 (red circles), PAF118 (green squares), PAF112 (blue triangles), PAF119 (pink diamonds) and PAF26 (black circles), tested against *P. digitatum* (C and E), *B. cinerea* (D) and *E. coli* (F). Dose-response curves show mean ± SD percentage of control growth of triplicate samples after 72 h of incubation at 24 ºC for fungi (C to E) and 48 h at 37 ºC for bacteria (F).

Peptide PAF109 is cationic, spans the first loop L1 between β-sheets β1 and β2, and corresponds to the conserved γ-core motif. PAF118, also with a positive net charge, includes the β2 and β3 sheets linked by a small loop (L2). PAF112 extents over the largest surface-exposed cationic loop L3 of the protein located between β3 and β4. In this peptide, the central Cys35 in L3 has been replaced by an alanine in order to promote disulfide bond formation between the terminal cysteine residues (Figure 2A). An additional peptide derived from this largest surface-exposed cationic loop L3, called PAF113, was designed. PAF113 corresponds to the linear version of the peptide PAF112 in which all cysteine residues were substituted by alanines. Finally, PAF119 has a negative net charge as opposed to the other three peptides, and includes the last two β-sheets (β4 and β5) linked by the small C-terminal loop (L4).

These AfpB-derived peptides were tested towards a selection of filamentous fungi that include plant pathogens (the citrus fruit specific *P. digitatum*, the polyphagous *B. cinerea*, and the vascular wilt pathogen *F. oxysporum*), the PAF producer *P. chrysogenum*, and a strain from *A. niger* particularly sensitive to the PAF protein. The cationic peptides PAF112 and PAF118 showed antifungal activity and were completely inhibitory to *P. digitatum* and *B. cinerea*, with MIC values between 64-128 μM (Figure 2C-D and Table 2).

Table 2: Minimum inhibitory concentration (MIC) of peptides studied in this work[1].

Microorganism	PAF26	PAF109	PAF112	PAF113	PAF118	PAF119	PAF116
P. digitatum CECT20796	8	NI[2]	64	64	64	NI	32
B. cinerea CECT2100	8	NI	128	128	64	NI	16
F. oxysporum 4287	16	NI	NI	NI	NI	NI	NI
A. niger CBS120.49	8	NI	>128	>128	>128	NI	_[3]
P. chrysogenum Q176	16	NI	>128	>128	>128	NI	_
E. coli JM109	NI	NI	NI	NI	NI	NI	_

[1] MIC values are shown in μM units for each fungus-peptide combination.
[2] NI, no significant inhibition at the highest concentration tested (128 μM).
[3] -, not tested.

At lower concentrations (8-32 µM), PAF112 and PAF118 showed partial inhibition of the growth of these two fungi (Figure 3 and data not shown). In the case of *A. niger* and *P. chrysogenum*, the PAF112 and PAF118 peptides significantly delayed fungal growth (Figure 3 and data not shown). Even at low concentrations (8-32 µM) an inhibition was observed at short incubation times (24 h), although the peptides were not completely inhibitory at the highest concentration tested (128 µM) (Figure 3). In some experiments, PAF118 showed slightly higher antifungal activity than PAF112 according to the dose-response effect (see Figures 2 and 3).

Regarding other peptides (Figure 2), PAF109 derived from the conserved ɣ-core did not show inhibitory activity at any of the concentrations tested. The anionic peptide PAF119 did not show activity either. None of the AfpB-derived peptides tested showed inhibitory effect against *F. oxysporum* at the maximum concentration used. This strain of *F. oxysporum* has shown comparatively higher tolerance to most of the peptides studied in our laboratory over the years (López-García et al., 2015).

The synthetic hexapeptide PAF26 is an antifungal peptide that was identified by a combinatorial approach against *P. digitatum* (López-García et al., 2002). PAF26 is a cationic tryptophan-rich peptide (Table 1) that belongs to the cell-penetrating class of AMPs and whose mechanism of action has been characterized previously (Muñoz et al., 2013a). PAF26 has specific activity for filamentous fungi showing MIC values below 10 µM and was therefore chosen as an internal control in this study. We found that peptides PAF112 and PAF118 are around one order of magnitude less active than PAF26 (Figure 2E and Table 2). As occurs with PAF26, none of the AfpB-derived peptides showed antibacterial activity against *Escherichia coli* (Figure 2F), which demonstrated the selective antifungal activity of PAF112 and PAF118 over the range of concentrations assayed.

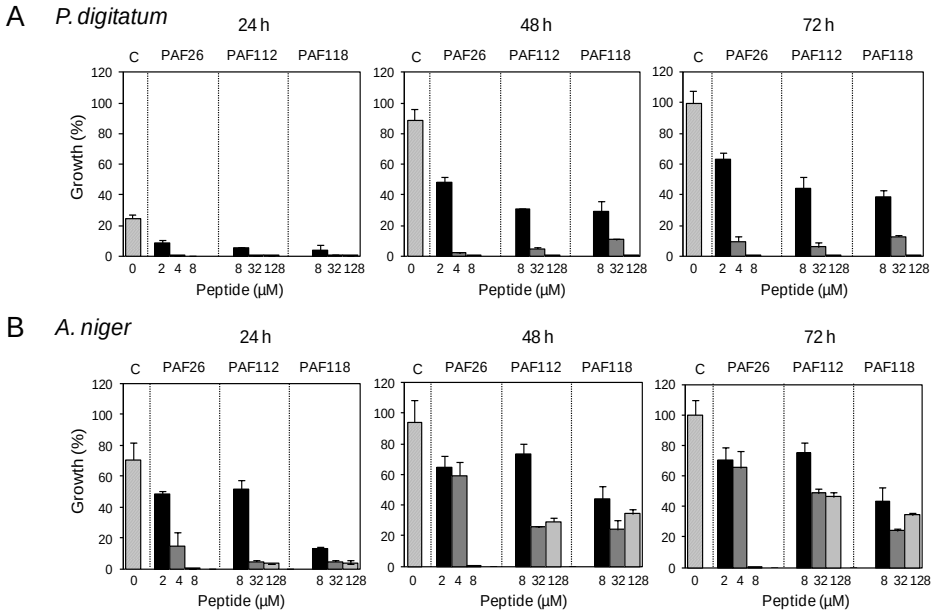

Figure 3: Time-course inhibition of growth of filamentous fungi by synthetic peptides. Time-course inhibition of growth of *P. digitatum* (A) and *A. niger* (B) in the presence of increasing concentrations of the synthetic peptides PAF26 (2, 4 and 8 µM), PAF112 (8, 32, and 128 µM), and PAF118 (8, 32, and 128 µM). Data are shown after 24, 48 and 72 h of incubation as indicated. Bars represent the mean value ± SD of the percentage of growth as compared to the 100% control that was defined for each fungus as the growth in the absence of peptide (C, control) at 72 h.

Peptide PAF116 derived from the *P. chrysogenum* antifungal protein PAF is less active than the native protein

Since it was impossible to detect AfpB in all the experiments conducted so far in our laboratory (Garrigues et al., 2016), we wanted to test whether peptides derived from AFPs could have antifungal activity similar to the native protein. Therefore, we designed the disulfide cyclized peptide PAF116 analogous to PAF112 but originating from the *P. chrysogenum* antifungal protein PAF, which was readily available (Figure 4). The sequence alignment of PAF112 and PAF116 showed low similarity (44 %) (Figure 4A). However, despite the sequence divergence between these two peptides, both shared comparable antifungal activity against *P. digitatum* (Figure 4B, top). As in the case of PAF112, PAF116 showed no antifungal activity against *F. oxysporum* (data not shown). In contrast, PAF116

exhibited higher antimicrobial activity than PAF112 against *B. cinerea* (Table 2).

Additional assays were conducted to compare the antifungal activity of the peptide PAF116 and the parent protein PAF (Figure 4B, bottom). The results showed that PAF116 is less active than the PAF protein against *P. digitatum*. The MIC values were 32 µM for the peptide PAF116 and 8 µM for the PAF protein. This result leads us to propose that AfpB would be also more active than the peptides PAF112 or PAF118 derived from its primary sequence. In addition, it is demonstrated that *P. digitatum* is sensitive to the PAF protein from *P. chrysogenum*, which as the producer fungus is tolerant to this protein (Table 3).

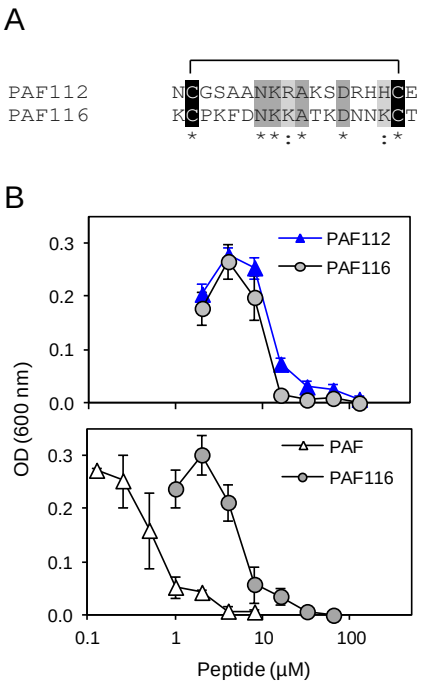

Figure 4: Comparative study of the AfpB-derived peptide PAF112 and the PAF-derived PAF116. (A) Amino acid sequence alignment of PAF112 and PAF116. Cysteine conserved residues are shadowed in black, conserved residues in dark gray, and similar in light gray. (B) Dose-response curves of *P. digitatum* inhibition by the synthetic peptides PAF112 (blue triangles) and PAF116 (gray circles) (top), and by the peptide PAF116 (gray circles) and protein PAF from *P. chrysogenum* (white triangles) (bottom). Curves show mean ± SD OD_{600} of triplicate samples after 72 h of incubation at 24 ºC.

Synergistic interactions

In view of the above data we hypothesized that the combined action of different AfpB-derived peptides could mimic the activity of the native protein. To address our prediction, we designed and conducted a series of experiments in which different peptides were tested for synergy against *P. digitatum* (Figure 5). From all possible combinations of the four AfpB-derived peptides in groups of two, only PAF112 and PAF118 showed positive synergistic interaction when combined (Figure 5 and data not shown). A multifactorial ANOVA test was conducted at different concentrations of PAF112 (from 1 to 32 µM), PAF118 (from 1 to 16 µM) and all possible concentration combinations of both, demonstrating the existence of an interaction ($p < 0.01$). As example, the data from the combined action of 16 µM PAF112 and 1 µM PAF118 is shown (Figure 5, left). Single treatments of PAF112 or PAF118 did not result in a fungal growth significantly different from the control. However, the combined activity of both peptides resulted in significant inhibition of *P. digitatum* ($p < 0.01$, Figure 5), in the range of the inhibition achieved by the PAF protein at 1 µM (Figure 4B). This observation leads us to suggest that these two motifs contribute to reach optimal activity of the native AfpB protein.

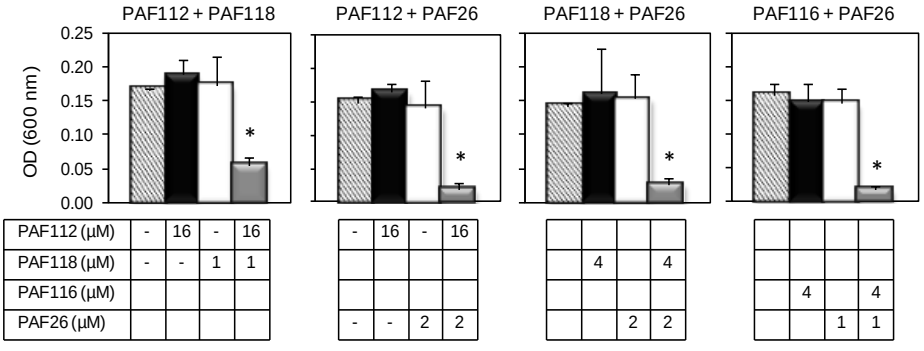

Figure 5: Synergistic interactions between AFP-derived peptides and PAF26**.** Each of the four panels show the synergistic interactions between PAF112 and PAF118; PAF112 and PAF26; PAF118 and PAF26; and PAF116 and PAF26, tested against *P. digitatum*. The graphics show mean ± SD OD_{600} values of triplicate samples after 48 h of incubation at 24 ºC. Asterisks show statistical significance for positive interaction (multifactorial ANOVA $p < 0.01$) of fungal growth inhibition for each peptide combination at the concentrations indicated below the panels.

To determine the possible interaction between the hexapeptide PAF26 and the antifungal peptides derived from the AfpB and PAF protein, additional combinations of peptides were tested. These experiments demonstrated that the AfpB-derived peptides PAF112 and PAF118, as well as the PAF-derived peptide PAF116, show a positive synergistic interaction with PAF26 (multifactorial ANOVA, $p < 0.01$) (Figure 5), reducing the fungal biomass when combined at non-inhibitory concentrations.

Given the synergy existing between PAF26 and the antifungal peptides derived from both AFPs, further antifungal assays were conducted to determine whether the protein PAF from *P. chrysogenum* could also interact synergistically with PAF26. Three fungi were chosen for these experiments that are representative of the sensitivity range to PAF, from the highly sensitive *A. niger* to the tolerant PAF-producer *P. chrysogenum* (Table 3).

Table 3: Synergistic interaction between PAF26 peptide and PAF protein.

Strain	PAF26 peptide[1]	PAF Protein	Interaction PAF26/PAF
P. digitatum CECT 20796	8	8	Positive synergy ($p < 0.01$)
P. chrysogenum Q176	16	NI[2]	No sinergy
A. niger CBS 120.49	8	0.08	Positive synergy ($p < 0.01$)

[1] MIC (µM) are shown for each combination.
[2] NI, no inhibition at the highes concentration tested (64 µM).

The results indicated no apparent synergy in assays with *P. chrysogenum*. For *P. digitatum* and the highly PAF-sensitive *A. niger*, PAF protein showed a positive synergistic interaction with the peptide PAF26 (Figure 6 and Table 3). Interestingly, the three fungi did not show major differences in their sensitivity to PAF26 while they did to the PAF protein (Table 3), suggesting that the PAF protein and the peptide PAF26 could have differences in their mechanism of action.

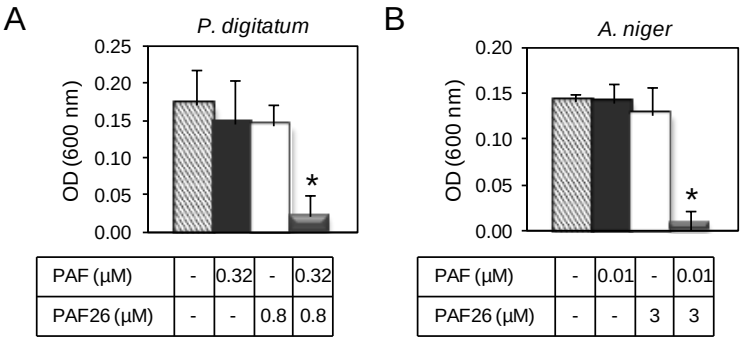

Figure 6: Synergistic interactions between the protein PAF from *P. chrysogenum* and the hexapeptide PAF26. The synergistic interaction was tested against *P. digitatum* (A) and *A. niger* (B). The graphics show mean ± SD OD_{600} values of triplicate samples after 48 h of incubation at 24 ºC. Asterisks show statistical significance for positive interaction (multifactorial ANOVA $p<0.01$) of fungal growth inhibition for each peptide/protein combinations at the concentrations indicated below the panels.

ECD spectroscopic analysis

Electronic circular dichroism spectroscopy is a sensitive tool for the study of the secondary structure of proteins and peptides. To investigate the structure of the AfpB-derived peptides, ECD spectroscopy was performed (Figure 7). ECD analyses revealed that peptides PAF109, PAF112 and PAF113 predominantly displayed unordered structure in water characterized by strong minima at 195-200 nm (Figure 7A). TFE is an organic solvent commonly used to induce intramolecular H-bonding, therefore inducing and stabilizing secondary structure of protein fragments in the absence of cooperatively stabilizing structural regions of the protein of origin. Upon addition of TFE, a minor helical contribution appeared in case of the linear peptide PAF113, reflected by a low intensity broad negative shoulder in the 220-225 nm region (Figure 7B). Since this peptide has no disulfide bond and therefore less conformational restraints, it can adapt more readily to changes in environment (for example addition of a helix-inducing solvent). PAF119 (Figure 7A) yielded a spectrum that reports on the dominance of random structures with a minor β-sheet contribution, and a more pronounced signal from the disulfide bridge (maximum around 230 nm). Addition of TFE to PAF119 did not increase, but rather diminished β-sheet contributions.

PAF118 displayed markedly different spectra compared to those of the rest of the peptides analyzed (Figure 7A, green line). The ECD spectrum of PAF118 is dominated by contributions from antiparallel β-structure (relative maximum near 195 nm and minimum near 215 nm) and disulfide bonds (maximum at 230 nm). Addition of TFE increased the β-character of the spectrum while the presence of a positive band at around 230 nm indicative of disulfide bonds was maintained (Figure 7B). Therefore, PAF118 represents an antifungal motif from AfpB that folds by itself in an antiparallel β-structure, consistent with the structural prediction within the context of the protein (see also Figure 2B).

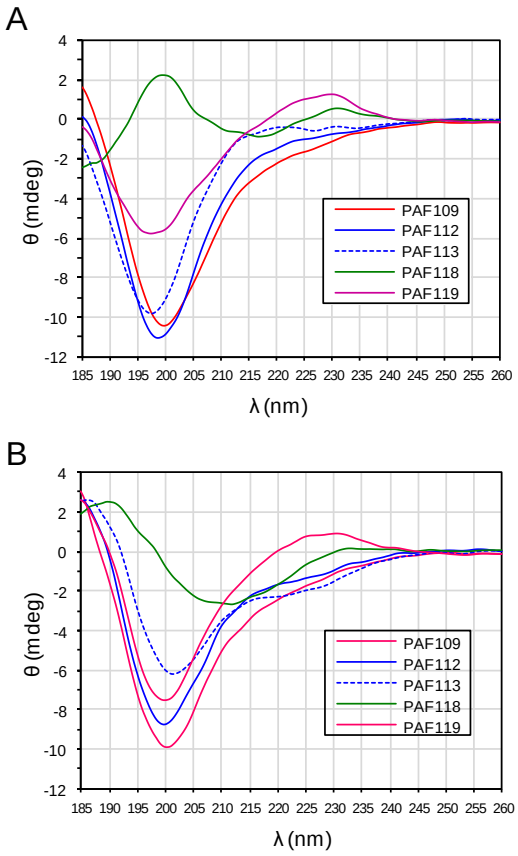

Figure 7: ECD spectra of AfpB-derived peptides**.** (A) Spectra obtained from peptides in H_2O. (B) Spectra obtained from peptides solved in TFE/H_2O (50:50) mixture. All experiments were conducted at 25ºC.

AfpB-derived peptides show resistance to proteolytic degradation

To exclude the possibility that increased sensitivity to proteolytic degradation could be the reason for limited antifungal activity, we tested the *in vitro* stability of the peptides derived from AfpB by incubation with proteinase K at different times and RP-HPLC analysis of the resultant digests. Again, we used peptide PAF26 as control as it is highly resistant to protease degradation (López-García et al., 2015). The RP-HPLC chromatograms (Supplementary Figure S3) showed that all the cyclic peptides derived from AfpB were, with varying degrees, resistant to protease treatment with a high dose of enzyme (5 µg/mL) (Figure 8). The antifungal peptides PAF112 and PAF118 show a degradation pattern similar to PAF26, with around 20 % of peptide left after 24 h of treatment. Consistently among the different repetitions of this experiment, PAF118 showed faster degradation than PAF112. Figure 8 shows a severe reduction of intact PAF118 after 1 and 2 h of treatment, which was different to the degradation of PAF112. Despite their similar antifungal activities and the presence of a disulfide bond in both sequences, PAF112 seems to be more resistant to protease degradation than PAF118. PAF113, which is a linear analog of PAF112, shows high sensitivity to proteinase K as it was completely degraded after only 1 h of treatment (Figure 8). We conclude that the presence of a disulfide bond increases PAF112 stability. Finally, the non-active peptides PAF109 and specially PAF119 were both remarkably resistant to degradation by proteinase K (Figure 8).

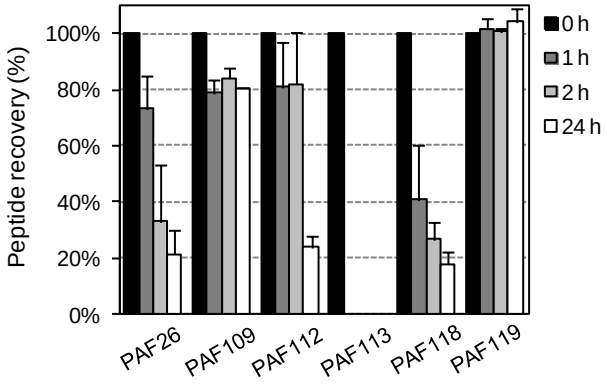

Figure 8: Time-dependent peptide degradation in the presence of proteinase K**.** Percentage of recovery of the input peptides (5 µM) represented as mean ± SD of two replicates, determined by the resulting chromatographic peak area of the corresponding peptide after treatment with proteinase K (5 µg/mL) for different times (0, 1, 2 and 24 h).

DISCUSSION

Most of the studies based on peptides derived from antifungal CRPs have focused on defensins and the highly conserved ɣ-motif (Sagaram et al., 2011; Avitabile et al., 2013; van der Weerden and Anderson, 2013; Muñoz et al., 2014; Kaewklom et al., 2016), but not on fungal AFPs. Our data identify for the first time synthetic peptides derived from fungal AFPs with antifungal activity. The peptides PAF112 and PAF118 derived from the *P. digitatum* AfpB and PAF116 from the *P. chrysogenum* PAF showed specific activity against selected filamentous fungi. PAF112 and PAF118 are not active against bacteria (*E. coli*) at the highest concentration tested (128 µM, Figure 2), which is consistent with the specificity profile of native AFPs that are antifungal but not antibacterial (Marx et al., 2008; Meyer, 2008). Importantly, none of the peptides overlap in neither of the two AFPs with the conserved ɣ-core motif that is present in virtually all known AFPs and other defensin-like antimicrobial peptides (Yount and Yeaman, 2004). There is no correlation between the GRAVY value and the activity of the peptides designed in our study, while all the active peptides are cationic (Tables 1 and 2). The importance of positive charge in the activity of AMPs including AFPs is well established (Brogden, 2005; Marcos et al., 2008). For example, three different mutations of the *P. chrysogenum* PAF in cationic lysine residues located at different positions resulted in loss of antifungal activity (Batta et al., 2009), and mutation of the arginine and/or lysine residues in the PAF26 sequence also abolished activity (Muñoz et al., 2013a). However, PAF112 and PAF116 show similar antifungal activity despite differences in charge (i.e., PAF116 is markedly more cationic). Therefore, the antifungal activity of these peptides can only be partially attributed to their positive net charge (see also below).

The *P. chrysogenum* PAF is the antifungal protein that has been most extensively characterized by site-directed mutagenesis, which allowed the identification of residues important for activity (Batta et al., 2009; Sonderegger et al., 2016; Sonderegger et al., 2017). Both PAF112 and PAF116 are located in the analogous large loop L3 of the corresponding class B protein AfpB and class A protein PAF, respectively. Although these peptides share only limited sequence identity, they represent one of the regions with highest similarity between both proteins. Mutations of Lys35 and Lys38 (Batta et al., 2009) and of Phe31 (Sonderegger et al., 2016), which are located in this L3 loop of *P. chrysogenum* PAF, negatively affect

antifungal activity. Therefore, by demonstrating the activity of isolated peptides from two different proteins (AfpB and PAF), our study contributes to establish L3 as an important domain in the antifungal activity of this class of AMPs. The peptide PAF112 is not structured (Figure 7), simulating the situation in the exposed loop L3 of the protein model, which lacks a defined canonical secondary structure (Figures 1 and 2). The structural data have shown that loop L3 is a dynamic and flexible region in PAF (Batta et al., 2009; Fizil et al., 2015).

The antifungal peptide PAF118 folds into an antiparallel β-sheet conformation that resembles the folding of this motif within the context of the protein. None of the previously reported mutations in residues of the PAF protein that affect the protein activity locates in the protein region analogous to PAF118 (Batta et al., 2009; Sonderegger et al., 2016). However, very recently it has been shown that the exchange of the negatively charged Asp19 to a neutral Ser in this PAF protein domain severely reduced protein activity (Sonderegger et al., 2017). This mutation does not disturb the overall 3D protein solution structure, but influences the surface charge distribution in distant regions of the PAF protein including the cationic L3, which most probably accounts for the loss of antifungal function (Sonderegger et al., 2017). The primary sequence of PAF and AfpB is different in this region as for instance no anionic residues are present in AfpB, contrary to PAF. Our demonstration of activity of the isolated motif represented by PAF118 identify this as a second putative region involved in the activity of AfpB. The only two cationic residues in this peptide are located in the turn that connects the two antiparallel β-sheets, and represent candidates to determine the antifungal properties of PAF118. Future work will attempt to confirm the importance of this domain in the activity of AFP-like proteins.

On the other hand, PAF109, which contains the minimal ɣ-core element, did not showed either antifungal or antibacterial activity with any of the microorganism tested in our study. Consistent with the archetypical ɣ-core motif (Yount and Yeaman, 2004), this peptide sequence spans two antiparallel β-sheets (Figure 2B). Plant defensins are related to AFPs in that they are small and cationic CRPs that have antifungal activity and contain the ɣ-core motif; however, their αβ fold is structurally distinct from that of AFPs (van der Weerden and Anderson, 2013; Silva et al., 2014).

Analogous ɣ-core peptides from the plant defensins MtDef4 from *Medicago*, SolyC07g007760 from tomato or BhDef2 from *Brassica* were antimicrobial (Sagaram et al., 2011; Avitabile et al., 2013; Kaewklom et al., 2016). However, other ɣ-core peptides such as the two motifs derived from the *Medicago* defensin MsDef1 did not show antifungal activity against *Fusarium graminearum* (Sagaram et al., 2011). In previous studies, a correlation between the cationic net charge of the ɣ-core-derivative peptides and their antimicrobial activity was demonstrated. The peptide derivative BhDEF12M from BhDef1 protein contains asparagine to arginine substitutions that promoted antibacterial and antifungal activity (Kaewklom et al., 2016). Yet, there are exemptions to this rule as other non-cationic ɣ-core peptides derived from plant defensins (i.e., BhDef23) were antimicrobial (Kaewklom et al., 2016). In this context, PAF109 is a peptide that has low positive charge (ca. +1.1 at pH 7.0) and did not show activity. In addition, PAF109 and PAF119 are extremely stable peptides. Based on our data, we propose that the ɣ-core in AfpB has mainly a structural significance important for the formation of the native structure and the stability of the protein but is not necessarily associated with antimicrobial activity. However, the ɣ-core in the *P. chrysogenum* PAF has a higher cationic charge than the AfpB ɣ-core and mutation of Lys9 in the PAF protein resulted in the decrease of protein activity suggesting that this motif is important for protein function (Batta et al., 2009). Future investigations will address this apparently contradictive issue in fungal AFPs.

Antifungal activity of PAF116 is about 5-10 times weaker than the activity of the corresponding PAF protein. This finding implicates that additional motifs/sequences in the protein are required to achieve full activity. Similarly, we predict that PAF112 and PAF118 also have significantly lower activity than the elusive (non-detectable) AfpB. In order to test this hypothesis, our current efforts are directed towards the heterologous expression of the *afpB* gene to achieve protein production and purification. As an alternative approach in this study, we conducted experiments to determine whether treatment with both PAF112 and PAF118 inhibits fungal growth with potency higher than the sum of both peptides acting individually. We could demonstrate a positive synergistic interaction (Figure 5), which supports our hypothesis that both domains could cooperate to achieve the activity of the full-length protein.

It is generally established that antimicrobial compounds that have a positive synergistic interaction act on different microbial targets or have mechanisms of action that complement each other (Glattard et al., 2016; Vriens et al., 2016). This would be the case for the different AFP-derived and PAF26 peptides on *P. digitatum* and also for the PAF26/PAF protein combination on *A. niger* and *P. digitatum* as described in this study. Different time-course inhibition by PAF26 or AfpB-derived peptides on either *P. digitatum* or *A. niger* indicate mechanistic differences (Figure 3). The existence of at least partially different mechanisms in the combination PAF26/PAF protein is also supported by the different profile of activity among different fungal strains (Table 3). Furthermore, the demonstration of synergy also opens up new alternatives to improve (i) the antifungal activity of AFPs by combined action with other unrelated peptides such as PAF26, or (ii) the rational design of novel AMPs by combination of distinct peptide sequences (Badosa et al., 2009; Rodriguez Plaza et al., 2014).

Besides, there are also common mechanistic features between AFP proteins and the unrelated synthetic peptide PAF26, such as the endocytic internalization and increased concentrations of intracellular ROS and cytoplasmic Ca^{2+} in sensitive fungal cells (Marx et al., 2008; Binder et al., 2010; Carmona et al., 2012; Muñoz et al., 2012). PAF26 has been proposed as a model peptide to study the mode of action of cell penetrating antifungal peptides (Muñoz et al., 2013a; Muñoz et al., 2013b). The mechanism of PAF26 has been divided in three stages: (i) interaction with the fungal cell surface (cell wall and membrane), (ii) cellular internalization and (iii) intracellular killing. This model framework could be useful to better refine and characterize the mechanism of AFPs and related proteins. Additionally, the identification of PAF112, PAF118 and PAF116 provides minimal motifs useful for the detailed mechanistic study of AFP-like proteins.

AFPs show remarkable thermal stability and resistance to protease degradation, which has been attributed to their tight structural packaging and disulfide bonding (Batta et al., 2009; Fizil et al., 2015). Consistently with this, all the AfpB-derived peptides with disulfide bonds were stable under restrictive *in vitro* degradation experiments (Figure 8). The stability of PAF109 and PAF119 indicates that their lack of antifungal activity cannot be attributed to propensity to degradation. By characterizing the linear

analog PAF113 of peptide PAF112 we confirmed that the disulfide bridge between the two cysteines greatly stabilizes the peptide towards proteolytic degradation by proteinase K (Avitabile et al., 2013). PAF113 shows initial antifungal activity comparable to PAF112, although the activity was gradually lost with time in some experiments (data not shown), probably as consequence of degradation. The stability of all four cyclized AfpB-derived peptides may suggest a reasonable stability of the putative protein AfpB. We therefore assume that other so far unidentified regulatory mechanisms in mRNA stability and/or protein translation could be responsible for the lack of AfpB detection in *P. digitatum* cultures.

In summary, this study identifies protein motifs involved in the specific antifungal activity of AFPs, and opens new strategies for the future use of AFP-related peptides and proteins, the development of rationally designed antifungal peptides and their use in the characterization of the antifungal activity of AFPs. Two synthetic peptides that span about the central half of AfpB, including the major L3 loop, would contribute to specific antifungal activity of the putative AfpB, whereas the ɣ-core motif seems to play a different functional role.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: SG, PM, JFM. Modeled the AfpB structure: SG. Designed the peptides: SG, MG, PM JFM. Performed antimicrobial experiments: SG, MG, FM. Provided the PAF protein: FM. Performed the ECD experiments: AB. Performed the degradation experiments: SG, PM. Run statistical analyses: SG. Wrote the paper: SG, PM, JFM. All authors approved the manuscript and agree to be accountable for the content of the work. Authorship is limited to those who have contributed substantially to the work reported.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22(2)**,** 195-201. doi: 10.1093/bioinformatics/bti770.

Avitabile, C., Capparelli, R., Rigano, M.M., Fulgione, A., Barone, A., Pedone, C., et al. (2013). Antimicrobial peptides from plants: Stabilization of the γ core of a tomato defensin by intramolecular disulfide bond. *Journal of Peptide Science* 19(4)**,** 240-245. doi: 10.1002/psc.2479.

Badosa, E., Ferré, R., Francés, J., Bardají, E., Feliu, L., Planas, M., et al. (2009). Sporicidal activity of synthetic antifungal undecapeptides and control of *Penicillium* rot of apples. *Applied and Environmental Microbiology* 75(17)**,** 5563-5569. doi: 10.1128/AEM.00711-09.

Batta, G., Barna, T., Gáspári, Z., Sándor, S., Kövér, K.E., Binder, U., et al. (2009). Functional aspects of the solution structure and dynamics of PAF - A highly-stable antifungal protein from *Penicillium chrysogenum*. *The FEBS Journal* 276(10)**,** 2875-2890. doi: 10.1111/j.1742-4658.2009.07011.x.

Binder, U., Chu, M., Read, N.D., and Marx, F. (2010). The antifungal activity of the *Penicillium chrysogenum* protein PAF disrupts calcium homeostasis in *Neurospora crassa*. *Eukaryotic Cell* 9(9)**,** 1374-1382. doi: 10.1128/EC.00050-10.

Brogden, K.A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* 3(3)**,** 238-250. doi: 10.1038/nrmicro1098.

Brown, G.D., Denning, D.W., Gow, N.A.R., Levitz, S.M., Netea, M.G., and White, T.C. (2012). Hidden killers: Human fungal infections. *Science Translational Medicine* 4(165)**,** 165rv113. doi: 10.1126/scitranslmed.3004404.

Carmona, L., Gandía, M., López-García, B., and Marcos, J.F. (2012). Sensitivity of *Saccharomyces cerevisiae* to the cell-penetrating antifungal peptide PAF26 correlates with endogenous nitric oxide (NO) production. *Biochemical and Biophysical Research Communications* 417(1)**,** 56-61. doi: 10.1016/j.bbrc.2011.11.050.

Ceroni, A., Passerini, A., Vullo, A., and Frasconi, P. (2006). Disulfind: A disulfide bonding state and cysteine connectivity prediction server. *Nucleic Acids Research* 34(WEB. SERV. ISS.)**,** W177-W181. doi: 10.1093/nar/gkl266.

Ferre, R., Badosa, E., Feliu, L., Planas, M., Montesinos, E., and Bardají, E. (2006). Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides. *Applied and Environmental Microbiology* 72(5)**,** 3302-3308. doi: 10.1128/AEM.72.5.3302-3308.2006.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484(7393)**,** 186-194. doi: 10.1038/nature10947.

Fizil, Á., Gáspári, Z., Barna, T., Marx, F., and Batta, G. (2015). "Invisible" conformers of an antifungal disulfide protein revealed by constrained cold and heat unfolding, CEST-NMR experiments, and molecular dynamics calculations. *Chemistry - A European Journal* 21(13)**,** 5136-5144. doi: 10.1002/chem.201404879.

Fjell, C.D., Hiss, J.A., Hancock, R.E.W., and Schneider, G. (2012). Designing antimicrobial peptides: Form follows function. *Nature Reviews Drug Discovery* 11(1)**,** 37-51. doi: 10.1038/nrd3591.

Galgóczy, L., Kovács, L., and Vágvölgyi, C. (2010). "Defensin-like antifungal proteins secreted by filamentous fungi," in *Current research, technology and education topics in applied and microbial biotechnology,* ed. A. Méndez-Vila. (Badajoz, Spain: Formatex), 550-559.

Galgóczy, L., Virágh, M., Kovács, L., Tóth, B., Papp, T., and Vágvölgyi, C. (2013). Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among Fusaria. *Peptides* 39(0)**,** 131-137. doi: 10.1016/j.peptides.2012.10.016.

Garrigues, S., Gandía, M., and Marcos, J.F. (2016). Occurrence and function of fungal antifungal proteins: a case study of the citrus postharvest pathogen *Penicillium digitatum*. *Applied Microbiology and Biotechnology* 100(5)**,** 2243-2256. doi: 10.1007/s00253-015-7110-3.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S.e., Wilkins, M.R., Appel, R.D., et al. (2005). "Protein Identification and Analysis Tools on the ExPASy Server," in *The Proteomics Protocols Handbook,* ed. J.M. Walker. (Totowa, NJ: Humana Press), 571-607.

Glattard, E., Salnikov, E.S., Aisenbrey, C., and Bechinger, B. (2016). Investigations of the synergistic enhancement of antimicrobial activity in mixtures of magainin 2 and PGLa. *Biophysical Chemistry* 210**,** 35-44. doi: 10.1016/j.bpc.2015.06.002.

Hancock, R.E.W., and Sahl, H.G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology* 24(12)**,** 1551-1557. doi: doi:10.1038/nbt1267.

Kaewklom, S., Euanorasetr, J., Intra, B., Panbangred, W., and Aunpad, R. (2016). Antimicrobial activities of novel peptides derived from defensin genes of *Brassica hybrid* cv Pule. *International Journal of Peptide Research and Therapeutics* 22(1)**,** 93-100. doi: 10.1007/s10989-015-9488-2.

Kovács, L., Virágh, M., Takó, M., Papp, T., Vágvölgyi, C., and Galgóczy, L. (2011). Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). *Peptides* 32(8)**,** 1724-1731. doi: 10.1016/j.peptides.2011.06.022.

López-García, B., Harries, E., Carmona, L., Campos-Soriano, L., López, J.J., Manzanares, P., et al. (2015). Concatemerization increases the inhibitory activity of short, cell-penetrating, cationic and tryptophan-rich antifungal

peptides. *Applied Microbiology and Biotechnology* 99(19)**,** 8011-8021. doi: 10.1007/s00253-015-6541-1.

López-García, B., Pérez-Payá, E., and Marcos, J.F. (2002). Identification of novel hexapeptides bioactive against phytopathogenic fungi through screening of a synthetic peptide combinatorial library. *Applied and Environmental Microbiology* 68(5)**,** 2453-2460. doi: 10.1128/AEM.68.5.2453-2460.2002.

Lovell, S.C., Davis, I.W., Arendall Iii, W.B., De Bakker, P.I.W., Word, J.M., Prisant, M.G., et al. (2003). Structure validation by Cα geometry: φ,ψ and Cβ deviation. *Proteins: Structure, Function and Genetics* 50(3)**,** 437-450. doi: 10.1002/prot.10286.

Marcos, J.F., Gandía, M., Harries, E., Carmona, L., and Muñoz, A. (2012). "Antifungal peptides: Exploiting non-lytic mechanisms and cell penetration properties," in *Small Wonders: Peptides for Disease Control,* eds. K. Rajasekaran, J.W. Cary, J. Jaynes & E. Montesinos. American Chemical Society), 337-357.

Marcos, J.F., Muñoz, A., Pérez-Payá, E., Misra, S., and López-García, B. (2008). Identification and rational design of novel antimicrobial peptides for plant protection. *Annual Review of Phytopathology* 46**,** 273-301. doi: 10.1146/annurev.phyto.121307.094843.

Marx, F., Binder, U., Leiter, É., and Pócsi, I. (2008). The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies. *Cellular and Molecular Life Sciences* 65(3)**,** 445-454. doi: 10.1007/s00018-007-7364-8.

Meyer, V. (2008). A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. *Applied Microbiology and Biotechnology* 78(1)**,** 17-28. doi: 10.1007/s00253-007-1291-3.

Muñoz, A., Chu, M., Marris, P.I., Sagaram, U.S., Kaur, J., Shah, D.M., et al. (2014). Specific domains of plant defensins differentially disrupt colony initiation, cell fusion and calcium homeostasis in Neurospora crassa. *Molecular Microbiology* 92(6)**,** 1357-1374. doi: 10.1111/mmi.12634.

Muñoz, A., Gandía, M., Harries, E., Carmona, L., Read, N.D., and Marcos, J.F. (2013a). Understanding the mechanism of action of cell-penetrating antifungal peptides using the rationally designed hexapeptide PAF26 as a model. *Fungal Biology Reviews* 26(4)**,** 146-155. doi: 10.1016/j.fbr.2012.10.003.

Muñoz, A., Harries, E., Contreras-Valenzuela, A., Carmona, L., Read, N.D., and Marcos, J.F. (2013b). Two functional motifs define the interaction, internalization and toxicity of the cell-penetrating antifungal peptide PAF26 on fungal cells. *PLoS ONE* 8(1)**,** e54813. doi: 10.1371/journal.pone.0054813.

Muñoz, A., Marcos, J.F., and Read, N.D. (2012). Concentration-dependent mechanisms of cell penetration and killing by the *de novo* designed

antifungal hexapeptide PAF26. *Molecular Microbiology* 85(1)**,** 89-106. doi: 10.1111/j.1365-2958.2012.08091.x.

Palicz, Z., Jenes, T., Gáll, T., Miszti-Blasius, K., Kollár, S., Kovács, I., et al. (2013). In vivo application of a small molecular weight antifungal protein of *Penicillium chrysogenum* (PAF). *Toxicology and Applied Pharmacology* 269(1)**,** 8-16. doi: 10.1016/j.taap.2013.02.014.

Petersen, T.N., Brunak, S., Von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nature Methods* 8(10)**,** 785-786. doi: 10.1038/nmeth.1701.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., et al. (2004). UCSF Chimera - A visualization system for exploratory research and analysis. *Journal of Computational Chemistry* 25(13)**,** 1605-1612. doi: 10.1002/jcc.20084.

Rodriguez Plaza, J.G., Morales-Nava, R., Diener, C., Schreiber, G., Gonzalez, Z.D., Ortiz, M.T.L., et al. (2014). Cell penetrating peptides and cationic antibacterial peptides: Two sides of the same coin. *Journal of Biological Chemistry* 289(21)**,** 14448-14457. doi: 10.1074/jbc.M113.515023.

Sagaram, U.S., Pandurangi, R., Kaur, J., Smith, T.J., and Shah, D.M. (2011). Structure-activity determinants in antifungal plant defensins MsDef1 and MtDef4 with different modes of action against *Fusarium graminearum*. *PLoS ONE* 6(4). doi: 10.1371/journal.pone.0018550.

Shah, D.M., and Read, N.D. (2013). Antifungal peptides come of age. *Fungal Biology Reviews* 26(4)**,** 107-108. doi: 10.1016/j.fbr.2013.01.001.

Silva, P.M., Gonçalves, S., and Santos, N.C. (2014). Defensins: Antifungal lessons from eukaryotes. *Frontiers in Microbiology* 5(MAR). doi: 10.3389/fmicb.2014.00097.

Sonderegger, C., Fizil, Á., Burtscher, L., Hajdu, D., Muñoz, A., Gáspári, Z., et al. (2017). D19S Mutation of the Cationic, Cysteine-Rich Protein PAF: Novel Insights into Its Structural Dynamics, Thermal Unfolding and Antifungal Function. *PLOS ONE* 12(1)**,** e0169920. doi: 10.1371/journal.pone.0169920.

Sonderegger, C., Galgóczy, L., Garrigues, S., Fizil, Á., Borics, A., Manzanares, P., et al. (2016). A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. *Microbial Cell Factories* 15(1)**,** 192. doi: 10.1186/s12934-016-0586-4.

Tóth, L., Kele, Z., Borics, A., Nagy, L.G., Váradi, G., Virágh, M., et al. (2016). NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization. *AMB Express* 6(1). doi: 10.1186/s13568-016-0250-8.

van der Weerden, N.L., and Anderson, M.A. (2013). Plant defensins: Common fold, multiple functions. *Fungal Biology Reviews* 26(4)**,** 121-131. doi: 10.1016/j.fbr.2012.08.004.

Váradi, G., Tõth, G.K., Kele, Z., Galgõczy, L., Fizil, A., and Batta, G. (2013). Synthesis of PAF, an antifungal protein from *P. chrysogenum*, by native chemical ligation: Native disulfide pattern and fold obtained upon oxidative refolding. *Chemistry - A European Journal* 19(38)**,** 12684-12692. doi: 10.1002/chem.201301098.

Virágh, M., Vörös, D., Kele, Z., Kovács, L., Fizil, Á., Lakatos, G., et al. (2014). Production of a defensin-like antifungal protein NFAP from *Neosartorya fischeri* in *Pichia pastoris* and its antifungal activity against filamentous fungal isolates from human infections. *Protein Expression and Purification* 94**,** 79-84. doi: 10.1016/j.pep.2013.11.003.

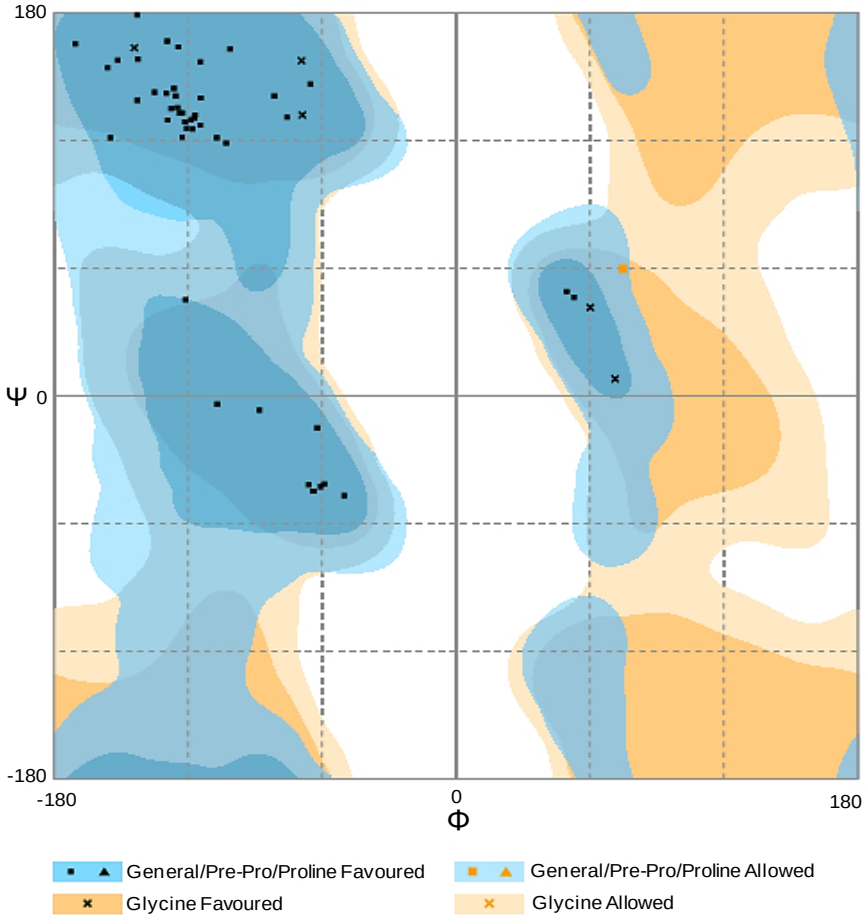
Vriens, K., Cools, T.L., Harvey, P.J., Craik, D.J., Braem, A., Vleugels, J., et al. (2016). The radish defensins RsAFP1 and RsAFP2 act synergistically with caspofungin against *Candida albicans* biofilms. *Peptides* 75**,** 71-79. doi: 10.1016/j.peptides.2015.11.001.

Xu, D., and Zhang, Y. (2011). Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. *Biophysical Journal* 101(10)**,** 2525-2534. doi: 10.1016/j.bpj.2011.10.024.

Yount, N.Y., and Yeaman, M.R. (2004). Multidimensional signatures in antimicrobial peptides. *Proceedings of the National Academy of Sciences of the USA* 101(19)**,** 7363-7368. doi: 10.1073/pnas.0401567101.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* 415(6870)**,** 389-395. doi: 10.1038/415389a

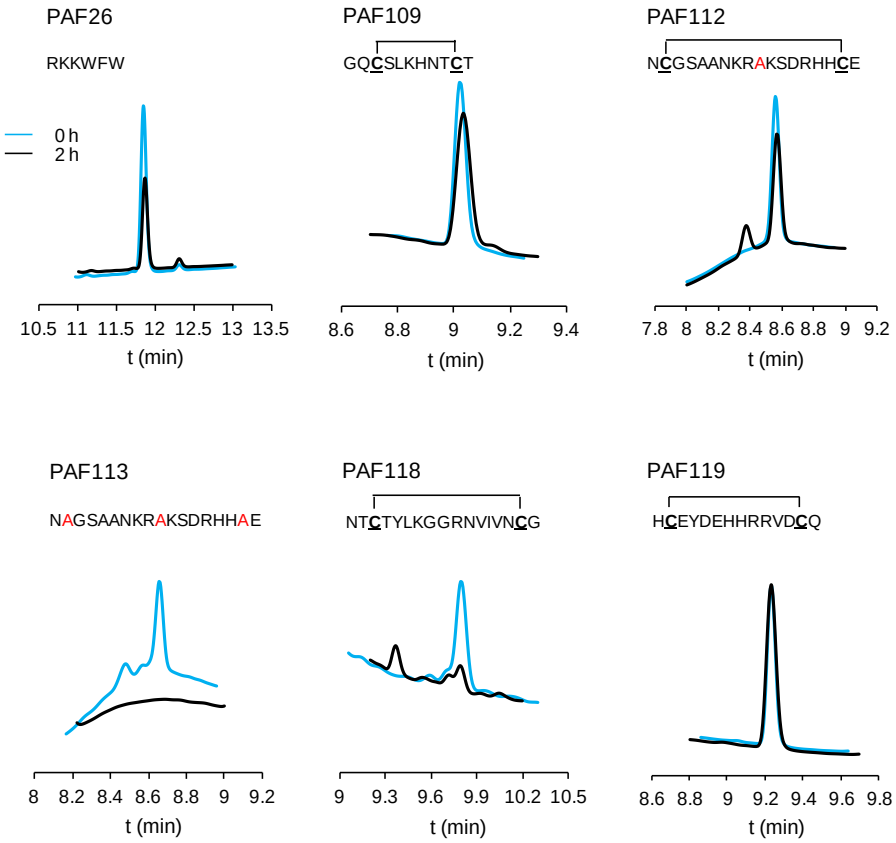
SUPPLEMENTARY MATERIAL



Supp. Fig. 1: Ramachandran Plot of the structural model of AfpB. Visualization of the energetically allowed regions for backbone dihedral angles ψ against φ of the amino acid residues in AfpB protein using RAMPAGE software tool. General amino acids are represented with a square. Proline residues are represented with a triangle. Glycine residues are represented with a cross. Energetically favored and allowed regions for all amino acids (excluding Glycine) are represented in dark and light blue respectively. Energetically favored and allowed regions for Glycine are represented in dark and light orange respectively. Amino acids located in energetically favored regions are colored in black. Amino acids located in energetically allowed regions are highlighted in orange. In AfpB refined molecular model, 56 amino acids out of 57 (98%) are located in energetically favored regions, while only 1 amino acid (2%) is located in energetically allowed regions.

Supp. File 2: A .pdb file of the predicted three-dimensional structure of AfpB. Download at:

https://www.frontiersin.org/article/10.3389/fmicb.2017.00592/full#supplementary-material



Supp. Fig. 3: RP-HPLC chromatograms of the AfpB-derived peptides after treatment with proteinase K. Superposition of representative RP-HPLC chromatograms of the corresponding peptides after treatment with proteinase K (5 µg/mL) for different times (0 and 2 h, blue and black, respectively). Retention times (min) are shown at the bottom. Black lines on the peptides sequences show the disulfide bonds in the AfpB-derived PAF109, PAF112, PAF118 and PAF119. Cysteine residues forming disulfide bonds are represented in bold and underlined.

CHAPTER III

CHAPTER III

Efficient production and characterization of the novel and highly active antifungal protein AfpB from *Penicillium digitatum*

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ABSTRACT

Filamentous fungi encode distinct antifungal proteins (AFPs) that offer great potential to develop new antifungals. Fungi are considered immune to their own AFPs as occurs in *Penicillium chrysogenum*, the producer of the well-known PAF. The *Penicillium digitatum* genome encodes only one *afp* gene (afpB), and the corresponding protein (AfpB) belongs to the class B phylogenetic cluster. Previous attempts to detect AfpB were not successful. In this work, immunodetection confirmed the absence of AfpB accumulation in wild type and previous recombinant constitutive *P. digitatum* strains. Biotechnological production and secretion of AfpB were achieved in *P. digitatum* with the use of a *P. chrysogenum*-based expression cassette and in the yeast *Pichia pastoris* with the α-factor signal peptide. Both strategies allowed proper protein folding, efficient production and single-step purification of AfpB from culture supernatants. AfpB showed antifungal activity higher than the *P. chrysogenum* PAF against the majority of the fungi tested, especially against *Penicillium* species and including *P. digitatum*, which was highly sensitive to the self-AfpB. Spectroscopic data suggest that native folding is not required for activity. AfpB also showed notable ability to withstand protease and thermal degradation and no haemolytic activity, making AfpB a promising candidate for the control of pathogenic fungi.

Keywords: antifungal protein (AFP); AfpB; *Penicillium digitatum*; *Penicillium chrysogenum*; *Pichia pastoris*; fungal cell factory; phytopathogenic fungi.

INTRODUCTION

Nowadays fungal infections have become a serious threat to human health and food security. Human fungal infections have severe consequences on the growing number of immune-compromised patients with high mortality rates. In addition, the control of plant diseases caused by phytopathogenic fungi represents a big challenge in agriculture. The emergence of antifungal resistant strains is in continuous growth, emphasizing the urgent need for the development of novel antifungal agents with properties and mechanisms of action different from existing ones[1].

Antifungal proteins (AFPs) secreted by filamentous fungi have been considered promising candidates for the development of novel antifungal compounds and therapies. AFPs are small, highly stable, cationic, cysteine-rich proteins (CRPs) stabilized by up to four disulphide bridges[2]. They are usually secreted in high amounts by filamentous Ascomycetes, mainly from the genera *Penicillium* and *Aspergillus*, and show potent antifungal activity against non-self fungi at micromolar concentrations.

It is increasingly clear that fungi have a complex repertoire of AFPs that offers a great potential to obtain new antifungal agents. In a previous study, fungal genome sequences were searched to identify AFP-like sequences and conduct detailed phylogenetic studies[3]. Based on phylogenetic clustering but also on sequence alignment, cysteine pattern, intron position and Pfam domain identification, we proposed the classification of fungal AFPs into three classes: A, B and C[3], expanding the two previously reported ones that divided the fungal AFPs into two clusters[4,5]. Recently, a fourth and distantly related group of AFPs has been described[6]. The fungal genomes that encode more than one AFP include those from *Penicillium chrysogenum* and *Penicillium expansum,* which have one AFP belonging to each of three different classes[3]. In the case of the three *P. chrysogenum* AFPs (class A PAF, class B PgAfp and class C Pc-Arctin) their antifungal activity has been experimentally demonstrated[7-9]. Another representative of the *Penicillium* genus is the citrus postharvest pathogen *Penicillium digitatum,* a necrotrophic filamentous fungus that is highly specific for citrus fruits and produces very important economic losses worldwide[10,11]. The recent sequencing of its genome allowed the identification of several potential AFP-like proteins[10], although further phylogenetic analyses confirmed the presence of only one *afp* gene (*afpB*)

whose corresponding protein has been classified into class B and was called AfpB[3].

The most characterized AFPs belong to class A: the AFP produced by *Aspergillus giganteus*[12-15], PAF from *P. chrysogenum*[16-19], and NFAP from *Neosartorya fischeri*[20,21]. Unfortunately, *P. digitatum* AfpB has not been experimentally characterized so far due to the lack of protein detection in *P. digitatum* cultures even in constitutive expression strains that produce up to 1,000 times more *afpB* mRNA than the wild-type strain[3]. In any case, the tertiary structure of the putative AfpB was predicted by *in silico* molecular modelling, which allowed the identification of antifungal peptides based on the AfpB primary sequence and structure[22].

There is a need for low cost and effective AFP-production platforms to produce these proteins at the scale and purity required for their different applications as new antifungal drugs. In this context, the *Pichia pastoris* expression system was applied to produce active recombinant *A. giganteus* AFP[23], *P. chrysogenum* PAF and PAF mutants[16] and *N. fischeri* NFAP and NFAP mutants[24,25]. Recently, a *P. chrysogenum*-based expression system for the production of AFPs was described, showing the feasibility of this approach for the overexpression of high amounts of PAF, PAF variants and NFAP[18,19]. The *P. chrysogenum* expression cassette consisted of the strong *paf* gene promoter, the *paf* pre-pro sequence (SP-pro sequence) for correct protein processing and secretion, and the *paf* gene terminator[8]. Moreover, this expression system was extended to heterologously express PAF in *P. digitatum* with similar yields to those obtained in *P. chrysogenum*, demonstrating the versatility of the system[19].

In this study, we have produced the protein AfpB from *P. digitatum* for the first time using two different expression systems: i) homologous expression in *P. digitatum*, using the *P. chrysogenum*-based expression cassette containing the *paf* gene promoter and terminator sequences, and either the *paf* or the native *afpB* SP-pro sequence, and ii) heterologous expression in *P. pastoris*, using an inducible promoter and the yeast α-factor signal peptide sequence (α-factor SS). Recombinant AfpB has been successfully purified to homogeneity and structurally and functionally characterized.

RESULTS

Recombinant production of AfpB in *P. digitatum* and *P. pastoris*.

We used the *P. chrysogenum*-based expression cassette[19] to express AfpB in *P. digitatum* under the regulation of the strong *paf* promoter and terminator sequences (Fig. 1). In our previous work, the presence of the *paf* SP-pro sequence warranted the secretion of PAF, PAF variants and NFAP proteins into the supernatant independently of the *Penicillium* species used as cell factory. Here, in order to study the functionality of the native SP-pro sequence of the AfpB protein, two different transformation vectors with two expression cassettes for the *afpB* gene insertion in *P. digitatum* were constructed.

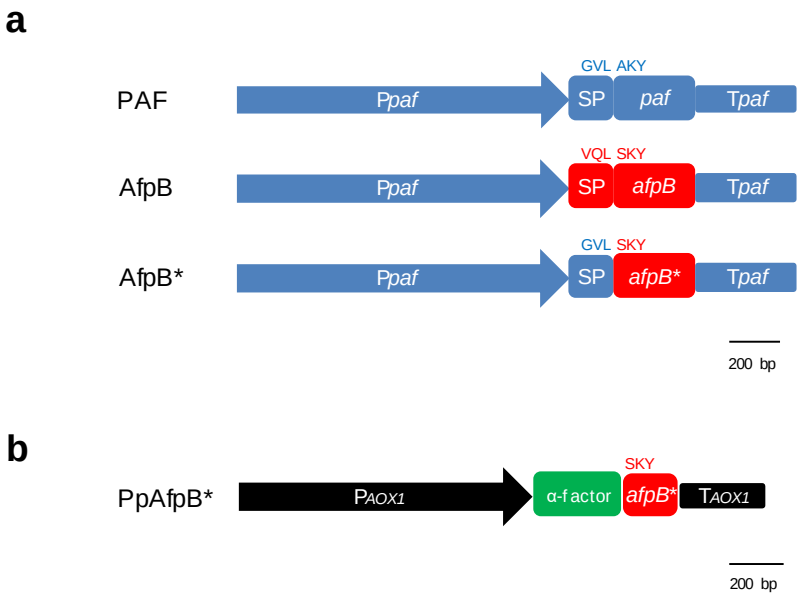

Figure 1: Schematic representation of the expression systems used to obtain AfpB producer strains. a) The PAF diagram constitutes the schematic representation of the *P. chrysogenum*-based expression cassette; in blue *paf* promoter (P*paf*), *paf* gene including the *paf* SP-pro sequence (*paf* SP), and *paf* terminator (T*paf*). Amino acids involved in the SP-pro peptide cleavage are coloured in blue. The AfpB diagram represents the genetic construction with the full-length AfpB coding sequence (in red) cloned under the control of the P*paf* and T*paf* sequences (in blue). Amino acids involved in the predicted SP-pro peptide cleavage are in red. The AfpB* diagram corresponds to the genetic construction with the in silico predicted AfpB coding sequence (*afpB**) cloned under the control of the P*paf*, *paf* SP-pro sequence and T*paf*. b) The PpAfpB* diagram represents the construction with the in silico predicted AfpB coding sequence (*afpB**, in red) cloned under the control of the AOX1 promoter for methanol-induced expression and terminator sequences (in black) and yeast α-factor signal sequence (α-factor SS) (in green).

Both constructions included the *paf* promoter and terminator sequences. However, in the first gene construction, the full-length *afpB* coding sequence (*afpB)* was cloned, while the second approach included the *in silico* predicted mature *afpB* coding sequence (*afpB*)* fused to the *paf* SP-pro sequence (Fig 1a). After evaluation of positive transformants for protein production, one clone with the highest production of the recombinant AfpB protein for each construction was selected for further characterization. The selected producer strains were PDSG2441 for AfpB and PDSG3543 for AfpB*. The genetic modification of these strains was confirmed molecularly (Supplementary Fig. S1).

The growth of the selected transformant strains in solid medium is shown in Fig. 2. Since the *P. chrysogenum* PAF was already expressed in *P. digitatum*[19], the PAF transformant strain (PDSG1521) was chosen as internal control in this study. AfpB transformants showed a moderate reduction of colony diameter on potato dextrose agar (PDA) plates (Fig. 2a,b), higher for the AfpB (PDSG2441) than for the AfpB* (PDSG3543) strain. However, the growth was indistinguishable from that of the parental strain in *P. digitatum* minimal medium (PdMM) (Fig. 2b). The PAF transformant did not show different phenotype to the parental strain in both media. The moderate reduction of growth shown in PDA plates for the transformants AfpB and AfpB* did not result in a difference in their pathogenicity and virulence shown during orange fruit infection (Supplementary Fig. S2).

Heterologous production of AfpB* was also addressed in *P. pastoris.* The genetic construct was designed for its gene inducible expression (*AOX1* promoter) and secretion of the corresponding protein (PpAfpB*, for AfpB* produced in *P. pastoris*) to the extracellular medium (Fig. 1b). With this purpose, *afpB** cDNA was fused in frame to a shortened yeast α-factor SS, in which the Ste13 protease cleavage site was removed and the Kex2 protease cleavage site maintained. This modification was introduced to avoid the Ste13 partial cleavage of the secretion signal resulting in the accumulation of an inactive protein, as previously reported for the AFP from *A. giganteous*[23]. The resulting construct was used to transform *P. pastoris* cells, and two independent transformant were selected for PpAfpB* production.

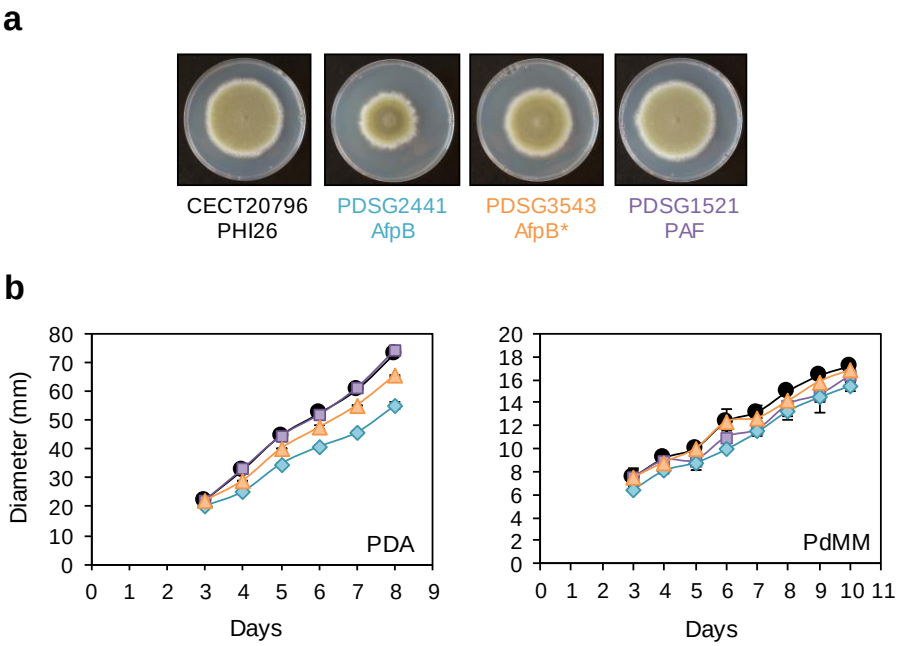

Figure 2: Growth of *P. digitatum* AfpB, AfpB* and PAF producer strains. a) Colony morphology after 7 days of growth in PDA plates. b) Growth on solid PDA and PdMM determined by the colony diameter from 3 to 10 days of growth at 25 ºC. Plotted data are mean values ± s.d. of triplicate samples. The strains shown are the parental CECT 20796 (isolate PHI26) (black), the AfpB producer strains PDSG2441 (AfpB; blue) and PDSG3543 (AfpB*; orange), and the PAF producer strain PDSG1521 (purple).

Single-step cationic exchange chromatography allowed AfpB purification from *P. digitatum* and *P. pastoris* culture supernatants.

Selected clones for AfpB production in *P. digitatum* were grown in PdMM and, after clearing the culture broth from insoluble matter, the proteins in the supernatant were purified by one-step cation-exchange chromatography. Optimal production was achieved after 11 days of growth, and the protein amounts varied between 12 (AfpB) and 20 (AfpB*) mg/L. Both proteins eluted as a single chromatography peak at 0.25 M NaCl, and SDS-PAGE analysis revealed a single-protein band in both, having the same apparent molecular mass of approximately 6 kDa in agreement with the AfpB predicted molecular mass (6.46 kDa) (Fig. 3a, top panel). When comparing with the slightly smaller PAF (6.2 kDa), AfpB showed faster migration than expected. Anomalous migration in SDS-PAGE was also observed for NFAP[19], PgAFP[9] and other fungal AFPs[5] and seems to be related to the extreme isoelectric points of these proteins[26].

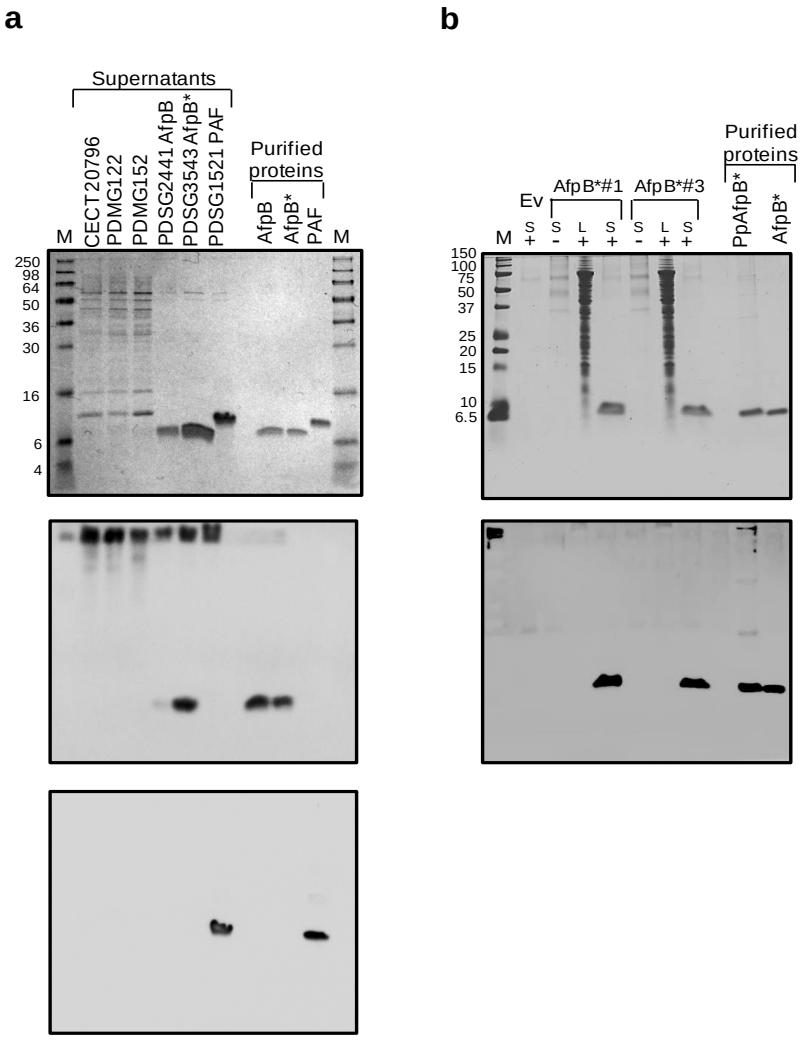

Figure 3: Analyses of supernatants of AfpB-producing strains and purified proteins by SDS-PAGE and Western blot. a) *P. digitatum* supernatants (4-8 µg of total protein loaded per lane) and purified proteins (1 µg loaded per lane) visualized by Coomassie blue staining (top panel); M: SeeBlue® Pre-Stained Protein Standard, supernatants of wild type strain CECT 20796, deletion strain PDMG122 Δ*afpB*, constitutive expression strain PDMG152 *afpB*^{c}, AfpB producer strain PDSG2441, AfpB* producer strain PDSG3543 and PAF producer strain PDSG1521. Immunoblot analyses of samples described above, using anti-PAFB (middle panel) and anti-PAF (bottom panel) antibodies. b*) P. pastoris* supernatants (15 µg loaded per lane), cell lysates (50 µg loaded per lane) and purified proteins (1 µg loaded per lane) visualized by Coomassie blue staining (top panel). M: Precision Plus Protein Standard; AfpB*#1 and AfpB*#3: selected PpAfpB* producer colonies; Ev: control strain transformed with empty vector pPICZαA. S: supernatant; L: cell lysate; under non-induced conditions (-) or induced conditions (+). Immunoblot analyses of samples were performed as described above using anti-PAFB antibodies (bottom panel).

Two independent *P. pastoris* colonies were assayed to evaluate the PpAfpB* accumulation upon methanol induction at different time points. A polypeptide showing similar electrophoretic mobility to the AfpB produced in *P. digitatum* was observed in the supernatant of *P. pastoris* cells harbouring *afpB** cDNA construct and grown under inducing conditions (Fig. 3b, top panel). The intensity of this band increased with time, reaching maximum levels at 48 hours of growth in methanol medium with strong aeration. This band was absent in the supernatant of cells grown under non-inducing conditions, as well as in cells transformed with the empty vector. It was not detected in the lysates of induced cells either, demonstrating its efficient secretion to the extracellular medium. The best producer colony was used to purify the secreted recombinant protein at large scale. The PpAfpB* was also easily purified to homogeneity by one-step cation-exchange chromatography (Fig. 3b, top panel) with yields of 1.2-1.4 mg/L.

Immunodetection confirmed the absence of AfpB in the parental strain

Western blot analyses of 11-days old *P. digitatum* culture supernatants of different fungal strains and purified AfpB proteins were performed with antibodies against *P. chrysogenum* PAF[27] and PAFB. PAFB is a protein from *P. chrysogenum* that is 88% identical to AfpB, and from which an antiserum was raised (unpublished). Culture supernatants of PDMG122 (null Δ*afpB*) and PDMG152 (constitutive *afpB*^{C}) were also included in the analyses as controls and to confirm previous absence of detection by Coomasie Blue staining[3]. Results indicated that both AfpB proteins and the corresponding supernatants reacted with anti-PAFB antibody (Fig. 2a, middle panel) while only purified PAF and the corresponding transformant strain PDSG1521 supernatant reacted with PAF antiserum (Fig. 2a, bottom panel). As expected, no protein band in the supernatant of the previous null transformant strain (Δ*afpB*) reacted with the anti-PAFB antibody. Remarkably, no immunoreaction was observed in the culture supernatant of either the parental strain (CECT 20796) or the constitutive *afpB*^{c} transformant strain (PDMG152), fully demonstrating our previous conclusions on the absence of detection of any differential band or AfpB-like protein in those *P. digitatum* culture supernatants[3].

Western blot analyses were also performed for the heterologously produced PpAfpB* protein with antibodies against PAFB (Fig. 3b, bottom

panel). Supernatants from *P. pastoris* pPICZαA-AfpB* containing colonies grown under inducing conditions and purified PpAfpB* reacted with the antiserum while, as anticipated by Coomassie staining, no protein was detected in the non-induced supernatants or in cell lysates.

Mass spectrometry revealed different processing of PAF and AfpB SP-pro sequence.

Molecular mass of AfpB proteins purified from *P. digitatum* was also determined by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Single peaks corresponding to average masses of 6570.53 and 6458.01 Da were detected for AfpB and AfpB*, respectively (Fig. 4). The experimental mass of AfpB* is consistent with the calculated theoretical mass of the oxidized protein predicted after cleavage from the PAF SP-pro sequence (6456.23 Da), indicating the presence of three intra-molecular disulphide bonds and the absence of other post-translational modifications. Interestingly the average mass detected for AfpB revealed the presence of an extra leucine residue at the N-terminus end of the protein (calculated theoretical mass 6575.39 Da) suggesting a different cleavage of the native AfpB SP-pro sequence.

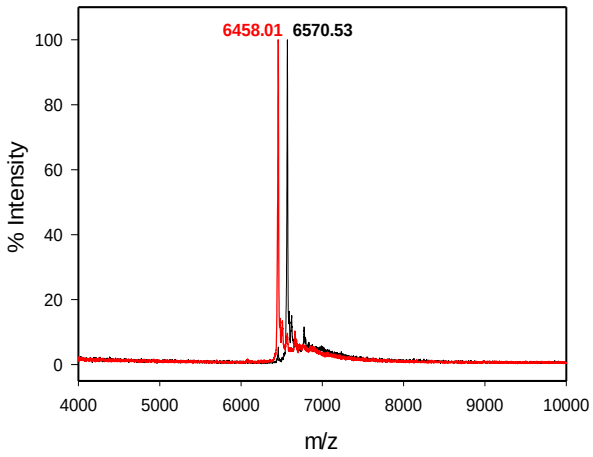

Figure 4: MALDI-TOF MS analyses. Data showing the isotopic average molecular mass (m/z) of the two recombinant protein variants AfpB* (red) and AfpB (black) produced in *P. digitatum*. The difference in the molecular mass fits with the extra leucine residue present at the N-terminus of the native protein AfpB.

The MS corresponding to PpAfpB* revealed one main signal of 6455.20 Da (Supplementary Fig. S3) in accordance with the calculated molecular mass of the oxidized protein form and the proper processing of the yeast α-factor SS. An additional minor signal of 7104.54 Da that did not correspond to any potential variable N-terminus was detected.

Electronic circular dichroism spectroscopy assays revealed incomplete refolding capability of the AfpB* variant.

Electronic circular dichroism (ECD) spectroscopy was used to determine protein conformation and proper folding of both *P. digitatum* AfpB and AfpB* variants. The ECD spectra recorded at 25 °C were nearly identical for both variants (Fig. 5 and Supplementay Fig. S4), and similar to those recorded for PAF, NFAP[19] and other disulphide bridged, β-structured proteins[28].

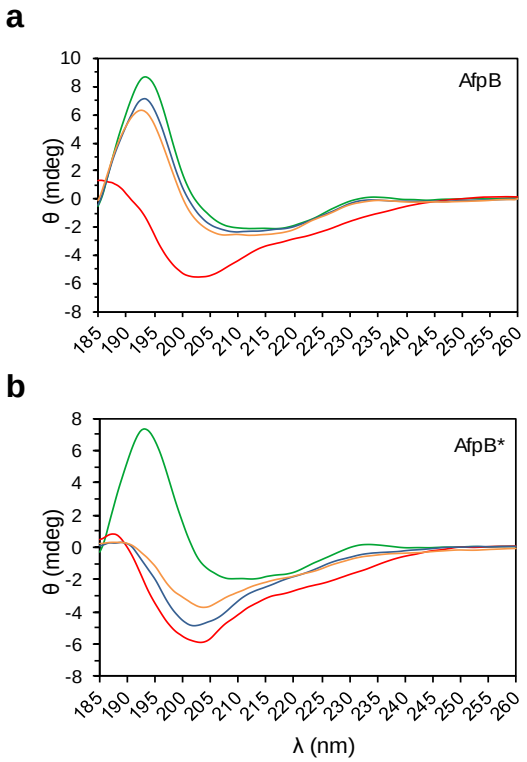

Figure 5: ECD spectra of the two recombinant protein variants produced in *P. digitatum*. a) AfpB, and b) AfpB* recorded at 25 °C (green), 95 °C (red), and at 25 °C immediately (blue), and 72 h (orange) after cooling from 95 °C to 25 °C.

The spectra had two maxima at 195 and 229 nm. The maximum at 229 nm was mainly attributed to the presence of disulphide bridges while the maximum centred at 195 nm reflected contributions from both β-pleated conformation and the electronic transitions of disulphide bridges. Spectra measured at 95 °C reflected the loss of ordered structure in both AfpB variants. After cooling back to 25 °C, the native fold of AfpB was restored completely and almost immediately (Fig. 5a). In contrast, a slow structural reorganization of AfpB* took place with incomplete refolding even after 72 h (Fig 5b). This observation may be attributed to the absence of the leucine residue at the N-terminus of this protein variant.

AfpB was highly active against *Penicillium* species including *P. digitatum*

Both AfpB protein variants, and PAF as an internal control, were tested for their antimicrobial activity towards *Escherichia coli*, *Saccharomyces cerevisiae* and a selection of filamentous fungi that include, in addition to the *P. digitatum* parental strain, several plant pathogens such as the citrus fruit specific *Penicillium italicum*, the main postharvest pathogen of pome fruit *P. expansum*, the polyphagous *Botrytis cinerea*, the rice blast fungus *Magnaporthe oryzae,* and the soilborne plant pathogen *Fusarium oxysporum*. The PAF producer *P. chrysogenum* strain, and a strain from *Aspergillus niger* which is particularly sensitive to the PAF protein were also tested. No differences in antimicrobial activity were observed among AfpB, AfpB* and PpAfpB* produced in either *P. digitatum* or *P. pastoris* (Supplementary Fig. S5). Therefore, only results from AfpB produced in *P. digitatum* are shown. AfpB was inactive against *E. coli* and *S. cerevisiae* at the highest concentration tested (200 µg/ml). AfpB showed antifungal activity and inhibited the growth of all fungi tested, with the exception of *M. oryzae* (Fig. 6). The minimum inhibitory concentration (MIC) values varied from 1.6 µg/ml in *P. italicum* to 100 µg/ml in *F. oxysporum*. The three plant pathogenic *Penicillium* species tested were considerably sensitive to the protein, including its producer strain. With the exception of the PAF-sensitive *A. niger*, AfpB showed higher antifungal activity than PAF, with MIC values at least one order of magnitude lower.

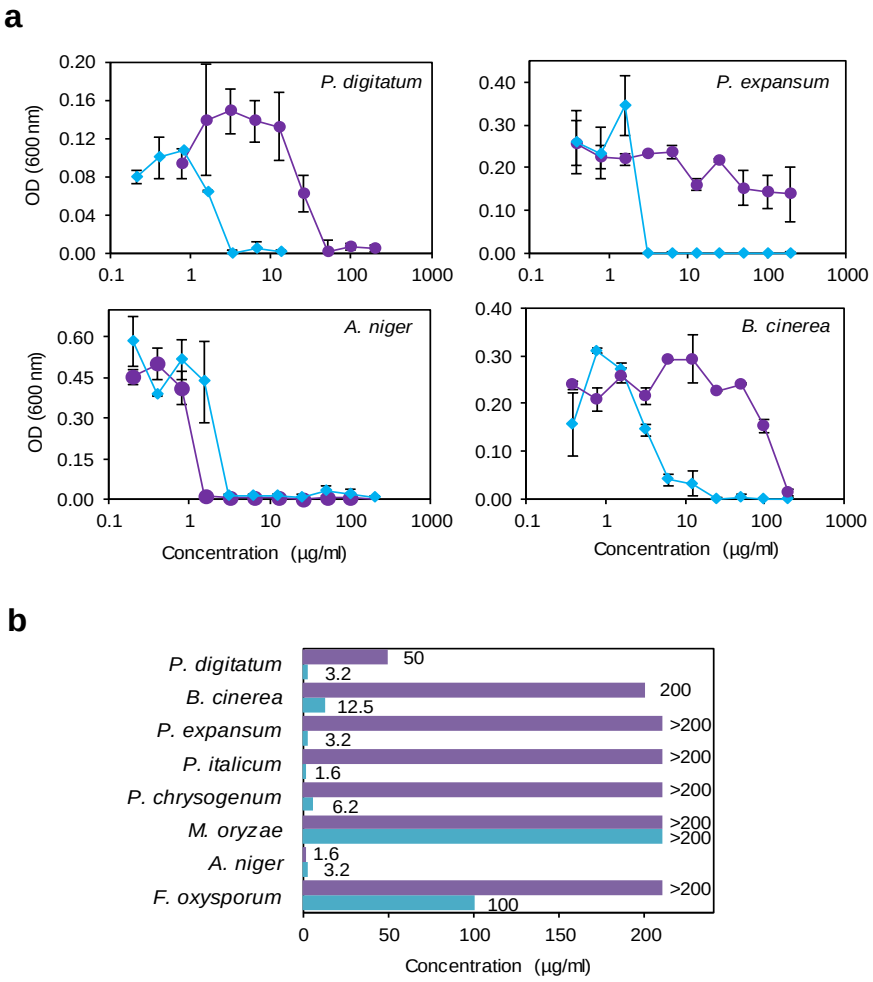

Figure 6: *In vitro* inhibitory activity of AfpB and PAF against filamentous fungi. a) Dose-response curves comparing the antifungal activity of PAF (purple circles) and AfpB (blue diamonds) against *P. digitatum*, *P. expansum*, *A. niger*, and *B. cinerea*. Dose-response curves show mean ± s.d. OD_{600} of triplicate samples after 72 h of static incubation at 25 °C, except for *A. niger*, which was incubated at 37 ºC. b) MIC values (µg/ml) of AfpB (blue) and PAF (purple) against all the fungi tested.

AfpB showed high protease and thermal resistance.

The sensitivity of AfpB to proteolytic digestion was tested using proteinase K and evaluating the residual antifungal activity against *P. digitatum*. Preincubation of AfpB and AfpB* with proteinase K did not decrease the antifungal activity of any of both protein variants (Supplementary Fig. S6), pointing out the high protease resistance of these antifungal proteins.

The incomplete refolding of AfpB* found with the ECD analyses allowed us to test and determine the effect of heat treatment and folding on the antifungal activity. After heat treatment at 95 °C for 5 min and cooling back to 25 °C (mimicking the ECD conditions) the antifungal activity of both protein variants was practically equal and comparable to that observed without treatment (Supplementary Fig. S7), suggesting that native AfpB refolding is not necessary for its antifungal activity.

Further experiments were conducted to compare the thermal stability of PAF and AfpB. After heat treatment at 80 ºC or 95 °C for 10 and 60 min and cooling back to 25 °C, the antifungal activity of PAF and AfpB were tested against *P. digitatum*. Fungal conidia were exposed to twofold MIC concentrations of heat-treated proteins (100 µg/ml for PAF, Fig. 7a, and 6.5 µg/ml for AfpB, Fig. 7b). The antifungal activity of PAF against *P. digitatum* at that concentration resulted in a growth reduction of 70-80% after 10 min of heat treatment at 95 ºC and 80 ºC, respectively in comparison with the untreated control (>95%). After 60 min of heat treatment, PAF showed a *P. digitatum* growth reduction of 60% at 80 ºC and 55% at 95 ºC. In the same conditions, AfpB showed total inhibition of the fungus growth (>95%, comparable to the non-treated control), demonstrating that AfpB is more resistant to heat treatment than PAF.

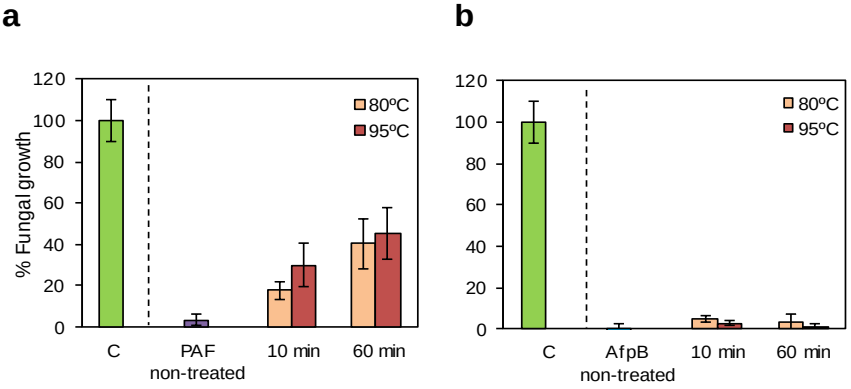

Figure 7: Effect of heat treatment on the antifungal activity of PAF and AfpB**.** PAF (a) and AfpB (b) (100 and 6.5 µg/ml, respectively) were exposed to 80 and 95 °C for 10 and 60 min, respectively. Values represent the percentage of growth (%) of *P. digitatum* in the presence of non-treated and treated protein compared to *P. digitatum* in the absence of protein (control, C). Data show mean ± s.d. OD_{600} of triplicate samples.

AfpB showed no haemolytic activity against human red blood cells.

Haemolytic activity assays are performed in order to determine the cytotoxicity of specific proteins and peptides against eukaryotic cells by their ability to lyse human red blood cells (RBCs)[29]. In this study, we determined the haemolytic activity of both AfpB variants at different concentrations from 1 to 100 µM (approximately 200 times the MIC against *P. digitatum*). In these experiments, PAF and melittin from honeybee[30] were included as negative and positive controls, respectively. Since the haemolytic properties of cationic peptides may show ionic strength dependence, haemolytic assays were conducted not only with a high ionic strength phosphate NaCl buffer (PBS) but also with a low ionic strength isotonic glucose phosphate buffer (PBG)[29].

As expected, the haemolytic peptide melittin caused 100% haemolysis at the highest concentration tested and around 80% at 10 µM (Fig. 8). By contrast, none of the AFPs showed haemolytic activity at any of the concentrations tested, neither in the presence of NaCl as in PBS (Fig. 8a) nor glucose (Fig. 8b), with values close to the control where no protein was added.

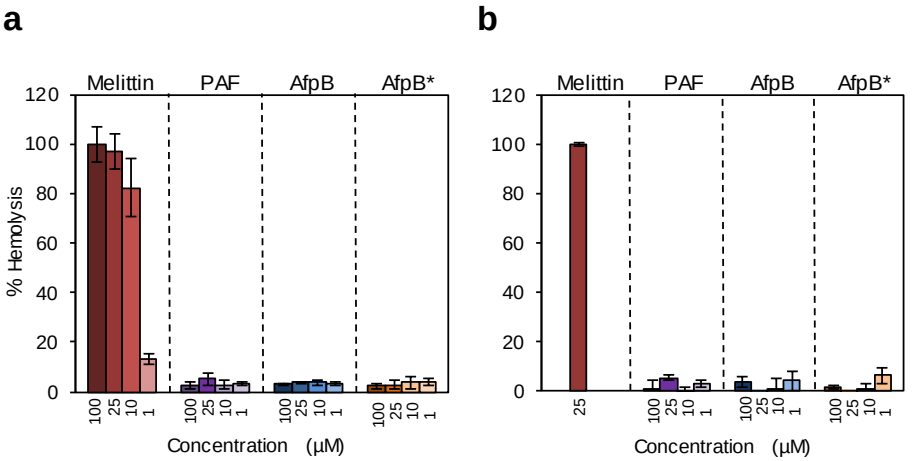

Figure 8: Haemolytic activity of the two AfpB variants and PAF. Analyses were conducted in PBS (150 mM NaCl) (a), and in PBG (250 mM glucose) (b). Proteins were used at the concentrations indicated (from 1 to 100 µM). The cytolitic peptide melittin was included for comparison. The haemolytic activity is given as the mean ± s.d. of the percentage of human RBCs haemolysis (three replicates), as compared with the positive control in the presence of the detergent Triton X-100 (regarded as 100% haemolysis).

DISCUSSION

In this study, we described the biotechnological production and characterization of AfpB, the only AFP encoded in the phytopathogenic fungus *P. digitatum.* This protein is a new member of AFPs grouped into the class B phylogenetic cluster[3]. For the first time, AfpB was produced, detected and purified from culture supernatants of recombinant *P. digitatum* and *P. pastoris* strains. The homologous production of AfpB in *P. digitatum* with the *paf*-derived expression cassette resulted in high yields around 12-20 mg protein/L, whereas the heterologous production in *P. pastoris* resulted in yields 10-fold lower (1.2-1.4 mg/L). Both approaches allowed one-step purification of fully active AfpB, and the purified protein from the two systems showed the same antifungal potency. Our results demonstrate that both filamentous fungi and yeasts are good production platforms for AFPs. The homologous system appears as the most efficient system reaching high yields; whereas the heterologous system has the advantage of time reduction since optimal production was achieved after 11 days of growth in *P. digitatum* while only 2 days were needed with the *P. pastoris* system.

Previously, three other class B AFPs were characterized: Anafp from *A. niger*[31], PgAFP from *P. chrysogenum*[32] and MAFP1 from *Monascus pilosus*[33]. They show an amino acid identity with *P. digitatum* AfpB of 79, 88, and 83%, respectively, much higher than the percentage of identity shared between AfpB and the class A PAF from *P. chrysogenum,* with only 33% identity[3]. The three of them were successfully isolated from the culture supernatants of the corresponding native and producer fungus. Notably, the concentration of purified PgAFP obtained from *P. chrysogenum* CECT 20922, which was isolated from cured meat, was up to 700 µg/ml[32]. Also, the class A PAF and AFP are secreted in large amounts by *P. chrysogenum*[17] and *A. giganteus*[13], respectively. However, previous attempts to detect AfpB in *P. digitatum* culture supernatants failed, even in *P. digitatum* transformants for a*fpB* constitutive expression under the strong *gpdA* promoter from *A. nidulans*[3]. Remarkably, those transformant strains showed a drastic reduction of axenic growth, abnormal hyphal morphology and delayed conidiogenesis[3]. Here, we have confirmed with immunodetection that AfpB is not produced by any of the previous strains. By contrast, *afpB* expression under the regulation of the strong *paf* promoter and the corresponding *paf* terminator warranted the production

and secretion of the protein at high levels, concomitant with only a moderate reduction of axenic growth. We showed that AfpB production is similar in the presence of either the *paf*- or the *afpB*-specific SP-pro sequences, indicating that the impossibility to detect AfpB in either the parental or constitutive strain cultures is not due to the native AfpB SP-pro peptide, but to the regulatory sequences. The *P. chrysogenum paf* promoter has been demonstrated to have greater efficiency than the *A. nidulans gpdA* constitutive promoter used as for the expression of reporter genes in *P. chrysogenum*[34], however, the approximately 2-fold difference in efficiency cannot account for the absence of AfpB accumulation when the *gpdA* promoter was used. In addition, differences in 5' untranslated regions (UTRs) in the mRNA have been demonstrated to play an important role in determining the translation efficiency of proteins in filamentous fungi[35]. Nevertheless, AfpB production was also achieved in the *P. pastoris* expression system under the control of the *AOX1* promoter using the yeast α-factor SS. Additional studies are required to elucidate the exact mechanism underlying the problematic of native *afpB* translation and protein accumulation in *P. digitatum*.

In accordance with the antimicrobial profile of native AFPs that possess antifungal but not antibacterial activity[1,17], AfpB displayed antifungal activity against selected filamentous fungi and it was inactive against bacteria (*E. coli*) or yeast (*S. cerevisiae*) However, the homologous protein AnAFP from *A. niger* inhibited the proliferation of *Candida albicans* and *S. cerevisiae*[31] whereas the other two class B representatives did not show any effect on different bacteria and yeast species[32,33]. Recently a new AFP from *N. fischeri* named NFAP2 proved to be highly effective against targeted yeasts including clinically relevant *Candida* species[6]. NFAP2, which seems to be the first member of a new, phylogenetically distinct fourth group among AFPs, showed MIC values in the range of 0.2–1.5 µg/ml. Potential AfpB anti-yeast activity against non-laboratory strains and/or pathogenic isolates deserves future research.

Until this study it was assumed that AFPs are not active against the producer fungus, and it has been speculated that fungi possess innate sensing or defense systems which enable them to discriminate between AFPs from self or non-self origin[36]. Whether the function of fungal AFPs is mainly defensive or associated with fungal growth and development is still

controversial. Null mutation of the *afpB* gene in *P. digitatum* did not affect spore production, growth or virulence[3], contrarily to that described for the null mutation of the *paf* gene in *P. chrysogenum* that severely affected asexual development, spore production and associated gene regulation[37]. Recently, novel functions related to nutrient recycling during starvation, autophagy, and development for the AnAFP produced by *A. niger* have been proposed[36]. It is very tempting to speculate that AFPs from different classes do have distinct biological functions in the producer fungus and also distinct mechanisms of antifungal action. This seems to be the case in the AfpB/PAF couple, according to the different phenotypes of the corresponding null mutants but also to the different activity profiles of both proteins against *P. digitatum*, *P. expansum* or *A. niger*.

AfpB is highly active against the own producer *P. digitatum* strain when added exogenously to the culture media (MIC = 3.2 µg/ml; Fig. 6), which is not the case of PAF against *P. chrysogenum* (MIC > 200 µg/ml). However, the fungal strain engineered to express the *afpB* gene produces high amounts of AfpB without deleterious effect to the axenic growth or pathogenicity of *P. digitatum*. This fungus is also susceptible to PAF protein (MIC = 50 µg/ml) but *P. digitatum* engineered strains were also able to produce PAF in high quantities[19]. In contrast, the heterologous expression of the *nfap* gene in a NFAP-sensitive *A. nidulans* strain only permitted the production of low amounts of protein (approximately 1.7 mg/L), but it provoked reduced hyphal growth and delayed and abnormal germination[38]. Other fungal AFPs such as AnAFP and *A. giganteous* AFP have been described as only moderate-active towards the producer strains[36] whereas the class B PgAFP was not active against its producing strain *P. chrysogenum* CECT 20922 while it showed antifungal activity against another *P. chrysogenum* strain, pointing out strain-dependent activity[32].

AfpB is unique for its very high antifungal activity against filamentous fungi that include the fungus from which the gene was identified. Our side-by-side experiments demonstrate that AfpB has higher levels of antifungal efficacy when compared to PAF (Fig. 6). With respect to other class B proteins already characterized, AnAfpB showed similar antifungal activity against *F. oxysporum*[31], and PgAfpB was as well active against other *P. expansum* and *A. niger* strains[32]. The activity of AfpB against the *Penicillium* species tested deserves to be emphasized. All of

them were highly sensitive to AfpB: MIC values in the range of 1.6-6.25 µg/ml that is equivalent to 0.25-1 µM. Remarkably, current fruit losses due to *Penicillium* decay are very important in agriculture since *Penicillium* species reproduce very rapidly and their spores are ubiquitous in the atmosphere, facilitating the dissemination on fruit surfaces. *P. digitatum and P. italicum* are the main causal agents of the citrus green and blue mold diseases, respectively[10]. On the other hand, *P. expansum,* the causal agent of the blue mold disease, is one of the most important pathogens of pome fruits, causing serious crop losses worldwide[11]. Unfortunately, there are only a few drugs available for the effective treating of fungal infections, and the development of resistance against fungicides used in agriculture is increasingly alarming. Thus, the use of AfpB as a powerful alternative in the control of pathogenic fungi could be of further interest.

In a previous work, we identified two cysteine-containing cationic peptides, PAF112 and PAF118, derived from two surface-exposed loops in AfpB, with moderate antifungal activity against *P. digitatum* with a MIC value of 64 µM[22]. Now our results show that AfpB is about 100 times more active than the peptides derived from its sequence suggesting that additional motifs/sequences in the protein are required to achieve full activity.

As generally described for fungal AFPs[16], AfpB showed a remarkable ability to withstand protease and thermal degradation. It has been assumed that the compact tertiary structure stabilized by disulphide bridges of AFPs favours protein stability. Although the tertiary structure of AfpB has not yet been determined, the predicted AfpB structure by *in silico* modelling showed a similar tertiary structure to those reported for A*. giganteous* AFP[12] and *P. chrysogenum* PAF[16,39]. In the present study ECD spectroscopic measurements, consistent with the previous modelling, suggested the presence of disulphide bridges and a β-pleated conformation, and proved that both AfpB variants have the same structural elements as other fungal AFPs[19,24]. Our data also demonstrate that the lack of the leucine residue at the N-terminal end of AfpB* does not affect neither the antifungal activity nor the protease and thermal tolerance, although this protein variant showed incomplete refolding in the conditions tested. Moreover, the demonstration that the heat treatment which denatures

AfpB*, mostly irreversibly, does not impair antifungal efficacy indicates that protein folding is not critical for the antifungal activity.

Finally, our results suggest that AfpB can be preliminary regarded as safe since it did not show haemolytic activity against human RBCs even in assays conducted at low ionic strength isotonic conditions, which are considered more sensitive for detecting the haemolytic activity of cationic peptides[29].

In summary, this study identifies AfpB from *P. digitatum* as a highly active antifungal protein against filamentous fungi, including the own producer fungus. This new member of class B AFPs was produced for the first time in two eukaryotic cell factories, the fungus *P. digitatum* and the yeast *P. pastoris*. Both expression systems allowed proper protein folding, efficient production and single-step purification from culture supernatants. The remarkable stability, absence of haemolytic activity and high levels of antifungal efficacy against filamentous fungi, especially *Penicillium* species, suggest the potential use of AfpB as an antifungal agent.

METHODS

Strains, media and culture conditions

The parental isolate *P. digitatum* CECT 20796 (PHI26)[10] and all of the transformants were cultured on PDA plates for 7-10 days at 25 ºC. To analyse the growth on solid medium, 5 µl of conidial suspension (5×10^4 conidia/ml) were deposited on the centre of PDA and PdMM plates[19], and the diameter of growth was monitored daily from 3 to 10 days. For AfpB production, strains were inoculated at a concentration of 10^6 conidia/ml in 500 ml of PdMM and incubated at 25 ºC with shaking for 14 days. For the antimicrobial assays, *B. cinerea* CECT 2100, *F. oxysporum* 4287, *P. expansum* CMP-1, *P. italicum* CECT 2294*, P. chrysogenum* Q176, and *M. oryzae PR9* were incubated at 25 ºC, *E. coli* JM109 and *A. niger* CBS 120.49 at 37 ºC, and *S. cerevisiae* BY4741 at 30 ºC. For the fungal transformation, vectors were propagated in *E. coli* JM109 grown in Luria Bertani (LB) medium supplemented with 100 µg/ml ampicillin or 75 µg/ml kanamycin. *Agrobacterium tumefaciens* AGL-1 strain was grown in LB supplemented with 20 µg/ml rifampicin at 28 ºC. *P. pastoris* X-33 wild-type strain was cultured on yeast extract peptone dextrose (YPD) medium at 28 ºC.

Generation of the *P. digitatum* AfpB producer strains

To generate the *P. digitatum* AfpB producer strains, two different genetic approaches were performed. The first approach included the full-length AfpB coding sequence (*afpB)* cloned under the control of the *paf* gene promoter and terminator sequences from *P. chrysogenum*. The second approach included the *in silico* predicted mature AfpB coding sequence[3] (*afpB**) cloned under the control of the *paf* gene promoter, *paf* SP-pro sequence and the *paf* gene terminator (Fig. 1**a**). The specific primers used to generate the *P. digitatum* AfpB producer strains are described in Supplementary Table S1 and Supplementary Fig. S1**a**. The *afpB* gene was amplified from *P. digitatum* CECT 20796 genomic DNA, while the *paf* gene promoter, SP-pro and terminator sequences were generated by PCR amplification of the fragments from the vector pSK275*paf*[19]. PCR reactions were performed using AccuPrime High-Fidelity polymerase (Invitrogen) and the constructs verified by Sanger DNA sequencing. The two different DNA constructions were generated by Fusion PCR[40] and cloned into the pGEM-T® Easy vector system

(Promega) from where they were excised with *Xma*I and *Xba*I and inserted into the digested binary vector pBHt2[41] containing the hygromycin resistant cassette (*hph*) used as positive selection marker. The binary vectors were transformed into *A. tumefaciens* AGL-1, and the fungal transformation of *P. digitatum* parental strain was performed by *A. tumefaciens*-mediated transformation (ATMT)[41,42]. Positive transformants were confirmed by PCR amplification of genomic DNA[43] (Supplementary Fig. S1; Supplementary Table S1).

Generation of the *P. pastoris* AfpB producer strains

To generate the *P. pastoris* AfpB producer strain, the *P. digitatum* cDNA encoding the predicted AfpB protein sequence (*afpB**) was obtained as previously described[43] and amplified by PCR using the primers MO3 and MO4 (Supplementary Table S1). The *afpB** cDNA sequence was inserted into the *Xho*I and *Xba*I digested pPICZαA plasmid (Invitrogen) carrying *AOX1* promoter for methanol-induced expression of *afpB** in *P. pastoris*. The *afpB** cDNA was inserted in frame with a modified version of the yeast α-factor SS, lacking the Ste13 cleavage sites, but including the Kex2 signal cleavage site (Fig. 1**b**). Constructs were verified by Sanger DNA sequencing. The digested plasmid pPICZαA-*afpB* with *Avr*II was used to transform *P. pastoris* X-33 cells by electroporation. After 5 min of incubation at 4 ºC, cells were subjected to a pulse (1.5 kV, 200 Ω) in 0.2-cm cuvettes and were immediately diluted with 1 ml of ice-cold 1M sorbitol and plated on YPD medium containing 1M sorbitol and 100 µg/ml zeocin. Plates were incubated for 3-4 days at 28 ºC. Two independent colonies were selected (#1 and #3) and used for PpAfpB* production. Transformant cells were grown in buffered minimal glycerol medium BMG (0.1 M potassium phosphate buffer, pH 6; 1.34% yeast nitrogen base; 4 x 10^{-5}% biotin; 1% glycerol), and transferred to BMM (buffered minimal medium with 0.5% methanol instead of glycerol as carbon source) for *afpB** induction.

Protein production and purification

AfpB variants were purified from the supernatants of *P. digitatum* transformant strains (AfpB and AfpB*) and *P. pastoris* positive transformants (PpAfpB*). Cell-free supernatants of *P. digitatum* grown on PdMM for 11 days or *P. pastoris* grown on BMM for 2 days were collected by centrifugation and dialyzed (2 K MWCO,) against 20 mM phosphate buffer pH 6.6. Dialyzed solutions were applied to an AKTA Purifier system

equipped with a 6 ml RESOURCE S column (GE Healthcare) for *P. digitatum* supernatants or a 5 ml HiTrap SP HP column (GE Healthcare) for *P. pastoris* supernatants, equilibrated in phosphate buffer. Proteins were eluted with a linear NaCl gradient from 0 to 0.5 M in the same buffer.

Protein containing fractions were pooled, dialyzed against Milli-Q water and lyophilized. Protein concentrations were determined spectrophotometrically (A_{280}) considering the molar extinction coefficient (ε_{280} = 0.52). Purification was monitored by SDS-PAGE[44] using SDS-16% polyacrylamide gels calibrated with prestained protein size-standard SeeBlue® (ThermoFischer Scientific) or Precision Plus Protein Standards (Bio-Rad) and Coomassie stained.

Western blot

Total proteins from supernatants, lysates and purified AfpB variants were separated by SDS-16% polyacrylamide gels and transferred to Amersham Protran 0.20 µm NC nitrocellulose transfer membrane (GE Healthcare). Protein detection was accomplished using anti-PAFB (unpublished) and anti-PAF[27] antibodies diluted 1:1000 and 1:2000, respectively. As secondary antibody, ECL NA934 horseradish peroxidase donkey anti-rabbit (GE Healthcare) was used and chemiluminescent detection was performed with ECL™ Select Western blotting detection reagent (GE Healthcare) using a LAS-1000 instrument (Fujifilm). The experiments were repeated twice.

MALDI-TOF MS

The mass of the purified proteins was analysed on a 5800 MALDI TOF/TOF (AB Sciex) in positive linear mode (1500 shots every position) in a range of 2000-20000 m/z. The analysis was carried out in the proteomics facility of SCSIE University of Valencia (Spain).

ECD spectroscopy

ECD spectroscopic measurements were performed in the 185-260 nm wavelength range (far–UV) to determine the secondary structure and examine the structural stability of the two AfpB variants. Protein samples were dissolved in pure H_2O at approximately 0.1 mg/ml concentration and measured in a 0.1 cm path-length quartz cuvette[19]. The spectra are

accumulations of 10 scans, from which the similarly recorded spectrum of H_2O was subtracted. Ellipticity data were given in mdeg units.

Protein stability assays

To investigate thermal stability, proteins (400 µg/ml) were dissolved in 10 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) pH 7 and were incubated at 80 ºC and 95 ºC for 5, 10 and 60 min. Proteins were cooled back to 25 ºC and directly used to determine the residual antifungal activity.

Proteolytic digestion assays were performed as previously described[45] with some modifications. Briefly, proteins (400 µg/ml) were dissolved in 10 mM MOPS pH 7 and digested with 100 µg/ml of recombinant proteinase K (2 U/mg; Sigma-Aldrich) at 30 ºC with shaking. Aliquots were withdrawn at 20 h of incubation and immediately heated at 80 ºC for 10 min. Treated proteins were used to perform antifungal activity assays.

Antimicrobial activity assays

Growth inhibition assays were performed in 96-well, flat-bottom microtiter plates in a total volume of 100 µl. Fifty µl of fungal conidia (5×10^4 conidia/ml) in 1/10 diluted potato dextrose broth (PDB) containing 0.02 % (w/v) chloramphenicol were mixed in each well with 50 µl of twofold concentrated protein from serial twofold dilutions (final concentrations from 0.2-200 µg/ml). Samples were prepared in triplicate. Plates were statically incubated for 96 h at 25 ºC. Growth was determined every 24 h by measuring the optical density (OD) at 600 nm using a Fluostar Omega plate spectrophotometer (BMG labtech), and the OD_{600} mean and standard deviation (s.d.) were calculated. Dose-response curves were generated from measurements after 72 h. These experiments were repeated at least twice. MIC is defined as the protein concentration that completely inhibited growth in all the experiments conducted.

Haemolytic assays

The haemolytic activity of the proteins was determined in a 96 round-bottom microtiter plate on human 1:40 diluted RBCs as previously described[29,46] with some modifications. Briefly, RBCs were harvested by centrifugation for 15 min at 100 × g and washed three times in 35 mM PBS (pH 7, 150 mM NaCl) or PBG (250 mM glucose as osmoprotectant). One

hundred µl of twofold protein concentration were mixed with 100 µl of RBCs in triplicate. Plates were incubated for 1 h at 37 ºC and centrifuged for 5 min at 300 × g. Supernatants (100 µl) were transferred to a new microtiter plate and the absorbance was measured at 415 nm.

No haemolysis and 100% haemolysis were determined in controls with a mixture of PBS or PBG, and 0.1% Triton X-100, respectively. The haemolytic activity was calculated as the percentage of total haemoglobin released compared with that released by incubation with 0.1% Triton X-100.

Fruit infection assays

The inoculation of *P. digitatum* strains on non-treated freshly harvested orange fruits (*Citrus sinensis* L. Osbeck cv Navelina) was conducted as previously described[47]. Briefly, three replicates of five fruits were inoculated with 5 µl of conidial suspension (5×10^4 conidia/ml) at four wounds around the equator. Orange fruits were stored at 20 ºC and 90% relative humidity. Each wound was scored daily for infection symptoms on consecutive post inoculation days.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

S.G., J.F.M., M.C. and P.M. conceived and designed the study. P.M. coordinated the study and prepared the first draft of the manuscript. S.G. and J.F.M. produced AfpB in P. digitatum, C.P. and M.C. produced AfpB in P. pastoris. S.G., C.P. and P.M. performed protein purification. S.G. and P.M. performed AfpB stability experiments. S.G., M.C. and C.P.

performed antimicrobial experiments. M.G. and C.P. performed Western blot analyses and molecular characterization of transformant strains. F.M. provided the anti-PAF and anti-PAFB antibodies and vector pSK275paf. A.B. performed the ECD experiments. All authors approved the manuscript and agreed to be accountable for the content of the work. Authorship is limited to those who have contributed substantially to the work reported.

REFERENCES

1 Meyer, V. A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. *Appl. Microbiol. Biotechnol.* 78, 17-28, doi:10.1007/s00253-007-1291-3 (2008).

2 Hegedüs, N. & Marx, F. Antifungal proteins: More than antimicrobials? *Fungal Biol. Rev.* 26, 132-145, doi:10.1016/j.fbr.2012.07.002 (2013).

3 Garrigues, S., Gandía, M. & Marcos, J. F. Occurrence and function of fungal antifungal proteins: a case study of the citrus postharvest pathogen *Penicillium digitatum*. *Appl. Microbiol. Biotechnol.* 100, 2243-2256, doi:10.1007/s00253-015-7110-3 (2016).

4 Galgóczy, L. *et al.* Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among Fusaria. *Peptides* 39, 131-137, doi:10.1016/j.peptides.2012.10.016 (2013).

5 Seibold, M., Wolschann, P., Bodevin, S. & Olsen, O. Properties of the bubble protein, a defensin and an abundant component of a fungal exudate. *Peptides* 32, 1989-1995, doi:10.1016/j.peptides.2011.08.022 (2011).

6 Tóth, L. *et al.* NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization. *AMB Expr.* 6, 1-13, doi:10.1186/s13568-016-0250-8 (2016).

7 Chen, Z. *et al.* Purification and characterization of a novel antifungal protein secreted by *Penicillium chrysogenum* from an Arctic sediment. *Appl. Microbiol. Biotechnol.* 97, 10381-10390, doi:10.1007/s00253-013-4800-6 (2013).

8 Marx, F. *et al.* Cloning, structural organization and regulation of expression of the *Penicillium chrysogenum paf* gene encoding an abundantly secreted protein with antifungal activity. *Gene* 167, 167-171, doi:10.1016/0378-1119(95)00701-6 (1995).

9 Rodríguez-Martín, A. *et al.* Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. *Peptides* 31, 541-547, doi:10.1016/j.peptides.2009.11.002 (2010).

10 Marcet-Houben, M. *et al.* Genome sequence of the necrotrophic fungus *Penicillium digitatum*, the main postharvest pathogen of citrus. *BMC Genomics* 13, 646, doi:10.1186/1471-2164-13-646 (2012).

11 Palou, L. *Penicillium digitatum*, *Penicillium italicum* (Green mold, Blue mold) in *Postharvest Decay. Control Strategies* (ed. Bautista-Baños, S.) 45-102 (Academic Press, 2014).

12 Campos-Olivas, R. *et al.* NMR solution structure of the antifungal protein from *Aspergillus giganteus*: evidence for cysteine pairing isomerism. *Biochemistry* 34, 3009-3021, doi:10.1021/bi00009a032 (1995).

13 Lacadena, J. *et al.* Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. *Arch. Biochem. Biophys* 324, 273-281, doi:10.1006/abbi.1995.0040 (1995).

14 Nakaya, K. *et al.* Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus*. *Eur. J. Biochem.* 193, 31-38, doi:10.1111/j.1432-1033.1990.tb19300.x (1990).

15 Wnendt, S., Ulbrich, N. & Stahl, U. Molecular cloning, sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*. *Curr. Genet.* 25, 519-523, doi:10.1007/bf00351672 (1994).

16 Batta, G. *et al.* Functional aspects of the solution structure and dynamics of PAF – a highly-stable antifungal protein from *Penicillium chrysogenum*. *FEBS J.* 276, 2875-2890, doi:10.1111/j.1742-4658.2009.07011.x (2009).

17 Marx, F., Binder, U., Leiter, É. & Pócsi, I. The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies. *Cell. Mol. Life Sci.* 65, 445-454, doi:10.1007/s00018-007-7364-8 (2008).

18 Sonderegger, C. *et al.* D19S mutation of the cationic, cysteine-rich protein PAF: novel insights into its structural dynamics, thermal unfolding and antifungal function. *PLOS ONE* 12, e0169920, doi:10.1371/journal.pone.0169920 (2017).

19 Sonderegger, C. *et al.* A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. *Microb. Cell. Fact.* 15, 192, doi:10.1186/s12934-016-0586-4 (2016).

20 Kovács, L. *et al.* Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). *Peptides* 32, 1724-1731, doi:10.1016/j.peptides.2011.06.022 (2011).

21 Virágh, M. *et al.* Insight into the antifungal mechanism of *Neosartorya fischeri* antifungal protein. *Protein Cell* 6, 518-528, doi:10.1007/s13238-015-0167-z (2015).

22 Garrigues, S. *et al.* Mapping and identification of antifungal peptides in the putative antifungal protein AfpB from the filamentous fungus *Penicillium digitatum*. *Front. Microbiol.* 8, doi:10.3389/fmicb.2017.00592 (2017).

23 López-García, B. *et al.* Production of the biotechnologically relevant AFP from *Aspergillus giganteus* in the yeast *Pichia pastoris*. *Protein Express. Purif.* 70, 206-210, doi:10.1016/j.pep.2009.11.002 (2010).

24 Galgóczy, L. *et al.* Structural determinants of *Neosartorya fischeri* antifungal protein (NFAP) for folding, stability and antifungal activity. *Sci. Rep.* 7, 1963, doi:10.1038/s41598-017-02234-w (2017).

25 Virágh, M. *et al.* Production of a defensin-like antifungal protein NFAP from *Neosartorya fischeri* in *Pichia pastoris* and its antifungal activity against

filamentous fungal isolates from human infections. *Protein Express. Purif.* 94, 79-84, doi:10.1016/j.pep.2013.11.003 (2014).

26 Monstadt, G. M. & Holldorf, A. W. Arginine deiminase from *Halobacterium salinarium*. Purification and properties. *Biochem. J.* 273 (Pt 3), 739-745 (1991).

27 Oberparleiter, C. *et al.* Active internalization of the *Penicillium chrysogenum* antifungal protein PAF in sensitive *Aspergilli*. *Antimicrob. Agents Chemother.* 47, 3598-3601, doi:10.1128/aac.47.11.3598-3601.2003 (2003).

28 Lees, J. G., Miles, A. J., Wien, F. & Wallace, B. A. A reference database for circular dichroism spectroscopy covering fold and secondary structure space. *Bioinformatics* 22, 1955-1962, doi:10.1093/bioinformatics/btl327 (2006).

29 Helmerhorst, E. J., Reijnders, I. M., van 't Hof, W., Veerman, E. C. I. & Nieuw Amerongen, A. V. A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. *FEBS Lett.* 449, 105-110, doi:10.1016/S0014-5793(99)00411-1 (1999).

30 Blondelle, S. E. & Houghten, R. A. Hemolytic and antimicrobial activities of the twenty-four individual omission analogs of melittin. *Biochemistry* 30, 4671-4678, doi:10.1021/bi00233a006 (1991).

31 Lee, D. G. *et al.* Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. *Biochem. Biophys. Res. Commun.* 263, 646-651, doi:10.1006/bbrc.1999.1428 (1999).

32 Delgado, J. *et al.* Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products. *Int. J. Food Microbiol.* 205, 23-29, doi:10.1016/j.ijfoodmicro.2015.03.029 (2015).

33 Tu, C.-Y. *et al.* Characterization and expression of the antifungal protein from *Monascus pilosus* and its distribution among various *Monascus* species. *J. Biosci. Bioeng.* 122, 27-33, doi:10.1016/j.jbiosc.2015.12.009 (2016).

34 Polli, F., Meijrink, B., Bovenberg, R. A. L. & Driessen, A. J. M. New promoters for strain engineering of *Penicillium chrysogenum*. *Fungal Genet. Biol.* 89, 62-71, doi:10.1016/j.fgb.2015.12.003 (2016).

35 Koda, A., Minetoki, T., Ozeki, K. & Hirotsune, M. Translation efficiency mediated by the 5′ untranslated region greatly affects protein production in *Aspergillus oryzae*. *Appl. Microbiol. Biotechnol.* 66, 291-296, doi:10.1007/s00253-004-1681-8 (2004).

36 Paege, N. *et al.* A transcriptome meta-analysis proposes novel biological roles for the antifungal protein AnAFP in *Aspergillus niger*. *PLOS ONE* 11, e0165755, doi:10.1371/journal.pone.0165755 (2016).

37 Hegedüs, N., Sigl, C., Zadra, I., Pócsi, I. & Marx, F. The *paf* gene product modulates asexual development in *Penicillium chrysogenum*. *J. Basic Microbiol.* 51, 253-262, doi:10.1002/jobm.201000321 (2011).

38 Galgóczy, L. *et al.* Investigation of the antimicrobial effect of *Neosartorya fischeri* antifungal protein (NFAP) after heterologous expression in *Aspergillus nidulans*. *Microbiology* 159, 411-419, doi:doi:10.1099/mic.0.061119-0 (2013).

39 Fizil, Á., Gáspári, Z., Barna, T., Marx, F. & Batta, G. “Invisible” conformers of an antifungal disulfide protein revealed by constrained cold and heat unfolding, CEST-NMR experiments, and molecular dynamics calculations. *Chem. Eur. J.* 21, 5136-5144, doi:10.1002/chem.201404879 (2015).

40 Szewczyk, E. *et al.* Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat. Protocols* 1, 3111-3120 (2007).

41 Khang, C. H., Park, S. Y., Rho, H. S., Lee, Y. H. & Kang, S. . Filamentous fungi (*Magnaporthe grisea* and *Fusarium oxysporum*). *Methods Mol. Biol.* 344, 403-420 (2006).

42 Harries, E., Gandía, M., Carmona, L. & Marcos, J. F. The *Penicillium digitatum* protein O-mannosyltransferase Pmt2 is required for cell wall integrity, conidiogenesis, virulence and sensitivity to the antifungal peptide PAF26. *Mol. Plant Pathol.* 16, 748-761, doi:10.1111/mpp.12232 (2015).

43 Gandía, M., Harries, E. & Marcos, J. F. The myosin motor domain-containing chitin synthase PdChsVII is required for development, cell wall integrity and virulence in the citrus postharvest pathogen *Penicillium digitatum*. *Fungal Genet. Biol.* 67, 58-70, doi:10.1016/j.fgb.2014.04.002 (2014).

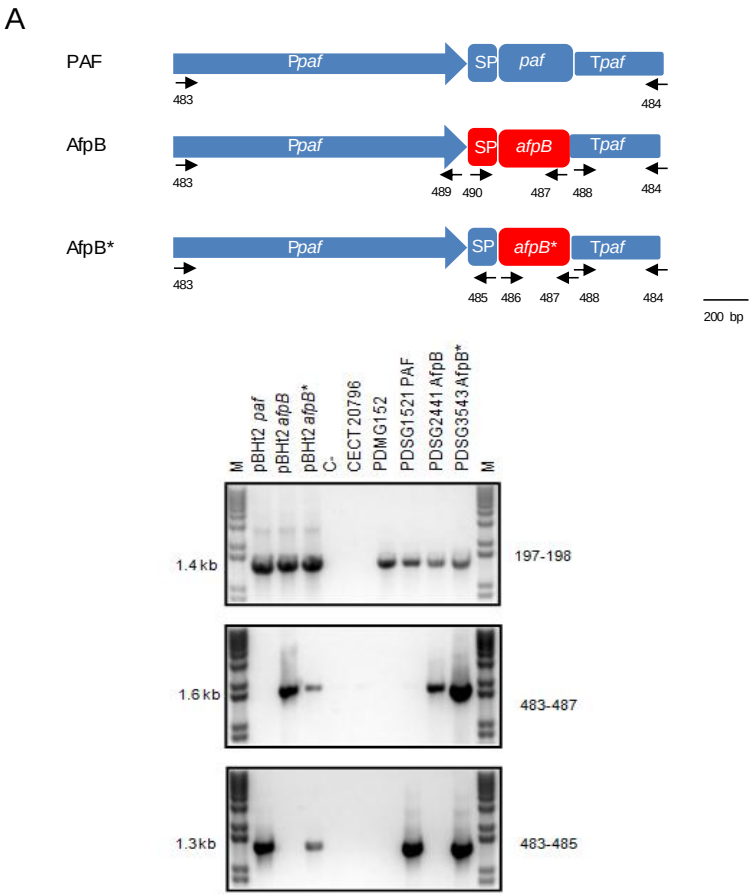
44 Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685 (1970).

45 López-García, B. *et al.* Concatemerization increases the inhibitory activity of short, cell-penetrating, cationic and tryptophan-rich antifungal peptides. *Appl. Microbiol. Biotechnol.* 99, 8011-8021, doi:10.1007/s00253-015-6541-1 (2015).

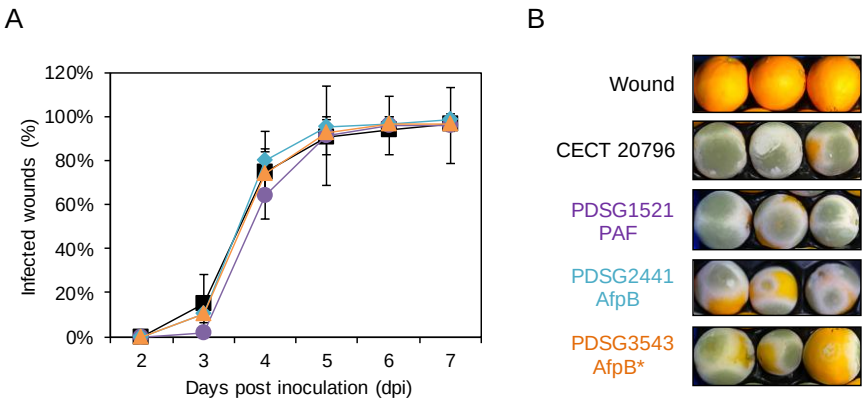
46 Muñoz, A., López-García, B. & Marcos, J. F. Studies on the mode of action of the antifungal hexapeptide PAF26. *Antimicrob. Agents Chemother.* 50, 3847-3855, doi:10.1128/aac.00650-06 (2006).

47 González-Candelas, L., Alamar, S., Sánchez-Torres, P., Zacarías, L. & Marcos, J. F. A transcriptomic approach highlights induction of secondary metabolism in citrus fruit in response to *Penicillium digitatum* infection. *BMC Plant Biol.* 10, 194, doi:10.1186/1471-2229-10-194 (2010).

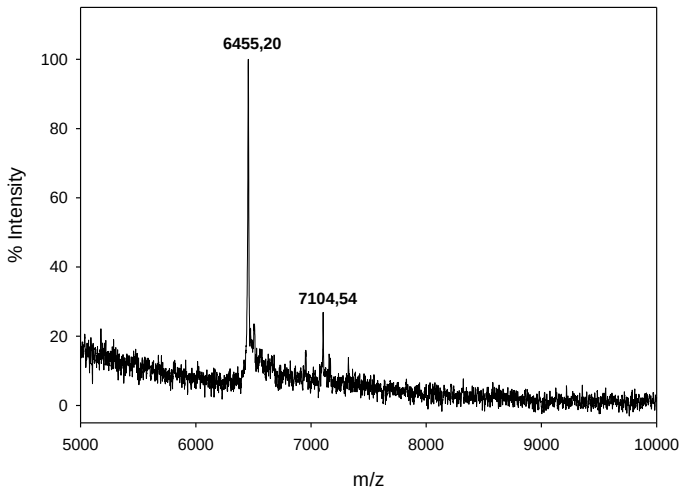
SUPPLEMENTARY MATERIAL



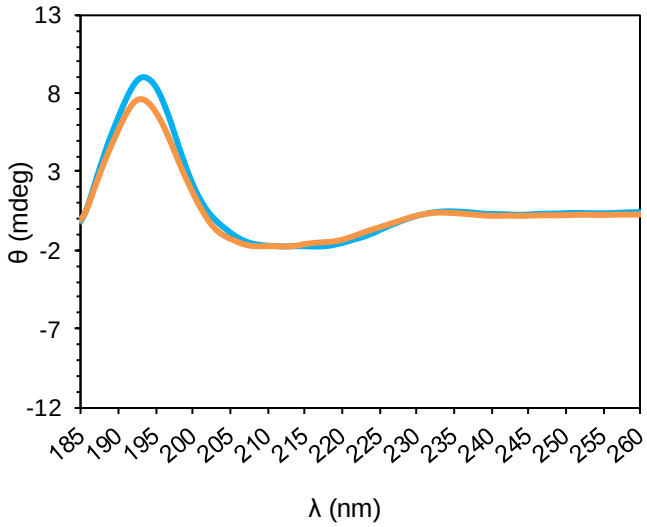
Supp. Fig. 1: Molecular characterization of *P. digitatum* AfpB and AfpB* producer strains. a) Representation of the expression systems used to obtain AfpB producer strains. The first diagram constitutes the schematic representation of the *P. chrysogenum*-based expression cassette (PAF); in blue*: paf* promoter (P*paf*), *paf* gene including the *paf* SP-pro sequence (*paf* SP), and *paf* terminator (T*paf*). The second diagram (AfpB) represents the genetic construction with the full-length AfpB coding sequence (in red) cloned under the control of the P*paf* and T*paf* from *P. chrysogenum*. The third part (AfpB*) corresponds to the genetic construction with the *in silico* predicted AfpB coding sequence (*afpB**) cloned under the control of the P*paf*, *paf* SP-pro sequence and T*paf*. All primers used for each construction generation and PCR analyses are located in the figure. b) PCR amplification of genomic DNA of the distinct *P. digitatum* strains with different primer pairs as indicated. Controls are the wild type strain CECT 20796, the *afpB*^{C} strain PDMG152, and the PAF producer strain PDSG1521. Additional controls were pBHt2 plasmids carrying the *paf*, *afpB* and *afpB** gene constructions and no DNA control (C-). The 1.4 kb bands (primers OJM197 and OJM198) correspond to the hygromycin resistant cassette (*hph*), which is present in all mutants and plasmid constructions. The 1.6 kb bands (primers OJM483 and OJM 487) correspond to the amplified region from the 5' P*paf* to the 3' *afpB* gene, only present in AfpB producer strains and afpB plasmid constructions. The 1.3 kb bands (OJM483-OJM485) correspond to the amplified region from 5' P*paf* to the 3' *paf* SP, only present in the PAF producer strain PDSG1521, AfpB* producer strain PDSG3543, and its corresponding plasmids.



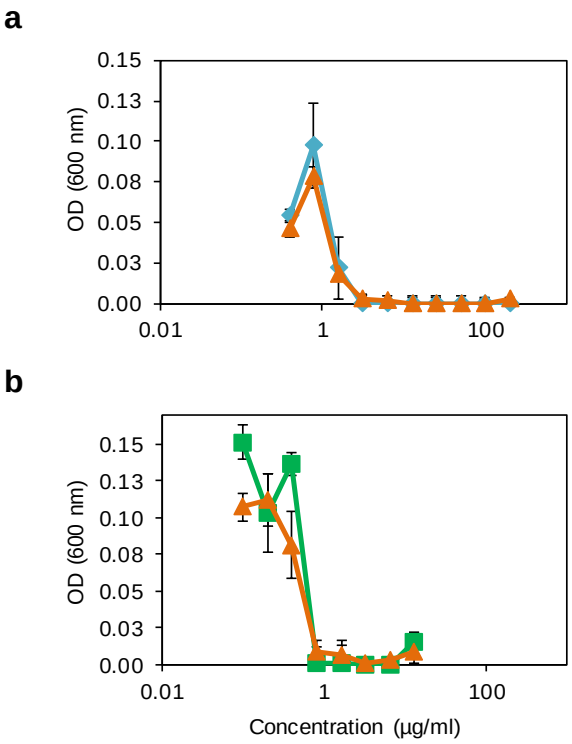
Supp. Fig. 2: Virulence assays of *P. digitatum* strains on orange fruits. a) Incidence of infection caused by the parental strain CECT 20796 (black squares), and transformants PDSG1521 (purple circles), PDSG2441 (blue diamonds), and PDSG3543 (orange triangles), which carry PAF, AfpB and AfpB* genetic constructions, respectively. Data are referred as mean values ± standard deviation of three replicates. b) Representative images of orange fruits infected by the indicated strains at 7 dpi.



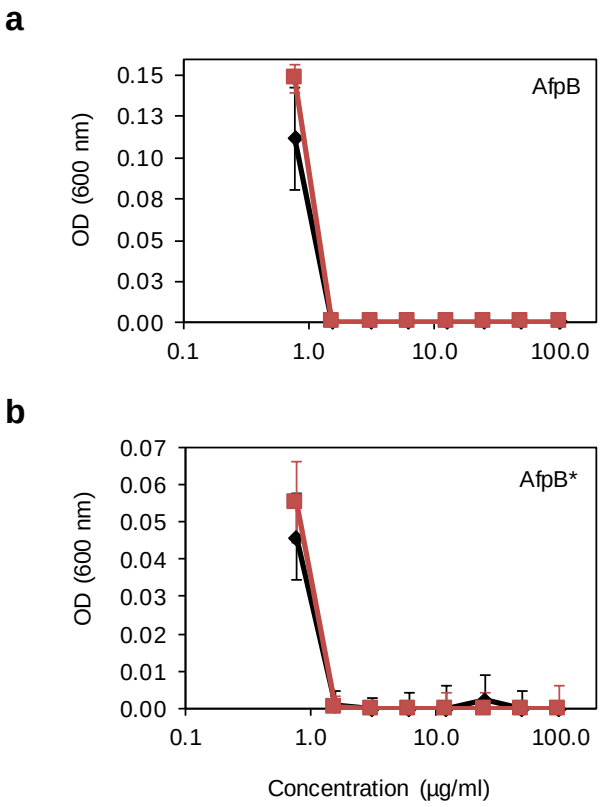
Supp. Fig. 3: MALDI-TOF MS data showing the isotopic average molecular mass (m/z) of AfpB* produced in the yeast *P. pastoris*. The predominant signal of 6455.20 Da corresponds to the calculated molecular mass of the oxidized protein AfpB* and the proper processing of the yeast α-factor signal peptide sequence (α-factor SS). The additional signal of 7104.54 Da cannot be explained by any incorrect processing of the α-factor SS in *P. pastoris.*



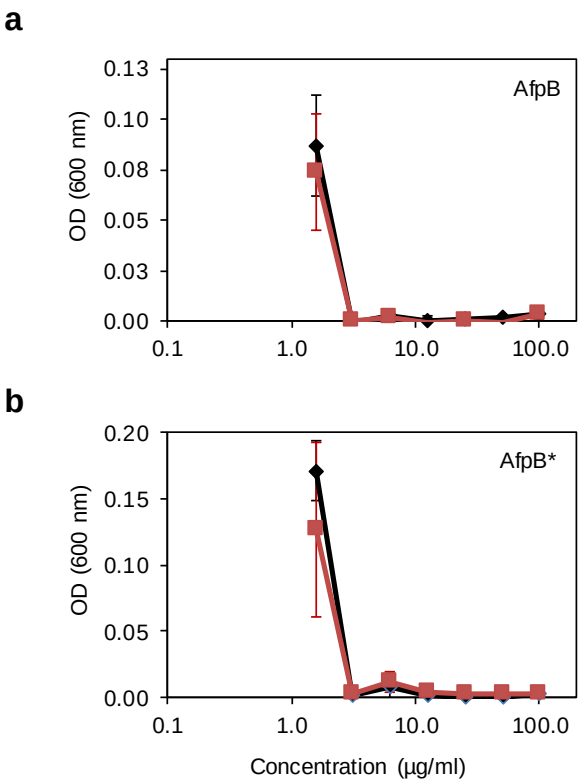
Supp. Fig. 4: ECD spectra of both *P. digitatum* AfpB variants at 25 ºC. Spectra obtained for AfpB (light blue) and AfpB* (orange) in H_2O at 25 ºC. Spectra show that both protein variants have practically the same folding at 25 ºC.



Supp. Fig. 5: Comparative study of the antifungal activity of AfpB and AfpB* variants . In this figure, two independent assays are shown. a) *In vitro* antifungal activity of AfpB (blue diamonds) and AfpB* (orange triangles) produced in *P. digitatum* against *P. digitatum* wild type strain after 72 h of growth at 25 ºC. b) Antifungal activity of AfpB* produced in *P. digitatum* (orange triangles) and PpAfpB* produced in *P. pastoris* (green squares) against *P. digitatum* wild type strain after 72 h of growth at 25 ºC. Data are referred as mean values ± s. d. of three replicates.



Supp. Fig. 6: Comparative study of the antifungal activity of both AfpB and AfpB* variants after treatment with proteinase K. a) Dose-response curve of *P. digitatum* CECT 20796 inhibition by AfpB (black diamonds) and AfpB after treatment with 100 µg/mL of proteinase K for 20 h (red squares). b) Dose-response curve of *P. digitatum* CECT 20796 inhibition by AfpB* (black diamonds) and AfpB* after treatment with 100 µg/mL of proteinase K for 20 h (red squares).



Supp. Fig. 7: Comparative study of the antifungal activity of both AfpB and AfpB* variants after heat treatment. a) Dose-response curve of *P. digitatum* CECT 20796 inhibition by AfpB (black diamonds) and AfpB after treatment at 95 ºC for 5 min (red squares). b) Dose-response curve of *P. digitatum* CECT 20796 inhibition by AfpB* (black diamonds) and AfpB* after treatment at 95 ºC for 5 min (red squares).

Supp. Table S1: Primers used in this study.

Name	Use*	Sequence 5´- 3´**	Tm*** (ºC)	Restriction sites	Gene	Description
OJM483	F	AT**CCCGGG**GAATTCAGAGAGCTTTTCGTACG	60	*Xma*I	*paf* promoter	Fungal transformation and transformant verification
OJM484	R	AT**TCTAGA**GCAGCAGTTTGATAGTTATCCCT	60	*Xba*I	*paf* terminator	Fungal transformation
OJM485	R	CAGGACACCGGCCTCAGCCC	60		*paf* pre-pro-sequence	Fungal transformation and transformant verification
OJM486	F	GGCTGAGGCCGGTGTCCTGAGTAAATACGGAGGAGTAAGTT	60		*afpB**	Fungal transformation
OJM487	R	GGTGATCGCAGAGACCATTCAAACTGGAGTCTGGCAGTC	60		*afpB*	Fungal transformation and transformant verification
OJM488	F	ATGGTCTCTGCGATCACCAGG	60		*paf* terminator	Fungal transformation
OJM489	R	TATGAAGGGCTTGAGATGATGATC	60		*paf* promoter	Fungal transformation
OJM490	F	CATCATCTCAAGCCCTTCATAATGCAGATTACCAGCATTGCC	60		*afpB*	Fungal transformation
OJM467	R	AGTCAACCCTCCTGTGGTG	56		*afpB*	DNA Sequencing
OJM491	F	CCACTTTAACCTTCTCCAGA	56		*paf* promoter	DNA Sequencing
OJM380	F	GTAATACGACTCACTATAGGG	56		*sp6* promoter	DNA Sequencing
OJM381	R	CATTTAGGTGACACTATAGAATAC	56		*T7* promoter	DNA Sequencing
OJM197	F	C**GTTAAC**TGATATTGAAGGAGCAT	60	*Hpa*I	*hph*	Fungal transformant verification
OJM198	R	T**GTTAAC**TGGTTCCCGGTCGG	60	*Hpa*I	*hph*	Fungal transformant verification
MO3	F	G**CTCGAG**AAAAGAAGTAAATAC	56	*Xho*I	*Kex2* signal cleavage + *afpB**	Yeast transformation and transformant verification
MO4	R	CC**TCTAGA**TCAAACTGGAGTCTG	62	*Xba*I	*Kex2* signal cleavage + *afpB**	Yeast transformation and transformant verification

* F: forward; R: reverse.
**Restriction sites are highlighted in bold.
***Tm: Temperature of annealing.

CHAPTER IV

CHAPTER IV

Three antifungal proteins from *Penicillium expansum*: different patterns of production and antifungal activity

Sandra Garrigues, Mónica Gandía, Laia Castillo, María Coca, Florentine Marx, Jose F. Marcos and Paloma Manzanares

Frontiers in Microbiology. Under second review (Manuscript ID: 415376)

ABSTRACT

Antifungal proteins of fungal origin (AFPs) are small, secreted, cationic, cysteine-rich proteins. Filamentous fungi encode a wide repertoire of AFPs belonging to different phylogenetic classes, which offer a great potential to develop new antifungals for the control of pathogenic fungi. The fungus *Penicillium expansum* is one of the few reported to encode one AFP belonging to each different phylogenetic class (A, B and C). In this work, the production of the putative AFPs from *P. expansum* was evaluated, but only the representative of class A, PeAfpA, was identified in culture supernatants of the native fungus. The biotechnological production of PeAfpB and PeAfpC was achieved in *Penicillium chrysogenum* with the *P. chrysogenum*-based expression cassette, which had been proved to work efficiently for the production of other related AFPs in filamentous fungi. Western blot analyses confirmed that *P. expansum* only produces PeAfpA naturally, whereas PeAfpB and PeAfpC could not be detected. From the three AFPs from *P. expansum*, PeAfpA showed the highest antifungal activity against all fungi tested, including plant and human pathogens. *P. expansum* was also sensitive to its self-AFPs PeAfpA and PeAfpB. PeAfpB showed moderate antifungal activity against filamentous fungi, whereas no activity could be attributed to PeAfpC at the conditions tested. Importantly, none of the PeAFPs showed haemolytic activity. Finally, PeAfpA was demonstrated to efficiently protect against fungal infections caused by *Botrytis cinerea* in tomato leaves and *Penicillium digitatum* in oranges. The strong antifungal potency of PeAfpA, together with the lack of cytotoxicity, and significant *in vivo* protection against phytopathogenic fungi that cause postharvest decay and plant diseases, make PeAfpA a promising alternative compound for application in agriculture, but also in medicine or food preservation.

Keywords: antifungal protein (AFP), *Penicillium expansum*, PeAfpA, *Penicillium chrysogenum, Penicillium digitatum*, *Botrytis cinerea*, postharvest, crop protection, pathogenic fungi.

Chapter IV

INTRODUCTION

Fungal infections are an emerging worldwide threat to animal, human and wildlife health (Fisher et al., 2012; Meyer et al., 2016). In medicine and agriculture, control of pathogenic fungi represents a serious challenge due to the increasing number of immunocompromised patients and the emergence of antifungal resistant strains. Accordingly, new antifungal strategies are needed, and current interests are focused on novel antifungal agents with properties and mechanisms of action different from existing ones. Ideally, newly developed antimycotics should also combine major aspects such as sustainability, high efficacy, limited toxicity and low costs of production (Marx et al., 2008; Meyer, 2008).

Antifungal proteins (AFPs) secreted by filamentous fungi meet the desired characteristics to fight fungal contaminations and infections. AFPs are small, cationic, cysteine-rich proteins highly stable to pH, high temperatures, and proteolysis, and exhibit broad antifungal spectra and different mechanisms of action against opportunistic human, animal, plant and foodborne pathogenic filamentous fungi (Marx et al., 2008; Hegedüs and Marx, 2013; Delgado et al., 2016). AFPs are coded with a signal peptide (SP) at the N-termini that includes a pre-sequence involved in AFP secretion to the extracellular space, and a pro-sequence, whose function is still controversial although it is assumed that might be involved in maintaining AFPs in an inactive form (Marx et al., 1995).

As shown by genome mining, fungi have a complex repertoire of AFP-like sequences, which are grouped in three major classes A, B and C (Garrigues et al., 2016). Noteworthy, filamentous fungi genomes encode more than one AFP from different classes. The *Penicillium chrysogenum* genome harbours three genes that code for AFPs belonging to each of three different classes while *Penicillium digitatum* has only one AFP in its genome (class B). The genome of *Neosartorya fischeri* encodes two AFPs (class A and C) but recently a new AFP has been characterized, which seems to be the first member of a fourth class (Tóth et al., 2016).

As new AFPs are being experimentally identified, differences regarding production, biological function, mode of action and antifungal spectrum are observed. Nowadays, the antifungal activity of at least one representative of all AFP classes has been experimentally demonstrated,

and lots of efforts are being made to further examine these proteins. Class A includes those AFPs described firstly, such as PAF from *P. chrysogenum* (Marx et al., 1995) and AFP from *Aspergillus giganteus* (Nakaya et al., 1990; Wnendt et al., 1994; Campos-Olivas et al., 1995; Lacadena et al., 1995) which have been deeply characterized (Meyer, 2008; Hegedüs and Marx, 2013). The first reported class B AFP was Anafp from *Aspergillus niger* (Lee et al., 1999) and currently representatives of class B also include those from *P. chrysogenum* (Delgado et al., 2015; Huber et al., 2018), *P. digitatum* (Garrigues et al., 2017) and *Monascus pilosus* (Tu et al., 2016). Only the antifungal activity of two class C representatives, the BP protein from *Penicillium brevicompactum* (Seibold et al., 2011) and the Pc-Arctin from *P. chrysogenum* (Chen et al., 2013), has been reported.

Some AFP-like proteins are yet uncharacterized, including those from the phytophatogenic fungus *Penicillium expansum*, whose genome contains three genes that code for three different AFP-like proteins, one of each class (Garrigues et al., 2016). Whether the distinct AFP-like proteins within a given fungus are differentially produced, perform different biological functions, or have different antifungal profiles and mode of action is still unknown, and *P. expansum* represents an opportunity to address these issues. In this study, the production of the putative AFPs from *P. expansum* was evaluated, and their antifungal activity demonstrated and described. Only the representative of class A, PeAfpA, was identified in culture supernatans of the native fungus whereas an heterologous expression system in *P. chrysogenum* allowed the production of PeAfpB and PeAfpC. Native and recombinat AFPs have been successfully purified to and their characterization showed distinctive antifungal profiles.

MATERIALS AND METHODS

Strains, media and growth conditions

Fungal strains used in this study were *P. expansum* CECT 20906 (CMP-1) (Ballester et al., 2015), *P. chrysogenum* wild type strain Q176, and *P. chrysogenum Δpaf* strain (Hegedüs et al., 2011), which was used as parental strain for fungal transformation. For the antimicrobial assays the following fungal strains were used: i) filamentous fungi: *P. digitatum* CECT 20796, *Botrytis cinerea* CECT 2100, *Fusarium oxysporum* 4287, *Penicillium italicum* CECT 2294, *A. niger* CBS120.49, *Magnaporthe oryzae* PR9, *Gibberella moniliformis* CECT 2987, *Aspergillus flavus* CECT 20802, *Trichophyton rubrum* CECT 2794 93 and *Arthroderma vanbreuseghemii* CECT 2958; ii) yeasts: *Saccharomyces cerevisiae* BY4741, *Candida albicans* CECT 1394, *Candida glabrata* CECT 1448, and *Candida parapsilopsis* CECT 1449. Filamentous fungi were cultured on Potato Dextrose Agar (PDA; Difco-BD Diagnostics, Sparks, MD, USA) plates for 7-10 days at 25 ºC except *A. vanbreuseghemii*, which was grown at 28 ºC. Yeasts were grown in Glucose Peptone Yeast extract Agar (GPYA) plates at 25 ºC except *S. cerevisiae*, which was grown at 30 ºC. For transformation, vectors were propagated in *Escherichia coli* JM109 grown in Luria Bertani (LB) medium supplemented with 100 µg/mL ampicillin or 75 µg/mL kanamycin. *P. chrysogenum Δpaf* was firstly grown in *P. chrysogenum* minimal medium (PcMM) agar (Sonderegger et al., 2016) supplemented with 200 µg/mL nourseotricin for 7 days at 25 ºC. Conidia were subsequently harvested with spore buffer (0.9 % NaCl, 0.01% Tween 80) and grown in *Aspergillus* complete medium (Sonderegger et al., 2016) for 36 h at 25 ºC with shaking. Transformants were grown on PcMM plates supplemented with 1 µg/mL pyrithiamine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA).

To analyse the growth of the *P. chrysogenum* transformant strains on solid media, 5 µL of conidial suspension (5 x 10^4 conida/mL) were placed on the centre of PDA and PcMM plates, and the colony diameter was monitored daily from 3 to 12 days. For protein production, 200 mL of Potato Dextrose Broth (PDB; Difco-BD Diagnostics) or PcMM were inoculated with a final concentration of 10^6 conidia/mL of either *P. expansum* CMP-1 or *P. chrysogenum* transformant strains and were incubated for 10 or 4 days, respectively.

Protein sequences and structure prediction

Sequences from the three different *Peafp* genes and the corresponding amino acid sequences were identified through BLAST searchers that were conducted at the National Center for Biotechnology Information (NCBI) server (https://www.ncbi.nlm.nih.gov/) (Ballester et al., 2015; (Garrigues et al., 2016). Multiple sequence alignments were performed with the Clustal Omega algorithm (https://www.ebi.ac.uk/Tools/msa/clustalo/), using the mature protein sequences without their signal peptide (SP). The I-TASSER software (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Yang et al., 2014) was used to predict the three dimensional (3D) structure of the *P. expansum* AfpA, AfpB and AfpC, using the *P. chrysogenum* antifungal proteins PAF and PAFB, and the *P. brevicompactum* bubble protein as templates, respectively (Protein Data Bank ID 2MHV, 2NC2 and 1UOY). Models obtained were refined using the ModRefiner software tool (https://zhanglab.ccmb.med.umich.edu/ModRefiner/) (Xu and Zhang, 2011) and were subsequently validated by RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) (Lovell et al., 2003) to ensure that all amino acids were located inside the favored and energetically allowed regions according to the Ramachandran Plot.

The theoretical molecular weight (MW) and isoelectric point (pI) of the mature proteins were examined with the Compute pI/MW and Protparam tools of the ExPASy Proteomics Server (http://web.expasy.org). All 3D models were visualized by UCSF Chimera software (Pettersen et al., 2004)

Vector constructions and *P. chrysogenum* transformant strains generation

Nucleotide sequences of *afpA, afpB and afpC* genes were PCR amplified from *P. expansum* CMP-1 genomic DNA, whereas the *paf* gene promoter, SP-pro, and terminator sequences were obtained from the vector pSK275*paf* (Sonderegger et al., 2016). All PCR procedures were performed using AccuPrime High-Fidelity polymerase (Invitrogen, Eugene, OR, USA), and the resulting DNA constructs were purified using High Pure PCR product Purification Kit (Roche, Mannheim, Germany), and verified by Sanger sequencing. Specific primers used for genetic amplification and

vector generation are listed in Supplementary Table 1. The three different DNA constructions were generated by fusion PCR (Szewczyk et al., 2007) and cloned into the pGEM-T® Easy vector system (Promega, Madison, WI, USA), from where they were excised using two internal restriction sites *Bsp*OI and *Not*I, and subsequently inserted into the previously digested vector pSK275*paf* (pSK275_*PeafpA,* pSK275_*PeafpB,* pSK275_*PexfpC*) containing the pyrithiamine hydrobromide resistant cassette as positive selection marker.

For the protein production of *P. expansum* AfpA, AfpB and AfpC in *P. chrysogenum,* the deletion strain ***Δpaf*** was used as recipient for the plasmids pSK275_*PeafpA,* pSK275_*PeafpB,* pSK275_*PexfpC.* Protoplast transformation was performed as previously described (Cantoral et al., 1987; Kolar et al., 1988) using 15 µg of *Sma*I linearized plasmids per transformation. Transformant strains were single spored four times on PcMM agar plates supplemented with 1 µg/mL pyrithiamine hydrobromide (Sigma-Aldrich). Positive transformants were confirmed by PCR amplification of genomic DNA (Supplementary Table 1, Supplementary Figure S1).

Protein production and purification

PeAfpA was purified from a 10-day PcMM supernatant of *P. expansum* CMP-1 strain. PeAfpB and PeAfpC were purified from supernatants of *P. chrysogenum* transformant strains growing in PcMM for 72-96 h. Cell-free supernatant containing PeAfpA was dialyzed (2 K MWCO, Sigma-Aldrich) against 20 mM phosphate buffer pH 6.6, and supernatants containing PeAfpB and PeAfpC were dialyzed against 20 mM acetate buffer pH 5.4. Dialyzed solutions were applied to an AKTA Purifier system equipped with 6 mL RESOURCE^{TM} S column (GE Healthcare Life Sciences, Little Chalfont, UK) equilibrated in the corresponding buffer. Proteins were eluted applying a linear gradient from 0-1 M NaCl in the same buffer.

Protein containing fractions were pooled, dialyzed against Milli-Q water, and lyophilized. Protein concentrations were determined by spectrophotometry (A_{280}) considering their molar extinction coefficients (ε_{280} = 0.64 for PeAfpA, and ε_{280} = 0.67 for PeAfpB and PeAfpC). The purity

was checked by SDS-PAGE (Laemmli, 1970) using SDS-16 % polyacrylamide gels calibrated with prestained protein size-standard SeeBlue® (ThermoFischer Scientific, Waltham, MA, USA) and Coomassie blue stained.

Matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS)

Analyses were performed in the proteomics facility of SCSIE University of Valencia (Spain). The mass of the purified proteins was analysed on a 5800 MALDI TOF/TOF (AB Sciex, Framingham, MA, USA) in positive linear mode (1500 shots every position) in a range of 2000-20000 m/z. For protein identification by peptide mass fingerprinting, samples were subjected to trypsin digestion and the resulting mixtures analysed on a 5800 MALDI TOF/-TOF in positive reflectron mode (3000 shots every position). Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4) were selected for every position for the MS/MS analysis. MS/MS data was acquired using the default 1kV MS/MS method. The MS and MS/MS information was sent to MASCOT via the Protein Pilot (AB Sciex).

Antibody generation and western blot

For PeAFPs detection, rabbit polyclonal antibodies were generated as described in (Mercader et al., 2017) with minor modifications. Procedures for animal immunization were approved by the Ethics Committee of the University of Valencia (Spain) for Animal Experimentation and Welfare (project2016/VSC/PEA/00136). Animal manipulation was performed according to Spanish and European laws and guidelines concerning the protection of animals used for scientific purposes (RD 1201/2005, Law 32/2007, and European Directive 2010/63/EU). Briefly, two white rabbits of around 2 kg were subcutaneously immunized with 300 µg of each PeAFP in a 1:1 emulsion of phosphate buffer solution and Freund´s adjuvant (complete for the first immunization, and incomplete for further boosts). The immunogen was given at least 4 times with intervals of 21 ± 1 days. Blood was taken 10 days after the final injection and it was allowed to coagulate overnight at 4 ºC. The antibody-containing sera were separated by centrifugation (270 × *g*, 15 min) and antibodies were precipitated twice

with 1 volume of saturated ammonium sulfate solution. Precipitated antisera were stored at 4 ºC until use.

Total proteins from supernatants and 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). Protein detection was accomplished using anti-PeAfpA, anti-PeAfpB, and anti-PeAfpC antibodies diluted 1:2500 for PeAfpA and PeAfpC, and 1:1500 for PeAfpC. As secondary antibody, 1:20000 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 a LAS-1000 instrument (Fujifilm, Tokyo, Japan). The experiments were repeated at least twice.

Antimicrobial activity assays

Growth inhibition assays were performed in 96-well, flat-bottom microtiter plates (Nunc, Roskilde, Denmark) as previously described (Garrigues et al., 2017) with minor modifications. Briefly, 50 µL of fungal conidia (5 x 10^4 conidia/mL) or yeast cells (2.5 x 10^5 cells/mL) in 10% PDB containing 0.02 % (w/v) cloramphenicol to avoid bacteria contamination were mixed with 50 µL of twofold concentrated proteins from serial twofold dilutions (final concentration 200 µg/mL). Plates were statistically incubated for 48 h at 25 ºC in case of yeasts (*S. cerevisiae* at 30 ºC), and 72 h at 25 ºC for filamentous fungi (*A. vanbreuseghemii* at 28 ºC) except dermatophytes which were incubated for 120 h. Growth was determined every 2 h and 24 h, respectively, by measuring the optical density (OD) at 600 nm using Fluostar Omega plate spectrophotometer (BMG labtech, Orlenberg, Germany), and the OD_{600} mean and standard deviation (SD) between three replicates were calculated. Dose-response curves were generated from measurements after 48 h in yeasts, and 72 h in filamentous fungi (120 h in dermatophytes). These experiments were repeated at least twice. Minimum Inhibitory Concentration (MIC) is defined as the protein concentration that completely inhibited growth in all the experiments performed.

Haemolytic activity assays

The haemolytic activity of the three PeAFPs was determined in a 96 round-bottom microtiter plate (Nunc) on 1:4 diluted rabbit red blood cells (RBCs) as described (Helmerhorst et al., 1999; Muñoz et al., 2006) with minor modifications. RBCs were harvested by slow centrifugation (100 × *g*, 15 min) and washed at least three times in 35 mM phosphate buffered saline (PBS, 150 mM NaCl, pH 7) or phosphate buffer glucose (PBG, 250 mM glucose as osmoprotectant). One hundred µL of twofold protein concentration were mixed with 100 µL of RBCs in triplicate. Plates were incubated for 1 h at 37 ºC and subsequently centrifuged (300 × *g*, 5 min). Eighty µL were transferred to a new microtiter plate and the absorbance was measured at 415 nm (Fluostar Omega, BMG labtech). Absence of haemolysis and 100 % haemolysis were determined in controls with a mixture of PBS or PBG, and 0.1 % Triton X-100, respectively. The haemolytic activity was calculated as the percentage of total hemoglobin released compared with that released by incubation with 0.1 % Triton X-100.

Protection assays against fungal infection caused by *P. digitatum* in citrus fruits

For protection assays, three replicates of five untreated, freshly harvested orange fruits (*citrus sinensis* L. Osbeck cv Navelina) were inoculated at four wounds around the equator with 5 µL of a *P. digitatum* PHI26 conidial suspension (10^4 conidia/mL) that were pre-incubated for 24 h with different concentrations of PeAfpA and *P. digitatum* AfpB (0.15, 1.5, and 15 µM). Orange fruits were stored at 20 ºC and 90 % relative humidity. The diameter of infection in each wound was measured daily for infection symptoms on consecutive days post-inoculation (dpi). Statistical analyses were performed using STATGRAPHICS Centurion 16.7.17. Fisher's minimum significant difference (LSD) procedure was performed to discriminate between means of % of infected wounds in each treatments with respect to the untreated control at each particular dpi with a 95 % confidence.

Protection assays against fungal infection caused by *B. cinerea* in tomato leaves

Tomato leaves (*Solanum lycopersici*, cv Marmande) from 21-days old plants grown at 22 ºC with 16 h light/8 h dark photoperiod were locally inoculated with conidial suspension of *B. cinerea* alone or in the presence of increasing amounts of AfpB from *P. digitatum* or PeAfpA from *P. expansum*. For this, two drops of 20 µL of the conidial suspension (5×10^5 conida/mL) together with the appropriate concentration of each AFP (1, 5, and 10 µg/mL) were applied onto leaf surfaces. Sterile water was used for the negative control. The plants were maintained with high humidity and the progression of symptoms was measured daily. Leaf damage was quantified by image analysis using the Fiji ImageJ2 package (Schindelin, et al., 2012). Statistical analyses were performed using Free Statistics Software, Office for Research Development and Education, version 1.2.1 (Wessa, 2018) to calculate the ANOVA and Tukey's HSD Test.

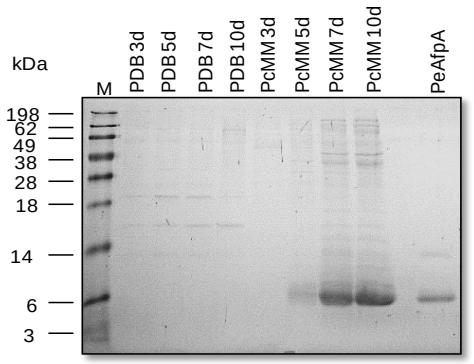
RESULTS

P. expansum encodes up to three distinct AFPs from different classes but only secretes AFP from class A

In order to detect and isolate any of the three putative *P. expansum* AFPs, called PeAfpA, PeAfpB and PeAfpC, from culture supernatants, the fungus was grown in either PDB or PcMM growth media, and time-course supernatants were analyzed by SDS-PAGE (Figure 1A). *In silico* studies predicted molecular masses of 6.64, 6.57 and 8.12 kDa and pI values of 9.5, 7.6 and 7.7 for PeAfpA, PeAfpB and PeAfpC, respectively. The largest amount of proteins was detected in PcMM supernatants, from which a protein band of apparent molecular mass of approximately 6 kDa was observed from day 5 till day 10 of growth. No band around 6 kDa was detected in PDB supernatants. To identify the putative PeAFPs produced in PcMM, peptide mass fingerprinting from an in-gel digestion of the 6 kDa band was performed. A Mascot database search resulted in a statistically significant hit for PeAfpA (score 125; E value $5.8e^{-11}$) with a sequence coverage of 78 % (Figure 1B).

According to its predicted chemical properties, PeAfpA purification was achieved from a 10 days *P. expansum* PcMM supernatant by one-step cation-exchange chromatography with yields of 125 mg/L. The protein eluted as a single broad chromatography peak at 0.1-0.5 M NaCl, and SDS-PAGE (Figure 1A) and MALDI-TOF-MS analyses (Figure 1C) revealed a single protein with a molecular mass of 6619.81 Da, which was very similar to that obtained by our previous *in silico* calculations.

A



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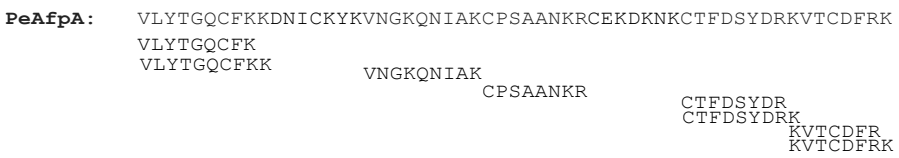

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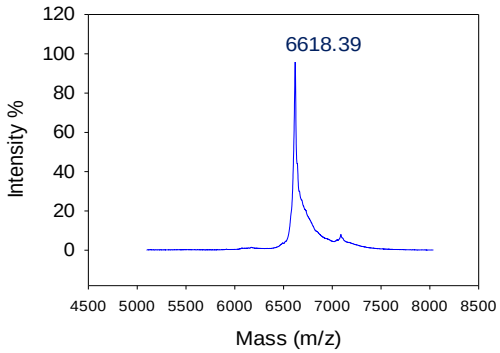

Figure 1: Production and identification of AFPs in *P. expansum* wild type strain. A) SDS-PAGE of 10 µL of 10X supernatants of *P. expansum* grown in rich medium (PDB) and Minimal Medium (PcMM) for 3, 5, 7 and 10 days and 2 µg of pure PeAfpA. M: SeeBlue® Pre-stained protein standard. (B) Peptide mass fingerprinting (PMF) of the class A AFP from *P. expansum* PeAfpA corresponding to the predominant 6 kDa band found in PcMM supernatants. Peptides obtained by PMF covered 78 % of PeAfpA primary sequence. (C) MALDI-TOF MS analysis. MS shows the isotopic average molecular mass (m/z) of pure PeAfpA produced in *P. expansum.*

Recombinant production of PeAFPs in *P. chrysogenum*

Since only PeAfpA was detected and isolated from the *P. expansum* culture supernatants, we used the *P. chrysogenum*-based expression cassette (Sonderegger et al., 2016; Garrigues et al., 2017) to produce the other two undetected AFPs from *P. expansum* PeAfpB and PeAfpC in *P. chrysogenum* under the regulation of the strong *paf* promoter and terminator sequences (Figure 2A). In addition, the PeAfpA production in *P. chrysogenum* was addressed as an internal control. Several positive transformants were obtained and evaluated for protein production in the case of proteins PeAfpB and PeAfpC, and one clone from each with the highest recombinant protein production was selected for further characterization. The selected producer strains were PCSGB14 for PeAfpB and PCSGC33 for PeAfpC. On the contrary, only one PeAfpA producer clone, PCSGA29, was obtained. The growth in solid medium of the selected transformants, the reference strain *P. chrysogenum* Q176 and the parental *P. chrysogenum* strain used for transformation (*Δpaf*) are shown in Figure 2B and C. The growth of PeAfpB and PeAfpC transformants was indistinguishable from those of the control strains independently of the medium used. In contrast, the PeAfpA transformant showed a significant reduction of colony diameter, more pronounced in PcMM plates and a drastic defect in conidia production (data not shown), and produced little amounts of PeAfpA, which hindered its use for purification of this recombinant protein.

Selected clones for PeAfpB and PeAfpC production in *P. chrysogenum* were grown in PcMM and, after clearing the culture broth from insoluble matter, the proteins in the supernatants were purified by one-step cation-exchange chromatography. Optimal production was achieved after 72 h with yields of 32 mg/L for PeAfpB and 62 mg/L for PeAfpC. PeAfpB eluted as a broad chromatography peak between 0.15-0.3 M NaCl while PeAfpC eluted as a sharp single peak at 0.075 M NaCl. SDS-PAGE analysis revealed a protein band in both protein samples, having apparent molecular masses higher than 6 kDa. PeAfpB showed less migration than expected from its predicted molecular mass (6.57 kDa) and in comparison to purified PeAfpA (Figure 3A top panel).

Molecular masses of both recombinant proteins were determined by MALDI-TOF-MS. Single peaks corresponding to average masses of

6576.07 and 6718.5 Da were detected for PeAfpB and PeAfpC, respectively (Supplementary Fig. S2).

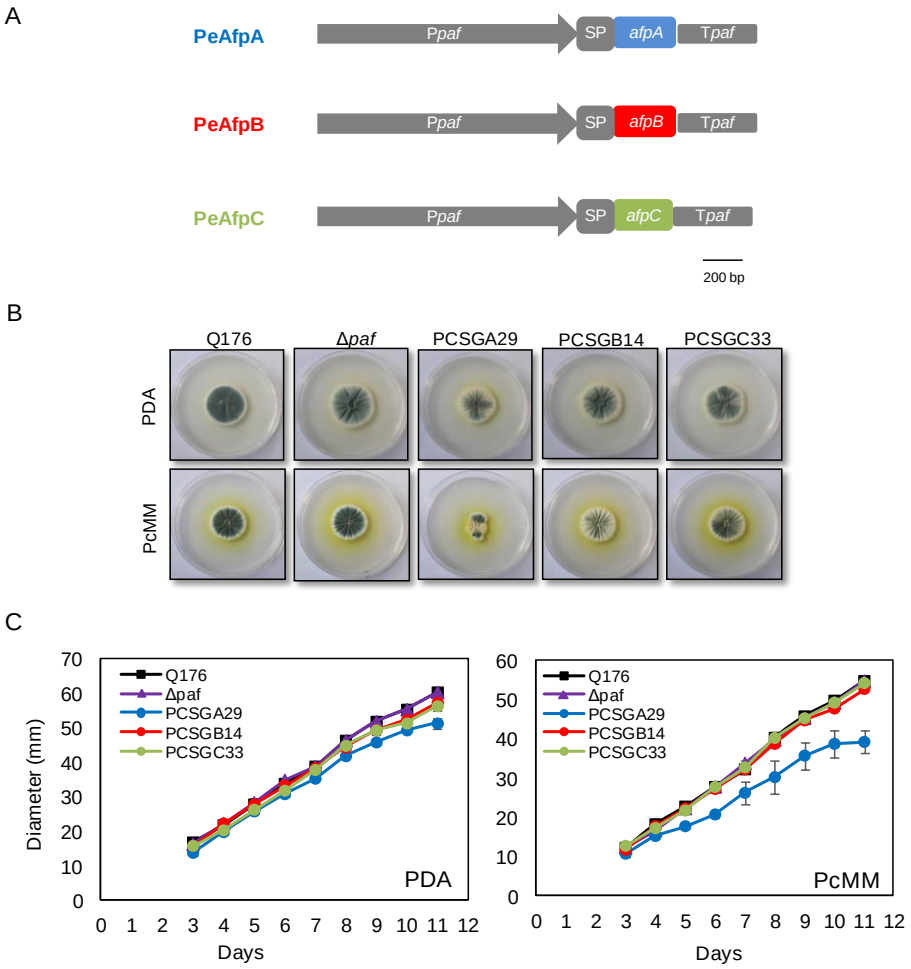

Figure 2: Phenotypical characterization of the *P. chrysogenum* transformant strains producing recombinant PeAFPs. (A) Schematic representation of the expression systems used to produce proteins PeAfpA (blue), PeAfpB (red) and PeAfpC (green) in *P. chrysogenum.* In grey: *paf* promoter (P*paf*), *paf* signal peptide (SP), and *paf* terminator (T*paf*). (B) Colony morphology of *P. chrysogenum* PeAfpA producer strain (PCSGA29), PeAfpB producer strain (PCSGB14), and PeAfpC producer strain (PCSGC33) compared to the wild type Q176 and the parental strain Δ*paf* after 5 days of growth on PDA and PcMM plates. (C) Growth in solid PDA and PcMM determined by the colony diameter from 3 to 11 days of growth at 25 °C in solid PDA and PcMM. Plotted data are mean values ± S.D of triplicate samples.

The experimental mass of PeAfpB is consistent with the calculated theoretical mass of the oxidized protein predicted after cleavage from the PAF SP-pro sequence (6572.2 Da), indicating the presence of three intra-molecular disulphide bonds and the absence of other post-translational modifications. By contrast, the average mass detected for PeAfpC was lower than the theoretical mass expected of 8123 Da, suggesting an inappropriate processing. To verify the identity of the recombinant PeAfpC produced in *P. chrysogenum*, peptide mass fingerprinting analysis of the purified protein was done. A Mascot database search resulted in a statistically significant hit for DUF1962 (protein with domains of unknown function) from *P. expansum* (score 280; E value $7.9e^{-21}$) with a sequence coverage of 53 % (Supplementary Fig. S3). This protein corresponded to PeAfpC, which in the genomic annotation included an internal insertion of eleven extra amino acids that were theoretically present in the three different *P. expansum* sequenced strains but absent in class C proteins from other fungi (Ballester et al., 2015; Garrigues et al., 2016) (Supplementary Figure S3A). Our data demonstrate that this insertion is absent in our purified PeAfpC (Supplementary Figure S3B). These results indicated that PeAfpC has a theoretical pI of 6.87 and a predicted molecular mass of 6.72 kDa, in accordance to that experimentally determined (6718.5 Da), and similar to that reported for other homologs 308 belonging to the same class.

Immunodetection confirmed the absence of PeAfpB and PeAfpC in *P. expansum* supernatants

Purified PeAFPs were used to generate polyclonal antibodies. The polyclonal anti-PeAfpA, anti-PeAfpB and anti-PeAfpC specifically recognized the corresponding purified protein while no cross reactivity among the three proteins was observed (Figure 3A bottom panel). Purified PAF from *P. chrysogenum* and AfpB from *P. digitatum* were also included as representatives of class A and B proteins, respectively. However, neither the polyclonal anti-PeAfpA recognized PAF nor anti-PeAfpB immunoreacted with *P. digitatum* AfpB (Figure 3A bottom). Specific signals were also detected in the supernatants of the selected PeAFP producer *P. chrysogenum* transformant strains PCSGA29, PCSGB14 and PCSGC33 (Supplementary Figure S4).

Polyclonal antibodies were then used to analyse the supernatants of *P. expansum.* In the *P. expansum* supernatants that were initially analysed by Coomasie blue staining (Figure 3B top panel), neither PeAfpB- nor PeAfpC-specific signals could be immunodetected in either PDB or PcMM culture supernatants. As expected, PcMM supernatants only reacted with the anti-PeAfpA antibody, and no immunoreaction was observed in the PDB culture supernatants (Figure 3B bottom panel), confirming that *P. expansum* only produces PeAfpA naturally in PcMM under the conditions tested.

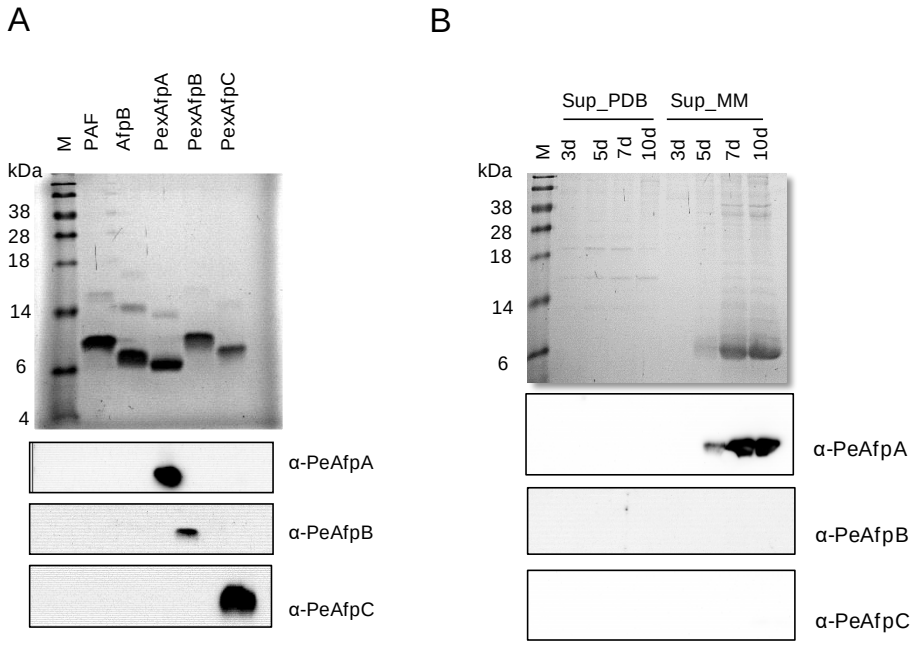

Figure 3: Western blot analyses of pure PeAFPs and growth supernatants of *P. expansum.* (A) SDS-PAGE (top) and western blot analyses (bottom) of pure PeAfpA, PeAfpB and PeAfpC (2 µg loaded per lane). Two µg of proteins PAF from *P. chrysogenum* and AfpB from *P. digitatum* were added as controls to test cross-reactivity among PeAFPs antibodies. Immunoblot analyses of these samples were performed using specific anti-PeAfpA, anti-PeAfpB and anti-PeAfpC antibodies generated in this work. (B) SDS-PAGE (top) and Western blot analyses (bottom) of *P. expansum* culture supernatants (10 µL of 10X supernatants loaded per lane) after 3, 5, 7 and 10 days of growth in PDB and MM. Immunoblot analyses of *P. expansum* supernatants were performed using the three specific PeAFPs antibodies. All SDS-PAGE analyses were visualized by Coomassie blue staining. M: SeeBlue® Pre-stained protein standard.

PeAFPs structural modelling

The 3D structure of mature PeAFPs was predicted by homology modelling using protein PAF (PDB ID 2MHV) (Fizil et al., 2015) and PAFB (PDB ID 2NC2) (Huber et al., 2018) from *P. chrysogenum* and BP protein from *P. brevicompactum* (PDB ID 1UOY) (Olsen et al., 2004) as templates for PeAfpA, PeAfpB and PeAfpC, respectively (Figure 4).

PeAfpA and PeAfpB show 53 % and 77 % amino acid identity with the *P. chrysogenum* PAF and PAFB, respectively. Tertiary structure of PeAfpA and PeAfpB were very similar to their classes A and B homologs, with five antiparallel β-strands forming a compact β-barrel that would be theoretically stabilized by three disulphide bonds following the abcabc pattern, as described for PAF and PAFB (Váradi et al., 2013; Huber et al., 2018).

PeAfpC shows 74 % amino acid identity with the BP protein used as template. However, PeAfpC predicted structure significantly differs from that of the BP. BP contains five antiparallel β-strands and four disulphide bonds connecting the two compacted β-sheets forming a basic accessible shallow funnel that may be relevant to the protein function (Olsen et al., 2004). Furthermore, BP contains a small α-helix structure absent in the other classes of AFPs. On the contrary, PeAfpC is predicted to have partially lost its tertiary structure if compared with BP. PeAfpC only contains three antiparallel β-strands forming a compacted β-sheet, whereas the second β-sheet and α-helix structures present in BP are missing in PeAfpC (Figure 4).

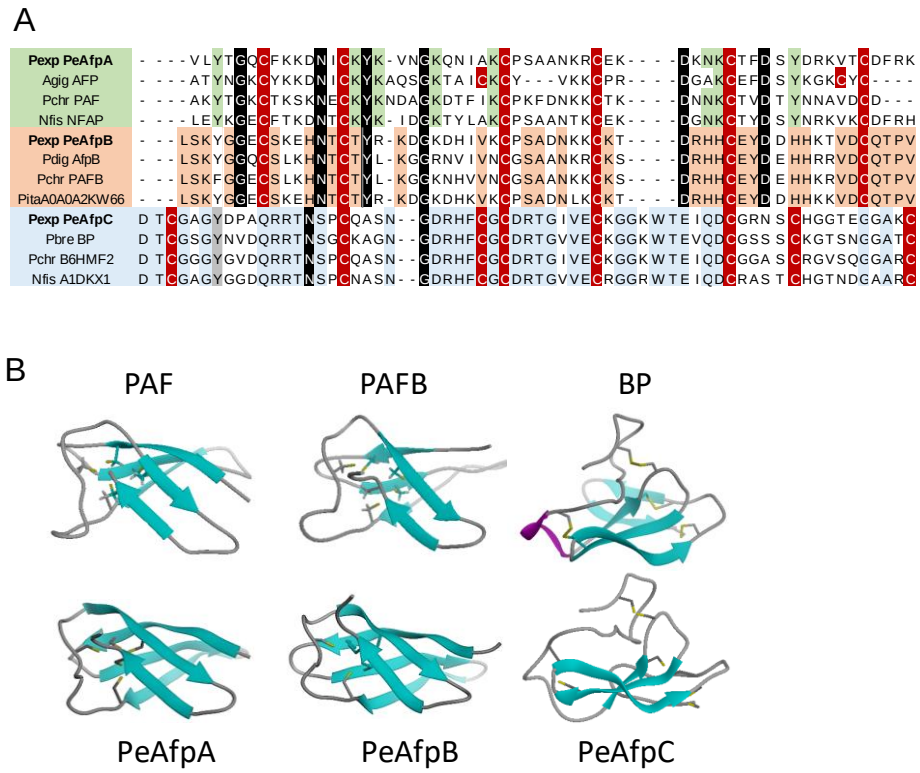

Figure 4: Molecular modeling of *P. expansum* AFPs of clases A, B and C. (A) Amino acid sequence aligment of a selection of AFPs and AFP-like proteins. Proteins belonging to different phylogenetic clases are highlighted in different colors. Proteins belonging to class A are represented in green, while classes B and C are shaded in orange and blue, respectively. Conserved intra-class motifs are shadowed following their colour code. Cysteine patterns are shadowed in red. Strongly conserved amino acids between clases are shadowed in black. (B) Comparison of the predicted tertiary structure of PeAfpA, PeAfpB and PeAfpC from *P. expansum* with the three-dimensional structures of the proteins PAF and PAFB from *P. chrysogenum* and BP from *P. brevicompactum* used as templates, respectively.

Antimicrobial activity assays

The three PeAFPs were tested for their antimicrobial activity towards a selection of filamentous fungi that include the *P. expansum* parental strain and several plant pathogens such as the citrus fruit specific *P. digitatum* and *P. italicum*, the polyphagous *B. cinerea*, the rice blast fungus *M. oryzae,* and the soilborne plant pathogen *F.oxysporum*. Furthermore, the mycotoxin producers *A. flavus* and *G. moniliformis*, and clinically relevant pathogens such as the skin pathogens *T. rubrum* and *A. vanbreuseghemii*, and the opportunistic human pathogens *C. albicans*, *C. glabrata* and *C. parapsilopsis* were also examined. Finally, *S. cerevisiae,* the PAF producer *P. chrysogenum* strain, and a strain from *A. niger* which is particularly sensitive to AFPs were also evaluated.

Table 1: Minimum inhibitory concentrations (MIC) (µg/mL) of PeAFPs.

Organism	PeAfpA	PeAfpB	PeAfpC
P. digitatum	1	12	>200
B. cinerea	4	50	>200
P. expansum	2	12	>200
P. italicum	2	12	>200
P. chrysogenum	2	50	>200
M. oryzae	16	>200	>200
A. niger	2	50	>200
T. rubrum	4	32	>64
A. vanbreuseghemii	4	>64	>64
F. oxysporum	4	>200	>200
G. moniliformis	4	>64	>64
A. flavus	4	>64	>64
C. albicans	8	>64	>64
C. glabrata	4	>64	>64
C. parapsilopsis	4	>64	>64
S. cerevisiae	4	>64	>64

48 h for yeasts
72 h for fungi (120 h for dermatophytes)

Differences in antimicrobial activity were observed among the three PeAFPs (Table 1 and Figure 5). PeAfpA showed high antifungal activity and inhibited the growth of all tested fungi. The minimum inhibitory concentration (MIC) values varied from 1 µg/mL against *P. digitatum* to 16 µg/mL against *M. oryzae*. The *Penicillium* species tested and *A. niger* were more susceptible to PeAfpA, including the producer parental strain *P.*

expansum. By contrast, PeAfpC was inactive against all the fungi at the highest concentration tested (200 µg/mL or 64 µg/mL), while PeAfpB showed a moderate antifungal activity with MIC values ranging from 12 µg/mL against the three phytopathogenic *Penicillium* species to 50 µg/mL against *P. chrysogenum*, *B. cinerea* and *A. niger*. PeAfpB was not active against either *M oryzae* and *F. oxysporum* at 200 µg/mL or against *G. moniliformis*, *A. flavus* or *A. vanbreuseghemii* at 64 µg/mL. PeAfpB was also inactive against *Candida* species.

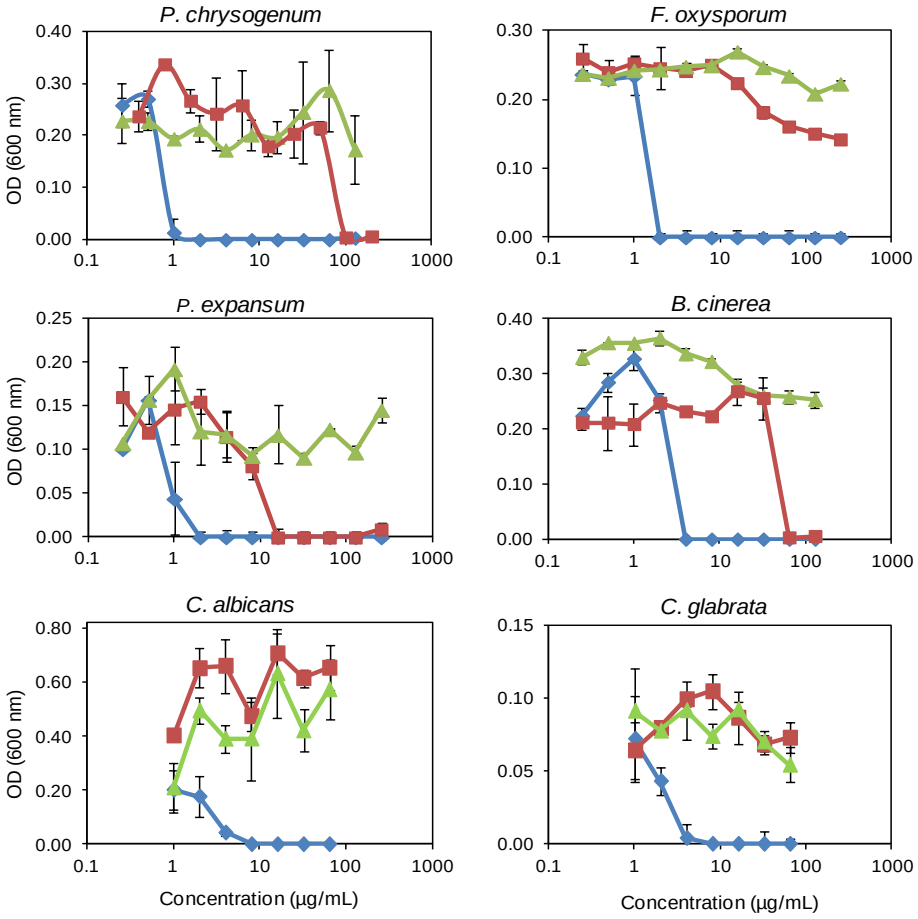

Figure 5: *In vitro* inhibitory activity of the three PeAFPs against filamentous fungi and yeasts. Dose-response curves comparing the antifungal activity of PeAfpA (blue diamonds), PeAfpB (red squares) and PeAfpC (green triangles) against the filamentous fungi *P. chrysogenum*, *F. oxysporum*, *P. expansum* and *B. cinerea,* and the pathogenic yeasts *C. albicans* and *C. glabrata*. Dose-response curves show mean ± S.D. OD_{600} of triplicate samples after 72 h at 25 º C for fungi and 48 h at 28 ºC for yeasts.

PeAFPs showed no haemolytic activity

Haemolytic activity assays are performed in order to determine the cytotoxicity of specific proteins and peptides against eukaryotic cells by their ability to lyse red blood cells (RBCs). The haemolytic activity of the three PeAFPs and of the cytolytic peptide melittin as positive control was determined using a high ionic strength phosphate NaCl buffer (PBS) and also with a low ionic strength isotonic glucose phosphate buffer (PBG) (Helmerhorst et al., 1999).

None of the PeAFPs showed haemolytic activity at any of the concentrations tested (1-100 µM), neither in the presence of NaCl as in PBS (Fig. 6A) nor glucose (Fig. 6B), in contrast to the haemolysis caused by melittin at 25 µM.

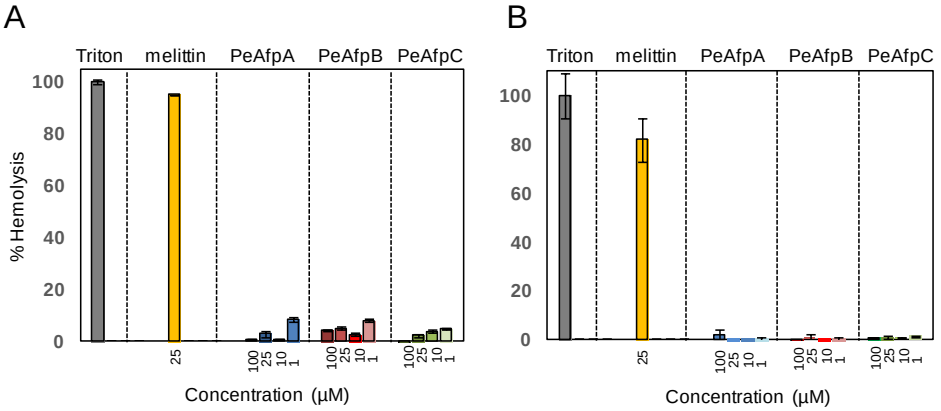

Figure 6: Haemolytic activity of the three AFPs from *P. expansum*. Analyses were conducted in PBS (150 mM NaCl) (A), and in PBG (250 mM glucose) (B). Proteins were used at the concentrations indicated (from 1 to 100 µM). For PeAFPs, 1, 10, 25, and 100 µM correspond to 6.6, 66, 166 and 662 µg/mL for PeAfpA; 6.5, 65, 164, and 657 µg/mL in PeAfpB; and 6.7, 67, 168, and 678 µg/mL in PeAfpC, respectively. The cytolytic peptide melittin (25 µM) was included for comparison. The haemolytic activity is given as the mean ± S.D. of the percentage of mammal red blood cells (RBCs) haemolysis (three replicates), as compared with the positive control in the presence of the detergent Triton X-100 (regarded as 100% haemolysis).

PeAfpA confers protection against *P. digitatum* infection in orange fruits

Based on the *in vitro* antimicrobial results, experiments were designed to evaluate PeAfpA ability to control the green mold disease caused by *P. digitatum* infection to citrus fruit. AfpB from *P. digitatum,*

which has been previously described as a highly active antifungal protein *in vitro* (Garrigues et al., 2017), was also included as a potential candidate to control green mold. Figure 7 shows the effects of different concentrations of AfpB and PeAfpA. The latter showed control of experimental *P. digitatum* infections when used at concentrations as low as 0.15 µM at late dpi. Contrarily, AfpB showed no significant protection at any of the concentrations tested ($p < 0.05$).

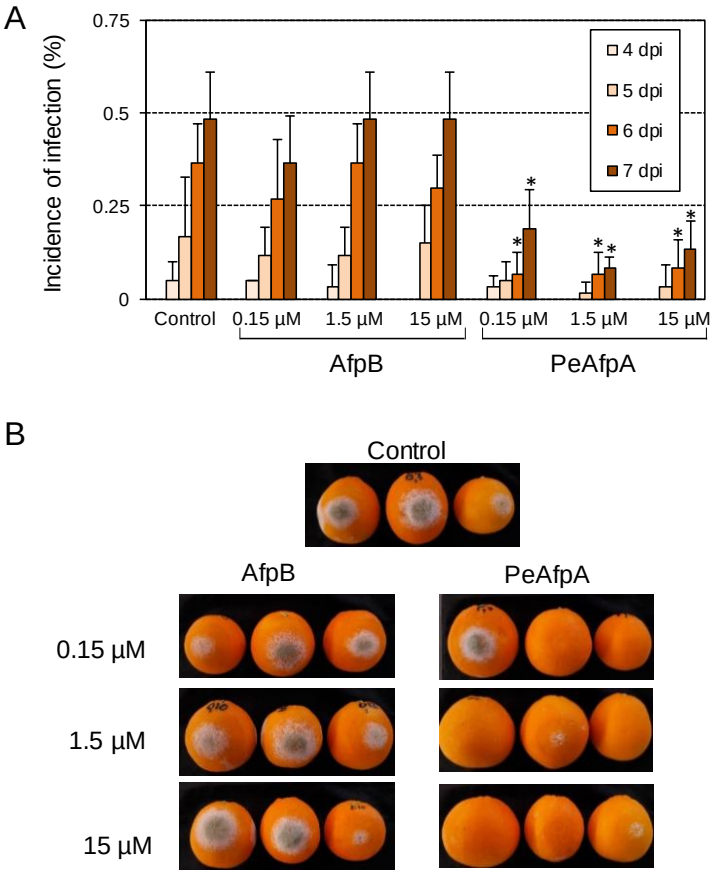

Figure 7: Effect of *P. digitatum* AfpB and *P. expansum* PeAfpA on the infection of citrus fruits caused by *P. digitatum.* (A) Incidence of infection of inoculated wounds. Orange fruits were inoculated with 10^4 conida /mL of *P. digitatum* either alone (Control) or in the presence of 0.15, 1.5 and 15 µM (1, 10 and 100 µg/mL, respectively) of AfpB and PeAfpA. Bars show the mean values of the percentage of infected wounds and S.D. of three replicates of five oranges at 4, 5, 6 and 7 days post-inoculation (dpi). Asterisks show statistical significance of the infection incidence compared to the control samples at each independent day (Fischer LSD test, $p < 0.05$). (B) Representative images of treated oranges with AfpB and PeAfpA at the indicated concentrations at 7 dpi.

PeAfpA confers protection against *B. cinerea* infection in tomato leaves

Experiments were designed to assess the effectiveness of PeAfpA against the infection caused by the polyphagous fungus *B. cinerea in vivo* in a detached leaf assay. Recently we have shown the effectiveness of *P. digitatum* AfpB at a concentration of 10 µM to control *B. cinerea* in tomato leaves (Shi et al., unpublished), and thus AfpB was included here as a positive control and for comparison of antifungal efficacy with PeAfpA. Development of disease symptoms on the detached leaves was monitored visually. Four days after inoculation, lesions were observed in the fungus-infected leaves that had not been treated with proteins (control) (Figure 8A). However, lesions were not observed or were significantly smaller when treated with PeAfpA (Figure 8A and B). This protective effect was dependent on protein doses, being still effective even at concentrations as low concentration as 1 µM, and greater than the caused by PeAfpB (Figure 8A and B). Interestingly, protection afforded by both PeAfpA and AfpB was also effective on established infection foci when proteins where applied 6 h after conidia (Figure 8C). This data suggests that both proteins can be used to treat already infected plants. The protective effect of these antifungal proteins was also observed in whole plant assays in which two leaves per plant were inoculated (Figure 8D). The control plants showed complete necrosis of the inoculated leaves and mild systemic signs of decay, while Afp-treated plants showed little or no infection symptoms.

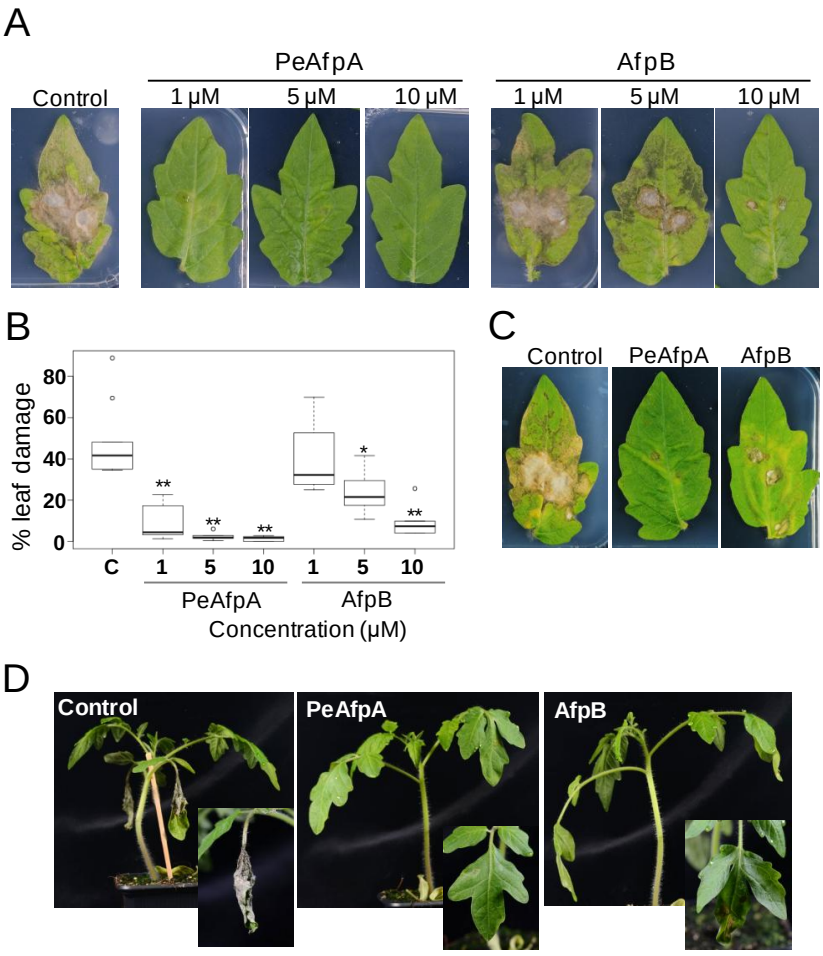

Figure 8: Tomato plants protection against *B. cinerea* infection by PeAfpA and AfpB. Cut leaves (A-C) or whole tomato plants (D) were infected in two different points per leaf with fungal conidia suspensions ($5x10^5$ conidia/mL) along with water (control) or the indicated amount of PeAfpA or AfpB, simultaneously (A, B, D) or with after 6 h (C). Concentrations of 1, 5 and 10 µM correspond to 6.6, 32 and 66 µg/mL, respectively. Ten µM (66 µg/mL) of AFPs were applied in C and D. Pictures were taken at 4 days post inoculation. Graph in B is a box plot of the percentage of leaf damage quantified from at least 6 leaves per treatments from two independent assays. Asterisks denote statistically significant differences in comparison to control values (ANOVA and Tukey's HSD test ** $p<0.001$; * $p<0.05$).

DISCUSSION

In this study, we detail the different patterns of production of the three AFPs from the phytopathogenic fungus *P. expansum*. PeAfpA, PeAfpB and PeAfpC are new members of classes A, B, and C, respectively and here we experimentally characterize their antifungal activity.

Only PeAfpA was detected in culture supernatants of *P. expansum* when grown in MM with sucrose as carbon source while in the nutritionally rich medium PDB (potato infusion + glucose) no protein was observed. The class A member PAF is also abundantly secreted by *P. chrysogenum* but its production depends on the type of carbon source present in the growth medium (Marx et al., 1995). AFP, another representative of class A, was successfully isolated from the culture supernatants of *A. giganteus* when grown in a rich medium based on corn starch and beef extract (Lacadena et al., 1995). Expression studies performed with *afp* and *paf* do not indicate a general pattern for both genes, except that the maximum mRNA and protein yield is reached during the stationary growth phase after 70-90 h of cultivation (Meyer and Stahl, 2002; Marx, 2004). Our time course experiments for protein production showed that PeAfpA was detected in MM *P. expansum* supernatants from day 5, and high yields of the protein (125 mg/L) were reached from 10 day-old supernatants. Thus, cultivation conditions seem to regulate PeAfpA production since the protein was neither detected by Coomassie staining nor by anti-PeAfpA antibodies in PDB supernatants. PeAfpA production at such long incubation times in MM suggests that it might be linked to nutrient limitation as described for PAF and AFP, and that glucose might supress production (Marx et al., 1995). Remarkably, a given fungal strain might produce different AFPs depending on culture broth as described for *N. fischeri* NRRL 181. The class A NFAP was isolated when the fungus was grown in a complex medium with starch, beef extract, peptone, NaCl and ethanol for seven days (Kovács et al., 2011) while NFAP2 but no NFAP was isolated from a 7-day old MM supernatant with sucrose as carbon source (Tóth et al., 2016).

PeAfpB and PeAfpC were not detected in any of the conditions tested. Instead, both proteins were produced using a *P. chrysogenum*-based expression system (Sonderegger et al., 2016), and they were purified from supernatants of recombinant *P. chrysogenum* strains. This expression system comprises the strong *paf* gene promoter, the *paf* pre-pro

sequence for correct protein processing and secretion, and the *paf* gene terminator (Marx et al., 1995). This system allowed the production of high amounts of several AFPs (Sonderegger et al., 2016; Sonderegger et al., 2017), including *P. digitatum* AfpB and *P. chrysogenum* PAFB which could not be isolated from the supernatants of the corresponding parental strains (Garrigues et al., 2016; Garrigues et al., 2017; Huber et al., 2018). Here, the heterologous production of PeAfpB and PeAfpC in *P. chrysogenum* resulted in yields of 32 and 62 mg/L, respectively, confirming the suitability of the system as a platform for the production of small cysteine-rich antifungal proteins. Further studies focussing on gene expression patterns will reveal whether the lack of PeAfpB and PeAfpC in the *P. expansum* culture broth results from a strict regulation during fungal growth, similar to the reports of AfpB (Garrigues et al., 2016) and PAFB (Huber et al., 2018), or in contrast , from non-functional or unexpressed genes.

Peptide mass fingerprinting of the recombinant PeAfpC revealed that this protein lacked the 11 amino acid insertion that was predicted by *in silico* annotation of three different sequenced strains of *P. expansum* (Ballester et al., 2015). Genes coding for AFPs from classes A and B have two introns, whereas genes coding for class C AFPs have only one (Garrigues et al., 2016). The predicted insertion within PeAfpC amino acid sequence correlates with an incorrect annotation of the single intron present in the class C AFP encoding gene from *P. expansum.* Thus, PeAfpC is similar to other characterized and putative class C AFPs regarding their size and chemical properties.

Two of the three PeAFPs are effective against filamentous fungi while PeAfpC did not show any antimicrobial activity under the conditions tested. One possible explanation for this different activity patterns could be explained with their distinct physico-chemical properties, especially the positive net charge at pH 7, which would correlate with their ability to bind fungal membranes. PeAfpA, which showed the highest antifungal activity, is a very cationic protein with a pI of 9.47. In contrast, PeAfpB (pI=7.4) showed a moderate antifungal activity against some of the fungi tested, but not against yeasts, and this protein showed a lower antifungal activity when compared to its class B homolog AfpB from *P. digitatum* (pI=9.06). On the contrary, PeAfpC (pI= 6.87) was inactive against all fungi and yeasts tested in this work. Only the antifungal activity of two other class C

representatives, the BP protein from *P. brevicompactum* (Seibold et al., 2011) and the Pc-Arctin from *P. chrysogenum* (Chen et al., 2013), have been reported. The former showed antifungal activity against *S. cerevisiae* and no other fungal species were evaluated (Seibold et al., 2011), while Pc-Arctin was effective against some plant pathogenic fungi (Chen et al., 2013).

The predicted 3D structure of PeAfpC significantly differs from the one experimentally determined for its class C homolog BP from *P. brevicompactum.* A loss of the three-dimensional organization in the PeAfpC *in silico* predicted structure might explain the loss of its antifungal activity. However, it has been reported that structural features of AFPs are not exclusively responsible for their antifungal activities. This is the case for *P. digitatum* AfpB, where we demonstrated that thermal denaturalization did not affect its antifungal activity (Garrigues et al., 2017), or for PAF from *P. chrysogenum*, where the change of a single amino acid did not affect its three-dimensional structure, but resulted in a complete loss of antifungal efficacy (Sonderegger et al., 2017). Recently, the anti-viral activity of some AFPs has been documented for the first time (Huber et al., 2018), suggesting that the properties of AFPs go beyond the traditional antifungal activity. Further structural and functional characterization of PeAfpC is currently in progress.

PeAfpA is the most potent antifungal protein from *P. expansum*. It is highly effective against relevant phytophatogenic fungi that cause postharvest decay and plant diseases. Moreover, we have shown that PeAfpA exerted significant protection against *P. digitatum* in oranges and against *B. cinerea* in tomato leaves. The application of antimicrobial peptides and proteins in postharvest conservation and crop protection has been described (Coca et al., 2004; Marcos et al., 2008). To our knowledge, AFP from *A. giganteus* is the only AFP that has been previously shown to successfully protect plants from fungal infection, albeit at higher protein doses than used in our assays. Similarly to the *in vivo* experiments described here, rice plants were protected from *Magnaporthe grisea* infection by direct application of 10 µM (58 µg/mL) AFP to rice leaves either by drops or spray (Vila et al., 2001), and geranium plants from *B. cinerea* (Moreno et al., 2003). *A. giganteus* AFP at a concentration of 100 µg/mL preincubated with tomato seedlings also prevented the infection of tomato

roots by the plant-pathogenic fungus *F. oxysporum* f. sp. *lycopersici* (Theis et al., 2005). Moreover, AFP sprayed on artificially infected-wounded bananas with *Alternaria alternata* was able to partly or totally inhibit the growth of the phytopathogen at concentrations in the range 15-50 µg/mL (Barakat, 2014). For crop protection, strategies based on the heterologous expression of the *A. giganteus afp* encoding gene conferred enhanced resistance to transgenic rice plants against the blast fungus *M. oryzae* (Coca et al., 2004), to transgenic wheat plants against the powdery mildew fungus *Erysiphe graminis* f.sp. *tritici* and the leaf rust fungus *Puccinia recondite* f.sp. *tritici* (Oldach et al., 2001) and to transgenic olive plants against the root infecting fungal pathogen *Rosellinia necatrix* (Narváez et al., 2018). Our results show that PeAfpA is highly effective in controlling *P. digitatum* and *B. cinerea* infections in citrus and tomato at concentrations as low as 0.15-1 µM (1-6.6 µg/mL). Both fungi have a considerable economic importance. Severe fruit losses due to *Penicillium* decay have an important impact in agriculture, especially decay caused by *P. digitatum,* one of the main postharvest pathogens of citrus fruits. *P. digitatum* specifically infects citrus fruits through peel injuries produced in the field, the packing house or during the fruit commercialization chain, causing the green mould disease (Palou, 2014). By contrast, the impact of *B. cinerea* in many areas is due to its broad host range, causing severe damage, both pre- and postharvest (Dean et al., 2012).

Despite the effectiveness of commercial chemical fungicides, concerns about environmental contaminations, the emergence of resistant strains and human health risks associated with fungicide residues lead to the search of new control strategies. Thus, PeAfpA might represent a powerful alternative in the control of phytopathogenic fungi. Moreover, considering the broad *in vitro* antifungal activity of PeAfpA against phytopathogenic fungi and against mycotoxins producers, it seems feasible that the protein may be effective also in other pathosystems not tested in this study. Our results also point to the heterologous expression of *P. expansum afpA* encoding gene in transgenic plants to confer disease resistance. Previously we described a very promising efficacy of the synthetic hexapeptide PAF26 and derivatives in citrus fruit protection (López-Garcia et al., 2003; Muñoz et al., 2007), although the high cost of synthetic peptide production and the failure to produce PAF26 through biotechnology (unpublished data) poses an obvious limit to postharvest

applications. By contrast, different expression systems, including the one used here, allow effective AFP-production (Sonderegger et al., 2016; Garrigues et al., 2017; Patiño et al., 2018), enabling the use of AFPs in crop and postharvest protection.

P. digitatum AfpB identified as an *in vitro* highly active antifungal protein against the own producer fungus (MIC = 3.2 µg/mL) (Garrigues et al., 2017) showed no *in vivo* effect in oranges as it did against *B. cinerea* in tomato leaves (MIC = 12.5 µg/mL). Until recently, it was assumed that AFPs were not active against the producer fungus. However, in addition to *P. digitatum* AfpB, PAFB (Huber et al., 2018) and now PeAfpA are effective towards *P. chrysogenum* and *P. expansum*, respectively. *In vitro* AFP growth inhibition against the own producer fungus is induced adding the protein exogenously to the culture media. Whether *in vivo* activity parallels that observed in *in vitro* tests deserves further studies.

Interestingly PeAfpA is also highly active against human fungal pathogens including dermatophytes (MIC 4 µg/mL), clinically important *Candida* species (MIC values 4-8 µg/mL), and also against mycotoxin-producer fungal strains (MIC 4 µg/mL), suggesting its potential application also in medicine and food preservation. The use of antimicrobial peptides for the prevention and treatment of fungal skin infections like those caused by *T. rubrum* and *A. vanbreuseghemii* has been proposed (López-García et al., 2007). AFPs such as PAF and PAFB from *P. chrysogenum* were active against *T. rubrum* with similar MIC values as that described here for PeAfpA (Huber et al., 2018). Nevertheless further characterization of AFPs in *in vivo* models are mandatory to confirm the potential of AFPs as novel therapies to treat dermatological diseases. Originally, AFPs were described as highly effective against filamentous fungi but not active against yeasts or bacteria (Marx et al., 2008; Meyer, 2008). However, anti-yeast activity of PAF was recently re-evaluated and its effectiveness against *S. cerevisiae* and *C. albicans* was reported, as well as that of PAFB (Huber et al., 2018). PAFB was the most active against *C. albicans* with a MIC value similar to that obtained here for PeAfpA. At present, NFAP2 is the most potent anti-yeast AFP described so far, with MIC values in the range of 0.2–1.5 µg/mL (Tóth et al., 2016). Remarkably, this protein, which seems to be the first member of a new, phylogenetically distinct fourth group among AFPs, was ineffective against filamentous fungal isolates whereas the opposite

antifungal profile was determined for the class A NFAP (Virágh et al., 2014).

Toxicity of antimicrobials should also be considered for successful application. The toxicity of PeAFPs has been measured as their cytolytic activity against red blood cells. The haemolytic activity of the three proteins was negligible in the conditions tested, even in assays conducted at low ionic strength isotonic conditions, which are considered more sensitive for detecting the haemolytic activity of cationic peptides (Helmerhorst et al., 1999). The lack of cytotoxicity was previously reported for PAF (Szappanos et al., 2005; Palicz et al., 2013) and *A. giganteus* AFP (Szappanos et al., 2006), and recently for *P. digitatum* AfpB (Garrigues et al., 2017) and *P. chrysogenum* PAFB (Huber et al., 2018), suggesting that AFPs can be regarded as safe.

To conclude, the high antifungal efficacy against human and plant pathogens and mycotoxin-producer fungi, together with the protection observed here upon application of PeAfpA for postharvest conservation of orange fruits and plant protection on tomato leaves, suggests that PeAfpA is a promising candidate for crop and postharvest protection and for its application in medicine or food security.

AUTHOR CONTRIBUTIONS

MC, FM, JFM and PM conceived and designed the study. PM coordinated the study and prepared the first draft of the manuscript. SG and FM produced AFPs in *P. chrysogenum*. SG and PM produced AFPs in *P. expansum*. SG and JFM performed antimicrobial experiments and structural modelling. MG and SG performed Western blot analyses. SG and MG performed haemolytic assays and protection assays in citrus fruits. LC and MC carried out protection assays in tomato plants. All authors read, revised and approved the final manuscript.

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CONFLICT OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Ballester, A.-R., Marcet-Houben, M., Levin, E., Sela, N., Selma-Lázaro, C., Carmona, L., et al. (2014). Genome, transcriptome, and functional analyses of *Penicillium expansum* provide new insights into secondary metabolism and pathogenicity. *Molecular Plant-Microbe Interactions* 28(3)**,** 232-248. doi: 10.1094/MPMI-09-14-0261-FI.

Barakat, H. (2014). Bio-control of *Alternaria alternata* during banana storage by purified AFP using isoelectric focusing technique. *Food and Nutrition Sciences* Vol.05No.15**,** 14. doi: 10.4236/fns.2014.515161.

Campos-Olivas, R., Bruix, M., Santoro, J., Lacadena, J., Martinez del Pozo, A., Gavilanes, J.G., et al. (1995). NMR solution structure of the antifungal protein from *Aspergillus giganteus*: evidence for cysteine pairing isomerism. *Biochemistry* 34(9)**,** 3009-3021. doi: 10.1021/bi00009a032.

Cantoral, J.M., Díez, B., Barredo, J.L., Alvarez, E., and Martín, J.F. (1987). High–frequency transformation of *Penicillium chrysogenum*. *Bio/Technology* 5**,** 494. doi: 10.1038/nbt0587-494.

Coca, M., Bortolotti, C., Rufat, M., Peñas, G., Eritja, R., Tharreau, D., et al. (2004). Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. *Plant Molecular Biology* 54(2)**,** 245-259. doi: 10.1023/b:plan.0000028791.34706.80.

Chen, Z., Ao, J., Yang, W., Jiao, L., Zheng, T., and Chen, X. (2013). Purification and characterization of a novel antifungal protein secreted by *Penicillium chrysogenum* from an Arctic sediment. *Applied Microbiology and Biotechnology* 97(24)**,** 10381-10390. doi: 10.1007/s00253-013-4800-6.

Dean, R.A., van Kan, J.A.L., Pretorius, Z.A., Hammond- Kosack, K.E., Di Pietro, A., P.D., S., et al. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* 13(4)**,** 414-430. doi: 10.1111/j.1364-3703.2011.00783.x.

Delgado, J., Acosta, R., Rodríguez-Martín, A., Bermúdez, E., Núñez, F., and Asensio, M.A. (2015). Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products. *International Journal of Food Microbiology* 205(0)**,** 23-29. doi: 10.1016/j.ijfoodmicro.2015.03.029.

Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A., and Núñez, F. (2016). Antifungal proteins from moulds: analytical tools and potential application to dry-ripened foods. *Applied Microbiology and Biotechnology* 100(16)**,** 6991-7000. doi: 10.1007/s00253-016-7706-2.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484(7393)**,** 186-194. doi: 10.1038/nature10947.

Fizil, Á., Gáspári, Z., Barna, T., Marx, F., and Batta, G. (2015). "Invisible" conformers of an antifungal disulfide protein revealed by constrained cold and heat unfolding, CEST-NMR experiments, and molecular dynamics calculations. *Chemistry European Journal* 21(13)**,** 5136-5144. doi: 10.1002/chem.201404879.

Garrigues, S., Gandía, M., and Marcos, J.F. (2016). Occurrence and function of fungal antifungal proteins: a case study of the citrus postharvest pathogen *Penicillium digitatum*. *Applied Microbiology and Biotechnology* 100(5)**,** 2243-2256. doi: 10.1007/s00253-015-7110-3.

Garrigues, S., Gandía, M., Popa, C., Borics, A., Marx, F., Coca, M., et al. (2017). Efficient production and characterization of the novel and highly active antifungal protein AfpB from *Penicillium digitatum*. *Scientific Reports* 7(1)**,** 14663. doi: 10.1038/s41598-017-15277-w.

Hegedüs, N., and Marx, F. (2013). Antifungal proteins: More than antimicrobials? *Fungal Biology Reviews* 26(4)**,** 132-145. doi: 10.1016/j.fbr.2012.07.002.

Hegedüs, N., Sigl, C., Zadra, I., Pócsi, I., and Marx, F. (2011). The *paf* gene product modulates asexual development in *Penicillium chrysogenum*. *Journal of Basic Microbiology* 51(3)**,** 253-262. doi: 10.1002/jobm.201000321.

Helmerhorst, E.J., Reijnders, I.M., van 't Hof, W., Veerman, E.C.I., and Nieuw Amerongen, A.V. (1999). A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. *FEBS Letters* 449(2)**,** 105-110. doi: 10.1016/S0014-5793(99)00411-1.

Huber, A., Hajdu, D., Bratschun-Khan, D., Gáspári, Z., Varbanov, M., Philippot, S., et al. (2018). New antimicrobial potential and structural properties of PAFB: a cationic, cysteine-rich protein from *Penicillium chrysogenum* Q176. *Scientific Reports* 8(1)**,** 1751. doi: 10.1038/s41598-018-20002-2.

Kolar, M., Punt, P.J., van den Hondel, C.A.M.J.J., and Schwab, H. (1988). Transformation of *Penicillium chrysogenum* using dominant selection markers and expression of an *Escherichia coli lacZ* fusion gene. *Gene* 62(1)**,** 127-134. doi: 10.1016/0378-1119(88)90586-0.

Kovács, L., Virágh, M., Takó, M., Papp, T., Vágvölgyi, C., and Galgóczy, L. (2011). Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). *Peptides* 32(8)**,** 1724-1731. doi: 10.1016/j.peptides.2011.06.022.

Lacadena, J., del Pozo, A.M.n., Gasset, M., Patiño, B., Campos-Olivas, R., Vázquez, C., et al. (1995). Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. *Archives of Biochemistry and Biophysics* 324(2)**,** 273-281. doi: 10.1006/abbi.1995.0040.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259)**,** 680-685.

Lee, D.G., Shin, S.Y., Maeng, C.-Y., Jin, Z.Z., Kim, K.L., and Hahm, K.-S. (1999). Isolation and characterization of a novel antifungal peptide from *Aspergillus*

niger. *Biochemical and Biophysical Research Communications* 263(3)**,** 646-651. doi: 10.1006/bbrc.1999.1428.

López-García, B., Ubhayasekera, W., Gallo, R.L., and Marcos, J.F. (2007). Parallel evaluation of antimicrobial peptides derived from the synthetic PAF26 and the human LL37. *Biochemical and Biophysical Research Communications* 356(1)**,** 107-113. doi: 10.1016/j.bbrc.2007.02.093.

López-Garcia, B., Veyrat, A., Pérez-Payá, E., González-Candelas, L., and Marcos, J.F. (2003). Comparison of the activity of antifungal hexapeptides and the fungicides thiabendazole and imazalil against postharvest fungal pathogens. *International Journal of Food Microbiology* 89(2-3)**,** 163-170.

Lovell, S.C., Davis, I.W., Arendall, W.B., de Bakker, P.I.W., Word, J.M., Prisant, M.G., et al. (2003). Structure validation by Cα geometry: ϕ,ψ and Cβ deviation. *Proteins: Structure, Function, and Bioinformatics* 50(3)**,** 437-450. doi: 10.1002/prot.10286.

Marcos, J.F., Muñoz, A., Pérez-Payá, E., Misra, S., and López-García, B. (2008). Identification and rational design of novel antimicrobial peptides for plant protection. *Annual Review of Phytopathology* 46(1)**,** 273-301. doi: 10.1146/annurev.phyto.121307.094843.

Marx, F. (2004). Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application. *Applied Microbiology and Biotechnology* 65(2)**,** 133-142. doi: 10.1007/s00253-004-1600-z.

Marx, F., Binder, U., Leiter, É., and Pócsi, I. (2008). The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies. *Cellular and Molecular Life Sciences* 65(3)**,** 445-454. doi: 10.1007/s00018-007-7364-8.

Marx, F., Haas, H., Reindl, M., Stöffler, G., Lottspeich, F., and Redl, B. (1995). Cloning, structural organization and regulation of expression of the *Penicillium chrysogenum paf* gene encoding an abundantly secreted protein with antifungal activity. *Gene* 167(1-2)**,** 167-171. doi: 10.1016/0378-1119(95)00701-6.

Mercader, J.V., Abad-Somovilla, A., Agulló, C., and Abad-Fuentes, A. (2017). Fluxapyroxad haptens and antibodies for highly sensitive immunoanalysis of food samples. *Journal of Agricultural and Food Chemistry* 65(42)**,** 9333-9341. doi: 10.1021/acs.jafc.7b03199.

Meyer, V. (2008). A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. *Applied Microbiology and Biotechnology* 78(1)**,** 17-28. doi: 10.1007/s00253-007-1291-3.

Meyer, V., Andersen, M.R., Brakhage, A.A., Braus, G.H., Caddick, M.X., Cairns, T.C., et al. (2016). Current challenges of research on filamentous fungi in relation to human welfare and a sustainable bio-economy: a white paper.

Fungal Biology and Biotechnology 3(1)**,** 6. doi: 10.1186/s40694-016-0024-8.

Meyer, V., and Stahl, U. (2002). New insights in the regulation of the afp gene encoding the antifungal protein of *Aspergillus giganteus*. *Current Genetics* 42(1)**,** 36-42. doi: 10.1007/s00294-002-0324-9.

Moreno, A. B., Del Pozo, A. M., Borja, M., San Segundo, B. (2003). Activity of the antifungal protein from *Aspergillus giganteus* against *Botrytis cinerea.* Phytopathology 93(11): 1344-1353.

Muñoz, A., López-García, B., and Marcos, J.F. (2006). Studies on the mode of action of the antifungal hexapeptide PAF26. *Antimicrobial Agents and Chemotherapy* 50(11)**,** 3847-3855. doi: 10.1128/aac.00650-06.

Muñoz, A., López-García, B., and Marcos, J.F. (2007). Comparative study of antimicrobial peptides to control citrus postharvest decay caused by *Penicillium digitatum*. *Journal of Agricultural and Food Chemistry* 55(20)**,** 8170-8176. doi: 10.1021/jf0718143.

Nakaya, K., Omata, K., Okahashi, I., Nakamura, Y., Kolkenbrock, H., and Ulbrich, N. (1990). Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus*. *European Journal of Biochemistry* 193(1)**,** 31-38. doi: 10.1111/j.1432-1033.1990.tb19300.x.

Narváez, I., Khayreddine, T., Pliego, C., Cerezo, S., Jiménez-Díaz, R.M., Trapero-Casas, J.L., et al. (2018). Usage of the heterologous expression of the antimicrobial gene *afp* from *Aspergillus giganteus* for increasing fungal resistance in olive. *Frontiers in Plant Science* 9(680). doi: 10.3389/fpls.2018.00680.

Oldach, K.H., Becker, D., and Lörz, H. (2001). Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. *Molecular Plant-Microbe Interactions* 14(7)**,** 832-838.

Olsen, J.G., Flensburg, C., Olsen, O., Bricogne, G., and Henriksen, A. (2004). Solving the structure of the bubble protein using the anomalous sulfur signal from single- crystal in- house Cu *K*α diffraction data only. *Acta Crystallographica Section D* 60(2)**,** 250-255. doi: doi:10.1107/S0907444903025927.

Palicz, Z., Jenes, Á., Gáll, T., Miszti-Blasius, K., Kollár, S., Kovács, I., et al. (2013). *In vivo* application of a small molecular weight antifungal protein of *Penicillium chrysogenum* (PAF). *Toxicology and Applied Pharmacology* 269(1)**,** 8-16. doi: 10.1016/j.taap.2013.02.014.

Palou, L. (2014). *Penicillium digitatum*, *Penicillium italicum* (green mold, blue mold). *Postharvest Decay. Control Strategies. Academic Press, Elsevier Inc., London, GB***,** 45-102.

Patiño, B., Vázquez, C., Manning, J.M., Roncero, M.I.G., Córdoba-Cañero, D., Di Pietro, A., et al. (2018). Characterization of a novel cysteine-rich antifungal protein from *Fusarium graminearum* with activity against maize fungal

pathogens. *International Journal of Food Microbiology*. doi: 10.1016/j.ijfoodmicro.2018.06.017.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., et al. (2004). UCSF Chimera—A visualization system for exploratory research and analysis. *Journal of Computational Chemistry* 25(13)**,** 1605-1612. doi: 10.1002/jcc.20084.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji - an Open Source platform for biological image analysis. *Nature Methods* 9(7)**,** 10.1038/nmeth.2019. doi: 10.1038/nmeth.2019.

Seibold, M., Wolschann, P., Bodevin, S., and Olsen, O. (2011). Properties of the bubble protein, a defensin and an abundant component of a fungal exudate. *Peptides* 32(10)**,** 1989-1995. doi: 10.1016/j.peptides.2011.08.022.

Sonderegger, C., Fizil, Á., Burtscher, L., Hajdu, D., Muñoz, A., Gáspári, Z., et al. (2017). D19S mutation of the cationic, cysteine-rich protein PAF: novel insights into its structural dynamics, thermal unfolding and antifungal function. *PLOS ONE* 12(1)**,** e0169920. doi: 10.1371/journal.pone.0169920.

Sonderegger, C., Galgóczy, L., Garrigues, S., Fizil, Á., Borics, A., Manzanares, P., et al. (2016). A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. *Microbial Cell Factories* 15(1)**,** 192. doi: 10.1186/s12934-016-0586-4.

Szappanos, H., Szigeti, G.P., Pál, B., Rusznák, Z., Szűcs, G., Rajnavölgyi, É., et al. (2006). The antifungal protein AFP secreted by *Aspergillus giganteus* does not cause detrimental effects on certain mammalian cells. *Peptides* 27(7)**,** 1717-1725. doi: 10.1016/j.peptides.2006.01.009.

Szappanos, H., Szigeti, G.P., Pál, B., Rusznák, Z., Szűcs, G., Rajnavölgyi, É., et al. (2005). The *Penicillium chrysogenum*-derived antifungal peptide shows no toxic effects on mammalian cells in the intended therapeutic concentration. *Naunyn-Schmiedeberg's Archives of Pharmacology* 371(2)**,** 122-132. doi: 10.1007/s00210-004-1013-7.

Szewczyk, E., Nayak, T., Oakley, C.E., Edgerton, H., Xiong, Y., Taheri-Talesh, N., et al. (2007). Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat. Protocols* 1(6)**,** 3111-3120.

Theis, T., Marx, F., Salvenmoser, W., Stahl, U., and Meyer, V. (2005). New insights into the target site and mode of action of the antifungal protein of *Aspergillus giganteus*. *Research in Microbiology* 156(1)**,** 47-56. doi: 10.1016/j.resmic.2004.08.006.

Tóth, L., Kele, Z., Borics, A., Nagy, L.G., Váradi, G., Virágh, M., et al. (2016). NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization. *AMB Express* 6(1)**,** 1-13. doi: 10.1186/s13568-016-0250-8.

Tu, C.-Y., Chen, Y.-P., Yu, M.-C., Hwang, I.-E., Wu, D.-Y., and Liaw, L.-L. (2016). Characterization and expression of the antifungal protein from *Monascus pilosus* and its distribution among various *Monascus* species. *Journal of Bioscience and Bioengineering* 122(1)**,** 27-33. doi: 10.1016/j.jbiosc.2015.12.009.

Váradi, G., Tóth, G.K., Kele, Z., Galgóczy, L., Fizil, Á., and Batta, G. (2013). Synthesis of PAF, an antifungal protein from *P. chrysogenum*, by native chemical ligation: native disulfide pattern and fold obtained upon oxidative refolding. *Chemistry – A European Journal* 19(38)**,** 12684-12692. doi: 10.1002/chem.201301098.

Vila, L., Lacadena, V., Fontanet, P., Martínez del Pozo, A., and San Segundo, B. (2001). A protein from the mold *Aspergillus giganteus* is a potent inhibitor of fungal plant pathogens. *Molecular plant-microbe interactions* 14**,** 1327-1331.

Virágh, M., Vörös, D., Kele, Z., Kovács, L., Fizil, Á., Lakatos, G., et al. (2014). Production of a defensin-like antifungal protein NFAP from *Neosartorya fischeri* in *Pichia pastoris* and its antifungal activity against filamentous fungal isolates from human infections. *Protein Expression and Purification* 94(0)**,** 79-84. doi: 10.1016/j.pep.2013.11.003.

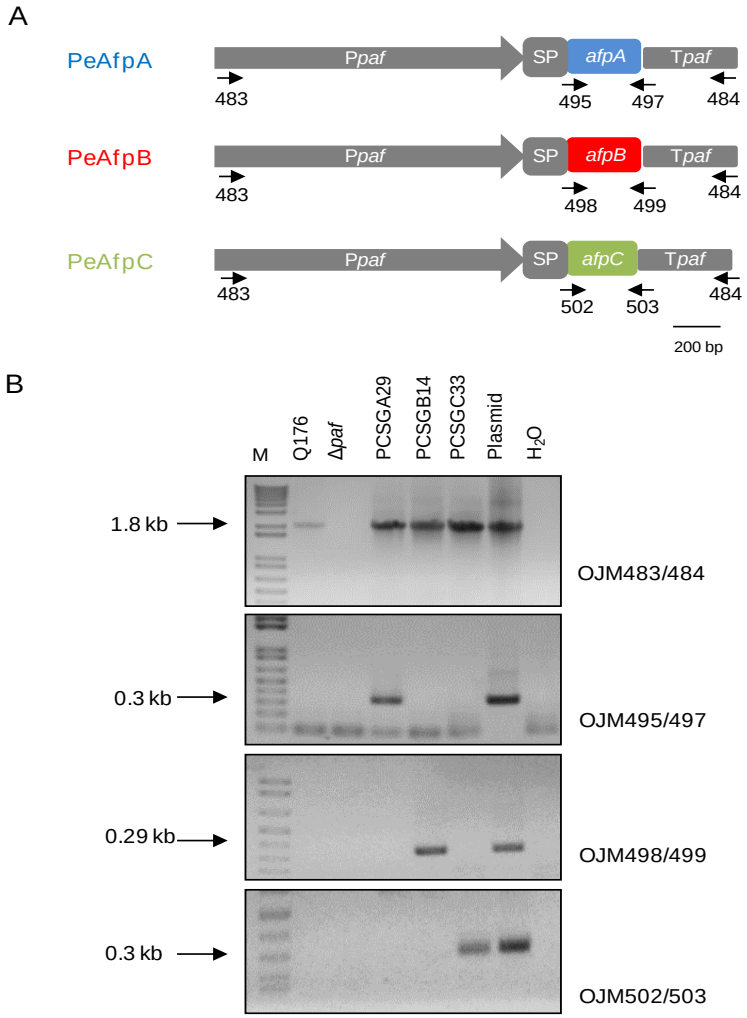
Wessa, P. (2018). Free Statistics Software, Office for Research Development and Education, version 1.2.1, URL https://www.wessa.net/.

Wnendt, S., Ulbrich, N., and Stahl, U. (1994). Molecular cloning, sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*. *Current Genetics* 25(6)**,** 519-523. doi: 10.1007/bf00351672.

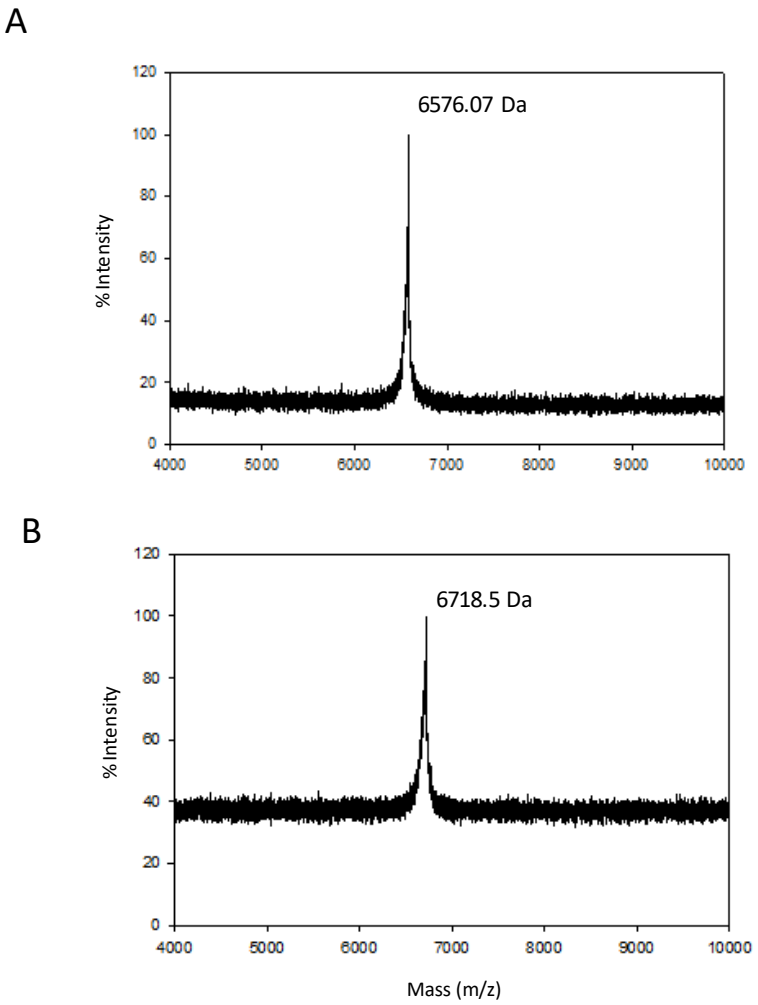
Xu, D., and Zhang, Y. (2011). Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. *Biophysical Journal* 101(10)**,** 2525-2534. doi: 10.1016/j.bpj.2011.10.024.

Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., and Zhang, Y. (2014). The I-TASSER Suite: protein structure and function prediction. *Nature Methods* 12**,** 7. doi: 10.1038/nmeth.3213

SUPPLEMENTARY MATERIAL

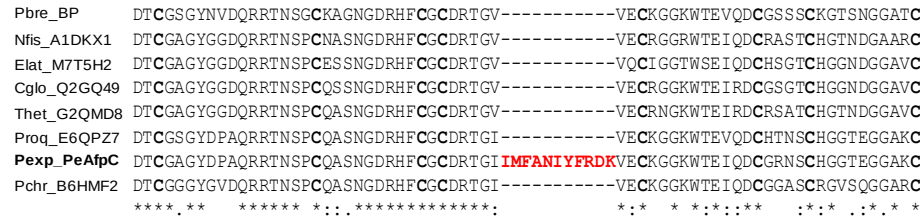


Supp. Fig. 1: Molecular characterization of *P. chrysogenum* transformant strains for PeAFP production. (A) Schematic representation of gene expression systems used to produce proteins PeAfpA (blue), PeAfpB (red) and PeAfpC (green) in *P. chrysogenum.* In grey: *paf* promoter (P*paf*), *paf* SP-pro sequence (SP), and *paf* terminator (T*paf*). Arrows show localization of primers used for PCR confirmation of the positive transformants for each gene construction. (B) PCR amplifications of genomic DNA for the confirmation of the different *P. chrysogenum* transformant strains, using the primer pairs indicated in the figure. The strains in the figure are: the PAF producer *P. chrysogenum* wild-type strain Q176, the null Δ*paf* strain used as recipient for PeAFP constructions, and *P. chrysogneum* transformant strains PCSGA29, PCSGB14 and PCSGC33, which produce PeAfpA, PeAfpB and PeAfpC, respectively.

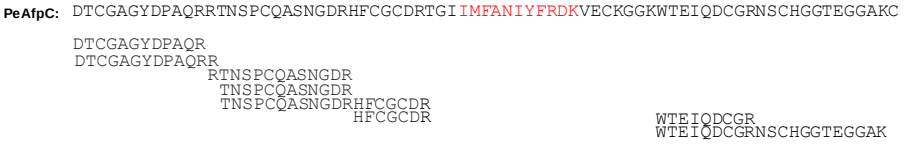


Supp. Fig. 2: MALDI-TOF MS analyses. Isotopic average molecular mass (m/z) of the two AFPs from *P. expansum* PeAfpB (A) and PeAfpC (B) produced in *P. chrysogenum*

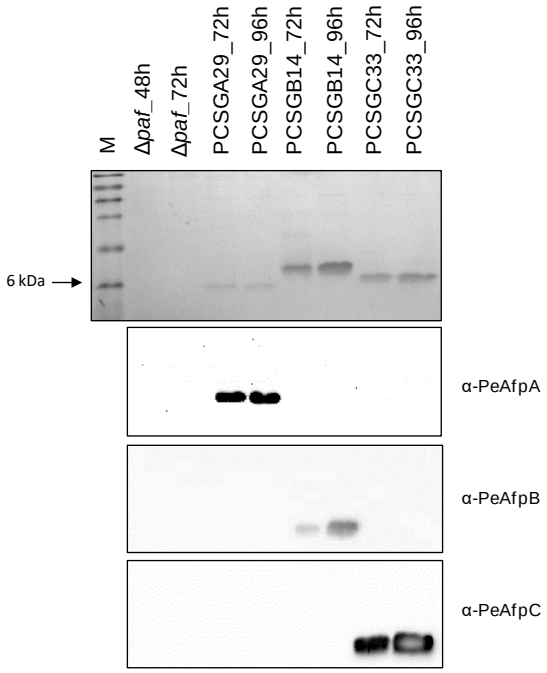
A



B



Supp. Fig. 3: Characterization of *P. expansum* class C AFP PeAfpC. (A) Sequence alignment of class C AFPs and AFP-like mature sequences. Conserved cysteine residues are highlighted in bold. The eleven extra amino acids predicted in PeAfpC but absent in the rest of class C homologs are highlighted in red. (B) Peptide fingerprint of pure PeAfpC. It revealed that PeAfpC lacks the predicted eleven amino acid insertion.



Supp. Fig. 4: Analyses of supernatants of *P. chrysogenum* PeAFP producing strains by SDS-PAGE and Western blot. SDS-PAGE of 10 µL of PcMM supernatants of *P. chrysogenum* transformant strains (top) ordered as follows: the parental strain Δ*paf,* PeAfpA producer strain PCSGA29, PeAfpB producer PCSGB14, and PeAfpC producer strain PCSGC33. Protein production was analized after 72 and 96 h of fungal growth. M: Seeblue® Pre-stained protein standard. Western blot confirmation of the proteins produced by *P. chrysogenum* transformants using specific antibodies against PeAfpA, PeAfpB and PeAfpC (bottom).

Supp. Table 1: Oligonucleotides used in this work.

ID	Use*	Sequence 5´- 3´**	Tm*** (ºC)	Restrict. sites	Genetic fragment	Description
OJM380	**F**	GTAATACGACTCACTATAGGG	**56**		*T7* promoter	DNA sequencing
OJM381	**R**	CATTTAGGTGACACTATAGAATAC	**56**		*sp6* promoter	DNA sequencing
OJM483	F	ATCCCGGGGAATTCAGAGAGCTTTTCGTACG	60	XmaI	*paf* promoter	Gene constructions generation and transformant verification
OJM484	R	ATTCTAGAGCAGCAGTTTGATAGTTATCCCT	60	*Xba* I	*paf* terminator	Gene constructions generation and transformant verification
OJM485	R	CAGGACACCGGCCTCAGCCC	60		sequence	Gene constructions
OJM488	F	ATGGTCTCTGCGATCACCAGG	60		*paf terminator*	Gene constructions and transformant verification
OJM495	F	**GGCTGAGGCCGGTGTCCTG**GTTCTGTACACCGGAGTGAG	60		*PeafpA*	Genomic DNA amplification
OJM497	R	**GGTGATCGCAGAGACCAT**TTACTTGCGGAAGTCACAGGTG	60		*PeafpA*	Genomic DNA amplification
OJM498	F	**GGCTGAGGCCGGTGTCCTG**AGTAAATACGGAGGAGTAAGTTC	60		*PeafpB*	Genomic DNA amplification
OJM499	R	**GTGATCGCAGAGACCAT**TTAAACCGGTGTTTGGCAGTC	60		*PeafpB*	Genomic DNA amplification
OJM500	R	CAGTGAGCGCGCGTAATACG	60		*PSK_λPAF v*ector	transformation vector generation and sequencing
OJM502	F	**GGCTGAGGCCGGTGTCCTG**GACACCTGCGGAGCCGGC	60		*PeafpC*	Genomic DNA amplification
OJM503	R	**GGTGATCGCAGAGACCAT**CTAGCACTTGGCTCCACCCTC	60		*PeafpC*	Genomic DNA amplification

* F: forward; R: reverse.
** Restriction sites are underlined. Complementary sequences for fusion PCR are in bold.
*** Tm: Temperature of annealing.

GENERAL DISCUSSION

The so-called cysteine-rich antifungal proteins of fungal origin (AFPs) have been studied for a long time, and have been proposed as promising compounds for the development of new antifungal alternatives in different fields, including medicine, agriculture, and food industry (Barakat et al., 2010; Hegedüs and Marx, 2013). In this work, we have focused on the study of four AFPs of interest for their potential application in agriculture as alternatives to the current use of chemical fungicides. Proteins PAF from *Penicillium chrysogenum* and AFP from *Aspergillus giganteus* are the most studied and characterized antifungal proteins to date (Lacadena et al., 1995; Marx et al., 1995). However, the number of experimentally characterized AFPs and of predicted AFP-like sequences from filamentous fungi increases continuously due to the number of sequenced genomes currently available. In this doctoral thesis, we produced and characterized the only AFP encoded in the citrus phytopathogen *Penicillium digitatum,* as well as the three AFPs encoded in *Penicillium expansum* for the first time, with the use of a highly efficient expression system for AFP production in filamentous fungi. In addition, new potential applications of these proteins in postharvest conservation and plant protection have been proposed.

1. A new and extended classification of AFPs based on phylogenetic analyses

Before this work, not so many studies had been made to establish a classification of the diversity of AFPs. Previous classifications indicated the existence of only two different groups that divided AFPs into the PAF-cluster and the BP-cluster (Seibold et al., 2011; Galgóczy et al., 2013b). In the present work, phylogenetic analyses of the available AFP and AFP-like sequences were conducted and a new classification is proposed, contributing to the characterization of the different AFP profiles in filamentous fungi in more detail. In this classification, we proposed three different classes of AFPs (A, B and C). This study revealed that different fungi might encode different numbers of AFPs from different classes, as it is the case of *P. expansum,* which encodes one AFP belonging to each class (PeAfpA, PeAfpB and PeAfpC), as opposed to *P. digitatum,* which only encodes one AFP belonging to class B (AfpB). *P. expansum and P. chrysogenum* are the only fungi that encode an AFP of each different class. The three AFPs from *P. chrysogenum* had already been studied, although these proteins were each isolated from three different strains (Marx et al., 1995; Rodriguez-Martín et al., 2010; Chen et al., 2013). On the contrary,

none of the three AFPs encoded in the phytopathogenic fungus *P. expansum* had been studied prior to this work, which made this fungus a very suitable candidate to produce the three AFPs from a phytopathogenic fungus for the first time, and to study the functional role of each different *afp* gene in the same fungal strain.

Throughout the development of this thesis, a new AFP from *Neosartoria fischeri* (NFAP2) has been described (Toth et al., 2016). This would be the first member of an additional "class D" of AFPs distant from classes A, B and C in a new and improved phylogenetic tree that we present in this Discussion for the first time (Figure 1).

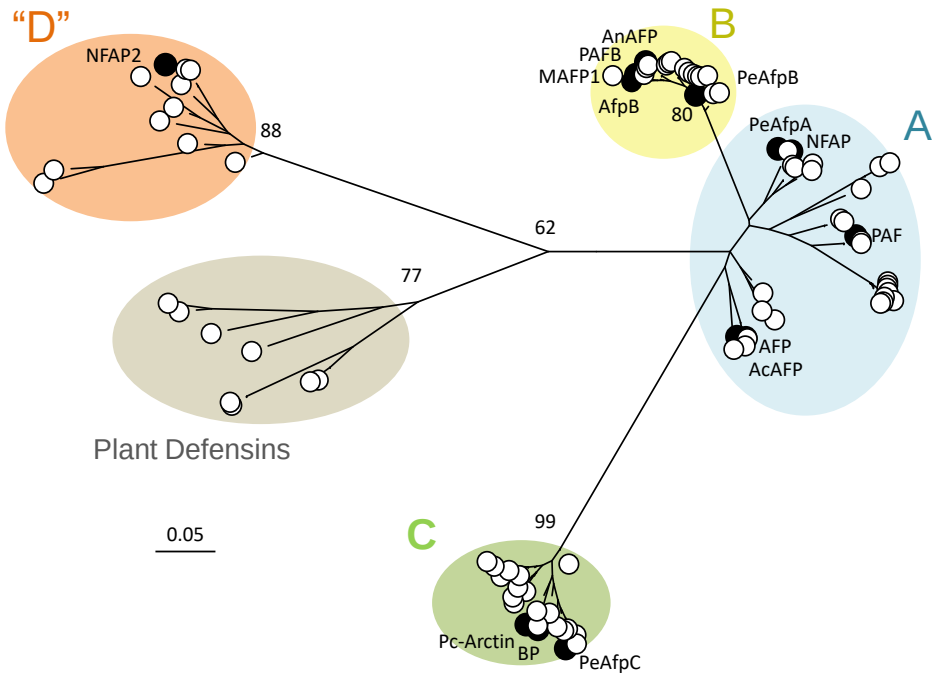


Figure 1: Maximum Likelihood phylogenetic analysis of the different classes of AFPs currently described. Classes A, B and C reported in this thesis are represented in blue, yellow, and green, respectively. The new and potential class "D" of AFPs with potent anti-yeast activity (Toth et al., 2016) is highlighted in orange. The structurally-related plant defensins were added as outgroup and are highlighted in grey. Black circles represent AFPs with demonstrated antifungal activity. Analyses were carried out with MEGA 7.0. and bootstrap values were calculated using 10,000 replicates. Bootstraps higher than 60 are shown. Updated from Garrigues et al., 2016.

This protein is the first member of a new class of AFPs with potent anti-yeast activity, which seems to be spread in many different ascomycetes, such as *Alternaria, Aspergillus, Fusarium, Neosartorya,* and *Penicillium,* among others (Toth et al., 2016). This fact suggests the existence of new unidentified classes of AFPs yet to be discovered.

The reason(s) why fungi encode distinct number of AFPs of different classes is still unknown. Beyond their antifungal activity, some AFPs have been described to play different biological roles or have alternative functions (see section 6 for more detail). Thus, distinct classes might play different functions that would explain the discrepancy in the number and/or classes of AFPs encoded in each fungus.

Even though AFPs belonging to different classes are considered homologous proteins (percentage of amino acid identity greater than 30 %), they show different sequence motifs, cationicity, length and number of cysteine residues and disulphide bond patterns (Figure 2). In these analyses (Figure 1 and 2), class A may divide into two subclasses (in blue, separated with a line in Figure 2). In this new classification, class A proteins with 8 cysteine residues, as it is the case of AFP from *A. giganteus*, would separate of that of class A with 6 cysteines, as it is the case of PAF from *P. chrysogenum.* Regarding to sequence motifs, class B shows a histidine-rich motif close to the C-terminus protein region, which is unique among proteins from class B. The position of the conserved ɣ-core motif also varies between classes. While classes A and B have their ɣ-cores at the N-termini, in classes C and "D" they are positioned at the C-termini. These observations would correlate with their antimicrobial activities, since classes A and B have mainly antifungal activity and classes C and "D" are mainly anti-yeast. All these features might have caused a differentiation in their biological roles in their producer fungi that would also explain why the same fungus does not encode more than one AFP belonging to the same class. Additionally, gene loss might also explain this variation in the number of AFPs encoded in each fungus. For instance, the *paf* gene locus of *P. chrysogenum* is located in a chromosomal region that is lost in the related genome of *P. digitatum*, providing an explanation for the lack of a class A gene in *P. digitatum*.

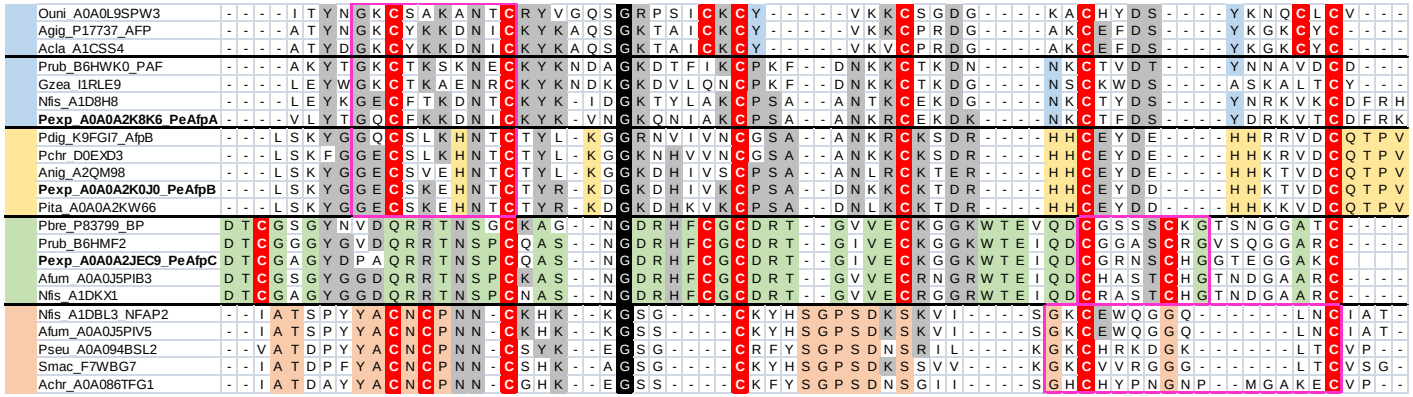


Figure 2: Amino acid sequence alignment of a selection of AFPs and AFP-like proteins. Proteins belonging to different phylogenetic classes are highlighted in different colours following colour codes in Figure 1. Proteins belonging to class A are represented in blue, classes B and C are shaded in yellow and green, respectively, and the new reported class of AFPs ("class D") is highlighted in orange. Conserved intra-class motifs are shadowed following their colour code. Cysteine patterns are shadowed in red. Strongly conserved Glycine is shadowed in black. The conserved ɣ-core is in a pink box.

Further characterization of new AFPs and AFP-like proteins would reveal whether different classes would play distinct roles in their producer strains. What is clear is that filamentous fungi have a complex repertoire of AFPs and cysteine-rich AFP-like proteins that offer a potential source of antimicrobial compounds to be explored, which is one of the purposes of this doctoral thesis.

2. The AfpA from *P. expansum* is produced in very high yields by the parental fungus

The diversity of AFPs currently known is not always correlated with protein production. A major finding of this work is the natural production of the class A AFP from *P. expansum* (AfpA) in very high amounts (up to 125 mg/L) in minimal medium and at late stages of growth when nutrients are restricted. On the contrary, AFPs from classes B and C are not naturally produced by *P. expansum* in the conditions tested.

AFPs were initially considered as proteins that were abundantly produced by their parental fungi. In fact, there are many examples of filamentous fungi that naturally produce AFPs in high yields. For example, the class B protein PgAFP from *P. chrysogenum* CECT 20922 is produced in very high amounts (up to 617 mg/L) when growing in malt extract broth after 14 days (Delgado et al., 2015). The class A AFP from *A. giganteus* can be also produced with yields up to 10 mg/L after 4 days in rich medium (Lacadena et al., 1995). In addition, PAF from *P. chrysogenum* Q176 is naturally produced in high yields (representing up to 30 % of total protein production) when growing for 48 h with sucrose as sole carbon source (Marx et al., 1995). However, other AFPs such as NFAP and NFAP2 from *Neosartoria fischeri,* were reported to be naturally produced in very low amounts (1.25 and 0.368 mg/L, respectively) (Galgóczy et al., 2013a; Toth et al., 2016). None of these AFPs were active against their producers.

In this work, we have demonstrated for the first time that expression of an *afp* gene is not accompanied by protein accumulation in culture supernatants, as it is the case of the *afpB* gene in *P. digitatum*. This fact is not unique among filamentous fungi. Very recently, the gene coding for the class B AFP from *P. chrysogenum* (PAFB) has also been described to be naturally expressed but the protein is not produced (Huber et al., 2018). One possible explanation for the extraordinary fact regarding the non-

production of AfpB and PAFB in the parental strains despite gene expression would be the potent antifungal activity of these two proteins. As we saw first in *P. digitatum* and was later described in *P. chrysogenum* (Garrigues et al., 2016; Huber et al., 2018), both fungi are sensitive to their self-AFPs from class B. For this reason, the production of these proteins may be strictly regulated and self-limited. However, this hypothesis is not applicable to any of the three AFPs of *P. expansum* CMP-1, since AfpA is naturally produced in high amounts although it shows potent antifungal activity against its producer fungus, with an inhibitory concentration as low as 2 µg/mL (see Table 1 below), and AfpB and AfpC are not produced in spite of their limited or null antifungal activity against their native fungus. Additional studies focused on gene expression would reveal whether *afpB* and *afpC* genes from *P. expansum* are expressed or not, or if the impossibility to detect the corresponding AfpB and AfpC in culture supernatants is due to a strict regulation during fungal growth, similar to what may be occurring with AfpB and PAFB in *P. digitatum* and *P. chrysogenum,* respectively.

3. Fungal biofactories for the biotechnological production of AFPs

Many different attempts have been carried out to produce AFPs biotechnologically. Initially, yeasts were the preferred biofactories for the heterologous production of AFPs. However, protein yields were not usually as high as desired, despite the fact that the AFPs produced do not show anti-yeast activity. For example, PAF mutated variants were heterologously produced in *Pichia pastoris* with yields around 5 mg/L (Batta et al., 2009). AFP from *A. giganteus* was also produced in *P. pastoris* with yields as low as 2.5 mg/L (López-García et al., 2010b). NFAP was also produced in the same system with protein yields of 6 mg/L (Viragh et al., 2014). We also produced AfpB from *P. digitatum* in *P. pastoris* and we obtained 1.4 mg/L of pure AfpB. An exception to these low AFP yields in yeast has been recently described for an AFP from *Fusarium graminearum* (FgAFP), with protein yields up to 20 mg/L (Patiño et al., 2018).

In this thesis, we have confirmed the suitability of the fungus *P. chrysogenum* as cell factory for the production of AFPs from different phylogenetic classes. In parallel works, *P. chrysogenum* was used for the

homologous and heterologous production of protein PAF, PAF variants, and NFAP in high yields (Sonderegger et al., 2016; Sonderegger et al., 2017), and we additionally showed that the *P. chrysogenum*-based expression cassette could be extended to express PAF in *P. digitatum*, demonstrating the versatility of the approach (Sonderegger et al., 2016). Due to the great efficiency of this cassette for AFP production, the *P. chrysogenum* null Δ*paf* strain, which does not produce any PAF, was chosen for the heterologous production of the two uncharacterized AFPs of classes B and C from *P. expansum.* PeAfpB and PeAfpC were successfully produced and purified in high amounts with this system. However, the PAF homologous class A protein PeAfpA, which was included as control, could not be produced in sufficient amounts for its subsequent purification. In addition, *P. chrysogenum* strain carrying *P. expansum afpA* gene showed a drastic reduction of growth and deficient conidiogenesis, which made this strain useless for protein production. It was very surprising to note that the transformation of the *paf* homologous class A *P. expansum afpA* gene in *P. chrysogenum*, which can be engineered to produce large amounts of PAF protein without any detrimental effect (Sonderegger et al., 2016), could have such negative impact in the biology of this fungus. Besides, *P. chysogenum afpA* transformant strain showed a phenotype reversion similar to the one previously reported for the *P. digitatum* strains constitutively expressing *afpB* (see below). This phenomenon is, at the very least, intriguing and we cannot provide reasonable explanation for the unstable phenotypes of these strains, since gene silence and DNA alterations seem to be unrelated. This has been the first time that three different AFPs of the same fungus have been produced on the same platform and using the same expression system.

In our previous attempts to produce AfpB in *P. digitatum,* we constitutively expressed the *afpB* gene under the regulation of the *gpdA* promoter from *A. nidulans,* which has been broadly used in filamentous fungi. However, we were not able to detect AfpB either in the culture supernatants of the parental or the constitutive expression strains, despite high gene expression. For this reason, we finally chose the *P. chrysogenum*-based expression system for the homologous production of AfpB in *P. digitatum*. With this approach, we could also study the effect of the production of this protein in the biology of *P. digitatum*. With this system, *P. digitatum* was able to produce detectable amounts of AfpB, with

yields ranging 12-20 mg/L after long incubation times with no detrimental effect. This was the first time AfpB could be produced and subsequently purified and characterized. Here, we confirmed that *P. digitatum* can be used as an effective cell factory for the production of AFPs in good yields, comparable to *P. chrysogenum*. However, it should be noted that *P. chrysogenum* is able to produce AFPs short after 2-3 days of incubation, (e.g. PAF, PAFB and other heterologous AFPs) (Sonderegger et al., 2016; Huber et al., 2018; Toth et al., 2018). On the contrary, *P. digitatum* needs at least 5 to 7 days to start producing detectable amounts of AFPs, and up to 10-11 days to reach optimal production. Another disadvantage of *P. digitatum* as biofactory is its phytopathogenicity. Pathogenic fungi, including phytopathogens, could hardly be accepted as GRAS biofactories for the bulk production of AFPs for their subsequent applications in medicine, agriculture or food industry. Therefore, *P. chysogenum* would be the best choice as fungal biofactory for AFP production given the high production yields achieved, short incubation times needed, and safety requirements, although other biofactories (e.g. plants) should be also taken into consideration for AFP production. In a recent work within the coordinated project in which our group collaborates, it has been demonstrated that a viral-based expression system worked very effectively for the transient expression of AFP-encoding genes in *Nicotiana benthamiana* (Shi et al., unpublished).

In parallel, we also demonstrated for the first time that not the signal peptide, but the regulatory elements (*afpB* promoter, UTRs, and/or terminator) would be involved in *afpB* mRNA translatability in *P. digitatum.* The *P. chrysogenum paf* promoter used for AfpB production in *P. digitatum* is regulated by glucose and nitrogen catabolite repression (Marx, et al., 1995) and was reported to have greater efficiency than the *gpdA* promoter in *P. chrysogenum* (Polli et al., 2016), and the same seems to happen in *P. digitatum*. In parallel, our laboratory successfully adapted the GoldenBraid (GB) cloning technology for its application in filamentous fungi. This new branch of GB was called FungalBraid, and it allows the exchange and assembly of DNA elements (e.g. promoters, genes, and terminators) in a very fast and efficient manner. We demonstrated that this system can be successfully applied in *P. digitatum,* and this technique was used to determine which regulatory element is responsible for AfpB production in *P. digitatum* (Hernanz-Koers et al., 2018). In this study, the production of AfpB

was tested under the regulation of either the *paf* promoter or an intronless shorter version of *gpdA* promoter from *A. nidulans*, leaving constant the *afpB* gene and *paf* terminator sequences. Data obtained confirmed our previous results in which *P. digitatum* strains carrying *gpdA* promoter did not produce AfpB, demonstrating that the promoter of the *P. chrysogenum paf* gene is necessary for high levels of AfpB production in *P. digitatum* (Hernanz-Koers et al., 2018). However, additional studies are required to elucidate the exact regulatory mechanism underlying the problem of AfpB production, which could be also taking place in the cases of the non-produced antifungal proteins AfpB and AfpC in *P. expansum.*

FungalBraid opens the way to apply synthetic biology in *P. digitatum* and other filamentous fungi, including GRAS organisms like *A. niger* or *Trichoderma ressei* in order to produce novel AFPs for future applications. Furthermore, FungalBraid will also allow us to test the suitability of different promoters and terminator sequences, for example the promising *afpA* promoter from *P. expansum,* for the development of new and more efficient expression systems for the production of AFPs.

4. Broad antimicrobial activity of AFPs and identification of antifungal motifs

AFPs had commonly been regarded as specific against filamentous fungi and innocuous against the producer fungus (Hegedüs and Marx, 2013). Here, we demonstrated for the first time that AFPs can also be highly active against the fungus from which these proteins are isolated, and also demonstrated anti-yeast activity.

To reach our conclusions regarding relative activity among the different AFPs, we conducted side-by-side experiments (Table 1). AfpA from *P. expansum* is one of the most potent AFPs against filamentous fungi described to date, even more active than the well-known PAF (Table 1) and AFP from *A. giganteus* (Barakat et al., 2010), with high effectiveness against economically important phytopathogenic fungi including the producer, dermatophytes, and clinically relevant yeasts, which makes this protein a very good candidate as antifungal and anti-yeast compound for medicine and agriculture. To date, only one AFP, the AcAMP from *Aspergillus clavatus*, has been reported to show antimicrobial activity against Gram+ and Gram- bacteria, including the pathogenic

Staphylococcus aureus (Hajji et al., 2010). Therefore, and given the great potency of this class A protein from *P. expansum*, this and other new antimicrobial functions deserve future studies.

AfpB from *P. digitatum* also showed high antifungal activity against a wide selection of fungi, including the parental and producer fungi (Table 1). AfpA from *P. expansum* is more active than AfpB against all the fungi tested, may be due to the high isoelectric point of this protein (pI 9.48 vs. 9.06). On the other hand, PeAfpB showed moderate antifungal activity, but lower than its class B homologs AfpB, AnAFP or PAFB (Gun et al., 1999; Huber et al., 2018), and anti-yeast activity could not be reported for this protein (Table 1). However, although anti-yeast activity has not been demonstrated yet for PeAfpB or AfpB, their homologs AnAFP, PAFB and PAF are active against *Saccharomyces cerevisie* and *Candida albicans* (Gun et al., 1999; Huber et al., 2018), suggesting a possible anti-yeast function yet to be discovered. PeAfpC showed neither antifungal nor anti-yeast activity and no function could be given to this protein, even though other class C homologs were described to have antifungal activity (Seibold et al., 2011; Chen et al., 2013). Finally, PAFB from *P. chrysogenum* has been recently reported to show antiviral activity. This led us to propose that AFPs could show novel antimicrobial actions that go beyond their classical antifungal activity.

Table 1: Antifungal activity of all AFPs studied in this work. MICs are shown in µg/mL.

Organism	PAF	AfpB	PeAfpA	PeAfpB	PeAfpC
P. digitatum	50	4	1	12	>200
B. cinerea	200	16	4	50	>200
P. expansum	>200	4	2	12	>200
P. italicum	>200	2	2	12	>200
P. chrysogenum	>200	8	2	50	>200
M. oryzae	>200	>200	16	>200	>200
A. niger	2	4	2	50	>200
T. rubrum	1.5[1]	ND[2]	4	32	>64
A. vanbreuseghemii	ND	ND	4	>64	>64
F. oxysporum	>200	100	4	>200	>200
G. moniliformis	ND	ND	4	>64	>64
A. flavus	ND	ND	4	>64	>64
C. albicans	25[1]	ND	8	>64	>64
C. glabrata	ND	ND	4	>64	>64
C. parasilopsis	ND	ND	4	>64	>64
S. cerevisiae	12.5[1]	>200	4	>64	>64

[1]Data from Huber et al., 2018
[2]ND: Not determined

The functional mapping of antimicrobial motifs within interesting protein sequences is an approach for the development of new antifungal molecules with improved antimicrobial activity, selectivity and efficacy. In this work, we predicted the three-dimensional structure of the AFPs from *P. expansum* and from AfpB from *P. digitatum*. This allowed us to identify antifungal motifs within an AFP (AfpB) for the first time, since most of the studies based on the search of antimicrobial motifs in cysteine-rich proteins have focused on defensins (Sagaram et al., 2011; Bleackley et al., 2016; Kaewklom et al., 2016).

Our results indicate that determinants for AfpB antifungal activity remain in the biggest cationic exposed loops L2 and L3, which are represented by the synthetic peptides PAF118 and PAF112, respectively. The γ-core motif from AfpB (PAF109) showed no antifungal activity, analogous to the γ-core from some plant defensins (Sagaram et al., 2011) and the recently described γ-core from the *N. fischeri* NFAP2 (Toth et al., 2018), suggesting a structural function in AfpB necessary for the correct protein folding. L3 is one of the most important domains among AFPs from classes A and B, since mutations in the analogous L3 in PAF negatively affected antifungal activity (Batta et al., 2009; Sonderegger et al., 2016). In addition, positive charge seems to play a crucial role in peptide activity, as it was also reported in PAF (Batta et al., 2009) and other antifungal peptides as PAF26 (Muñoz et al., 2013b). However, recent studies showed that the negatively charged residues also present in L2 are very important for the antifungal activity of PAF (Sonderegger et al., 2017), since a change of a negatively charged amino acid (Asp) by a neutral one (Ser) in this zone does not significantly modify the tertiary structure of PAF, but it completely loses the antifungal activity. This also suggests that PAF folding is not the only mechanism involved in its antifungal activity, although the presence of disulfide bonds have been reported to be necessary for PAF to exert its antifungal action (Batta et al., 2009). Our recent studies with AfpB also suggested that protein folding is not sufficient for displaying its antifungal effects since denatured AfpB displayed the same antifungal activity as that of the native protein, although in our conditions, thermal denaturation did not include disulfide bond reduction.

None of the antifungal peptides identified in AfpB were sufficient to reach the activity of the native protein, as we also demonstrated with a

direct comparison with protein PAF from *P. chrysogenum* and PAF116, a peptide derived from the loop L3 of PAF. PAF116 was much less active than native protein (Garrigues et al., 2017). A similar behaviour was observed with NFAP2-derived peptides when compared to native NFAP2 (Toth et al., 2018). AfpB is about 100 times more active than its derived peptides even when the peptides are mixed, suggesting that apart from the protein sequence, additional determinants are involved in AfpB full antifungal activity.

Although in this work the three-dimensional structures of the three AFPs from *P. expansum* have been predicted, the study and characterization of the antimicrobial motifs have not been experimentally addressed yet. Tertiary structure of PeAfpA and PeAfpB were very similar to their classes A and B homologs PAF and PAFB, respectively (Varadi et al., 2013; Huber et al., 2018). However, PeAfpC is predicted to have partially lost its tertiary structure if compared with BP (Olsen et al., 2004). These structural features along with the absence of antimicrobial activity and the non-natural production of this protein by its producer fungus could be indicating a gradual loss of *afpC* gene function in *P. expansum.*

In silico approaches for the prediction of antifungal peptides could be applied as a first try to find potential antifungal motifs within the AFPs from *P. expansum* (Agrawal et al., 2018). It is noteworthy that some of the features described for PAF and AfpB could be also extrapolated, for example, to the highly active AfpA from *P. expansum*, which is one of the most interesting AFPs that we have to date due to its potent antifungal activity, protein yields, and physicochemical properties. The analogous L3 in PeAfpA is even more cationic than L3 in AfpB, and show similar degree of cationicity to that shown for L3 in PAF, suggesting that this loop might also be involved in the antimicrobial activity of PeAfpA. However, future studies will be performed to characterize the antimicrobial motifs within PeAFPs. This will allow the development of improved rationally-designed peptides that could be used alone or in combination with other (un)related compounds in agriculture and other areas such as medicine, in which other peptides are currently approved or at very high stages of development (Duncan and O'Neil, 2013; Rautenbach et al., 2016).

5. Functional role and mechanism of action of AFPs in phytopathogenic fungi

Currently, the biological role of *afp* genes in filamentous fungi is not well understood. In the case of the class A protein PAF, it has been reported that this protein enhances conidiation in *P. chrysogenum* by modulating the expression of conidiogenesis-related genes, such as *brlA*, and that a *paf* deletion strain (Δ*paf*) showed a significant impairment of spore formation (Hegedüs et al., 2011b). Anisin1 from *Aspergillus nidulans* has been reported to be involved in fitness support in the producer fungus (Eigentler et al., 2012). In addition, a recent *in silico* meta-analysis in *A. niger* suggested novel functions for AnAFP in nutrient recycling during starvation, autophagy, and development (Paege et al., 2016). This led to propose that AFPs might act as cannibal toxins in fungi, similar to cannibalism described in bacteria, in which the sub-population of *Bacillus* cells that enters the sporulation program secretes toxins to kill the non-sporulated cells and recycle their nutrients (Meyer and Jung, 2018).

In this thesis work, we have characterized different AFPs from phytopathogenic fungi for the first time and we have contributed to the study of the biological role of AfpB in the phytopathogenic fungus *P. digitatum.* The *afpB* gene expression pattern significantly differs from the ones previously reported for other *afp* genes. For example, in *A. giganteus* AFP*,* gene expression is only detectable in mycelium, but not in asexual spores (Meyer and Stahl, 2002). Similarly, the *paf* mRNA is absent in conidia from *P. chrysogenum* (Hegedüs et al., 2011b). However, *afpB* mRNA is present in aerial hyphae, submerged mycelia, and is highly induced in conidia and rapidly decreases during germination. This expression pattern led us to initially propose a functional role for *afpB* in conidiogenesis in accordance with the *paf* deletion mutant of *P. chrysogenum* Δ*paf,* which showed up to a 70 % reduction in the concentration of produced conidia (Hegedüs et al., 2011b). However, null *afpB* mutants of *P. digitatum* showed no phenotypical differences when compared to the parental strain, and only little delay in conidia production was noticed at very early stages of growth. The absence of an *afpB* deletion phenotype could suggest that (i) its phenotype might be undetectable in the assays described, (ii) AfpB is involved in processes that have not been assessed yet, or (iii) other redundant proteins might have

taken over the function of AfpB. Nevertheless, our results indicated that although a potential role in conidiation cannot be discarded, this gene is dispensable for the biology, pathogenicity and virulence of this fungus.

Further experiments carried out in our laboratory demonstrated that the exposure of *P. digitatum* mycelia to AfpB produced a gradual increase of Hog1 and Slt2 phosphorylation, which are two mitogen activated protein kinases (MAPKs) responsible for the activation of transcription factors that modulate gene expression in filamentous fungi. In addition, these signal cascades activation do not seem to be related to a defense role, since *P. digitatum* null mutant strains Δ*hog1* and Δ*slt*2 did not show increased sensitivity to AfpB. This suggests that maybe AfpB would have a biological role involved in the regulation of gene expression through the activation of the MAPK cascades, although more studies are required to confirm this hypothesis (Gandía et al., unpublished).

The study of the biological role of the three different *afp* genes of *P. expansum* has not been deeply addressed yet. Preliminary attempts were conducted in our laboratory to determine the presence of any of the three AFPs from this phytopathogen during apple fruit infection, but none of these proteins were immunodetected, suggesting that none of the three AFPs would have a biological role during fruit infection (data not shown). In addition, the study of the gene expression pattern of the three *Peafp* genes is currently being performed. Future attempts to study the biological roles of the three different AFPs from *P. expansum* will include the generation of deletion strains of each of the three *afp* genes using the fast and efficient cloning system FungalBraid.

Regarding the effects of other AFPs on sensitive fungi, PAF from *P. chrysogenum* has been reported to be internalized inside fungal cells into an energy-dependent manner (Oberparleiter et al., 2003), to perturb calcium homeostasis (Binder et al., 2010), and to inhibit germination of conidia and growth of germinated hyphae in sensitive fungi (Kaiserer et al., 2003). PAF toxicity also triggers the generation of reactive oxygen species, permeabilizes intracellular membranes (Kaiserer et al., 2003) and induces cAMP/PKA signalling pathways (Binder et al., 2015). AFP from *A. giganteus* was reported to bind to the membranes of sensitive fungi and to cause permeabilization (Theis et al., 2003). AFP has a high affinity for

binding to chitin, which is a main constituent of fungal cell walls and might be also involved in protein-cell wall interaction (Liu et al., 2002). Moreover, AFP interferes with fungal cell wall biosynthesis (Meyer, 2008) and it is rarely internalized (Theis et al., 2005). AFP has also been described to have fungistatic activity at low concentrations, and fungicidal at high concentrations (Theis et al., 2005). Very recently, the class B AFP from *P. chrysogenum* PAFB has been described to have fungicidal activity and enter the fungal cells, providing evidence that antifungal toxicity of PAFB (as it also happens with PAF but not with AFP) is closely linked to its uptake into fungal cells (Huber et al., 2018). In addition, compared to PAF, PAFB prevented germination in a more efficient manner (Huber et al., 2018).

The mechanism of action of the three active AFPs produced in this work is not known yet. Preliminary studies on AfpB from *P. digitatum* suggest that this protein is also internalized and efficiently prevents spore germination and hyphae prolongation. In addition, AfpB from *P. digitatum* and AfpA from *P. expansum* show fungicidal activity *in vitro* (data not shown). However, further studies are needed to elucidate the mechanisms of action of these proteins.

6. AfpB and PeAfpA are potential candidates for plant protection and postharvest conservation

The final goal of this thesis is the application of AFPs for the control of plant and postharvest diseases. We have studied the feasibility of the two more effective AFPs that we have in our hands, AfpB and PeAfpA, for the control of the infection in two important pathosystem models: the citrus fruit specific *P. digitatum*, which is an extreme case of host specificity, and the infection of tomato plants by *B. cinerea*, which on the contrary is one of the most polyphagous fungus known to date.

Only one AFP of fungal origin, the well-known AFP from *A. giganteus,* has been reported to control plant infection caused by sensitive fungi, and always under laboratory conditions. Transgenic plants expressing the *A. giganteus afp* gene have enhanced resistance in wheat to the powdery mildew fungus (*Erysiphe graminis*) or the leaf rust fungus (*Puccinia recondite*) (Oldach et al., 2001), or in rice to *M. oryzae* (Coca et

al., 2004). Application of purified AFP to non-transgenic plants was also demonstrated to reduce experimental infection. AFP purified from *A. giganteus* was included in the liquid fertilizer of tomato plants to prevent the subsequent infection by the root pathogen *F. oxysporum* (Theis et al., 2005). Direct application of the AFP to leaves also controlled the *M. oryzae* infection to rice leaves (Vila et al., 2001) or *Botrytis* infection on geranium plants (Moreno et al., 2003). Sprayed AFP from *A. giganteus* reduced the postharvest infection incidence of *A. alternata* on harvested bananas (Barakat, 2014). To our knowledge, no other fungal AFP besides this from *Aspergillus* has been shown to control fungal infection on either plants or fruits.

In this thesis, we propose the use of AfpB or PeAfpA as promising alternatives for crop protection, since AfpB protected from *B. cinerea* infection in tomato plants, and PeAfpA was successfully applied against both *B. cinerea* and *P. digitatum* in tomato plants and orange fruits, respectively at very low protein concentrations. These results led to the development of two patents (Manzanares et al., 2018a; Manzanares et al., 2018b), and further applications of these AFPs will be addressed in the future. Considering the high *in vitro* potency of PeAfpA against other relevant phytopathogens, this protein could also be considered an effective alternative for the control of other fungal diseases that cause severe plant damage and fruit decay, although this has not been addressed in this work. For example, PeAfpA might prevent from *Fusarium oxysporum* infection in tomato, melon, and banana; *Magnaporthe oryzae* infection in rice; or *Penicillium italicum* or *P. expansum* in citrus and pome fruits, respectively.

Surprisingly, AfpB did not confer protection against *P. digitatum* infection in orange fruits despite the high protein concentrations used in our experiments (up to 100 µg/mL) and its high *in vitro* antifungal activity against this fungus (MIC above 4 µg/mL). A possible explanation for this might be that *P. digitatum* would recognize or interact differently with AfpB in the context of citrus fruit infection *in vivo*. Another possible explanation could be related to an unidentified AfpB positive role during the infection of *P. digitatum*, which has not been phenotypically revealed in the Δ*afpB* null mutant. In this regard, there are some intriguing “negative” data obtained in our fruit inoculation assays. The synthetic hexapeptide PAF26 shows extremely high protection activity when used in co-inoculation experiments

of *P. digitatum* onto citrus fruits (López-García et al., 2003; Muñoz et al., 2007a). The AfpB from *P. digitatum*, when added to the inoculum in addition to the conidia and PAF26, antagonizes the activity of PAF26 in a dose- dependent manner. Interestingly, this effect is not observed with PeAfpA, indicating some degree of specificity between the protein and the fungus and indirectly a role of AfpB during the infection. Future experiments where we use PeAfpA to protect apples from the infection caused by *P. expansum* will reveal whether the producer fungus itself is insensitive to the action of its protein *in vivo*, reinforcing the first hypothesis. The reason(s) why *in vivo* activity parallels that observed *in vitro* deserves future studies.

In our experiments, *in vivo* antifungal activity of PeAfpA and AfpB is comparable to that reported for other antifungal AMPs, such as PAF26, PAF38, BM0, cecropin, melittin, indolicidin, and others (Muñoz et al., 2007b), and even to the extensively used fungicides thiabendazole and imazalil (López-García et al., 2003) with the advantage that AFPs are natural compounds that can be efficiently produced through biotechnology. In agriculture, fungicides are typically applied in water as a dip, drench or spray, or in amended waxes. In our experiments, we have applied point-coinoculations of the purified AFPs in combination with the phytopathogen, which is far from the real form of application. A possible cost-effective way of apply AFPs for plant protection and postharvest conservation would be the direct spray of previously-heated culture supernatants enriched in our proteins of interest. However, a better characterization in terms of safety requirements, large-scale production, modes of antifungal action and ways of potential applications should be further explored.

7. Further considerations

AFPs are small, compacted, secreted proteins that present a high thermodynamic and chemical stability. They are synthesized in a single polypeptide chain format, and have surface exposed loop regions in their tertiary structure (Batta et al., 2009; Hegedüs et al., 2011a). These extraordinary properties, together with their ability to be (naturally or biotechnologically) produced in high amounts, make them perfect candidates for their further improvement by rational design. Rational design of peptides and proteins allows the improvement of their properties (selectivity, antimicrobial potency, protein stability, toxicity, etc.) for their

final applications. To date, many studies have been published for plant defensins undergoing rational modifications of their amino acid sequences in order to improve their antifungal activities, as it is the case, for example, of defensins MsDef1 and MtDef4, in which their γ motifs were exchanged, resulting in different intensities of antifungal activities (Sagaram et al., 2011). Another example would be the case of defensins NaD1 and NaD2. Different chimeras were produced by replacing loops 1-7 from NaD1, with the corresponding loops from NaD2, resulting in protein variants that sometimes increased the antifungal potency of the native proteins (Bleackley et al., 2016). However, nothing similar has been carried out with fungal AFPs to date, despite their suitability and their potential to be improved. For this reason, we propose that AFPs could also be great candidates for their rational modification.

In our laboratory, we explored the improvement of AfpB antifungal/fungicidal properties by the insertion of the hexapeptide PAF26 into the AfpB primary sequence. Not all attempts were successful, and the place where PAF26 is inserted seems to be determinant for protein stability and accumulation. In addition, PAF26 did not improve substantially AfpB potency. However, when loop L3 from AfpB was truncated by the insertion of PAF26, this protein lost its fungicidal activity, which allowed us to map the fungicidal motif in AfpB (Heredero et al, unpublished). Another example of what can be done with AFPs subjected to rational design would be the use of these proteins as scaffolds for the production of compounds with novel activities and functions. The use of monoclonal antibodies as scaffold proteins is widely used in clinic (Nuttall and Walsh, 2008), although not so many proteins have been reported to be used as scaffold in other fields. Further experiments need to be performed to make the most of AFPs, which offer an endless number of possibilities for their potential applications.

Undoubtedly, safety concerns regarding the application of AFPs not only in agriculture, but also in medicine or food industry to combat fungal infections and contaminations are crucial. The AFPs characterized in this work can be considered preliminary as safe since they show no erythrocyte lysing activity, although further toxicity studies to demonstrate the lack of detrimental effects are mandatory (Rautenbach et al., 2016). Nevertheless, the absence of cytotoxic effects of *A. giganteus* AFP and *P. chrysogenum*

PAF on plant (Vila et al., 2001) and mammalian cells (Szappanos et al., 2006) supports the safety of AFPs for their *in vivo* applications.

Overall, AFPs from filamentous fungi are considered promising candidates for their use as alternative antifungal compounds, and their study and in-depth characterization deserve to be further explored. In addition, AFPs are excellent candidates for undergoing rational design to improve their properties as desired. In this PhD thesis we have contributed to the efficient production and characterization of four new AFPs, two of them with very interesting properties for their real application in agriculture. Additional studies addressing safety requirements and to unravel their modes of action would also favour their use and future application in this and other relevant fields, leading to a new era of safe, biocompatible and sustainable antifungals yet to be exploited.

CONCLUSIONS

1. Fungal genomes encode a wide repertoire of AFP proteins. We have proposed a new classification of the diversity of AFPs in three classes (A, B and C). The recent discovery of a new fungal AFP led us to propose an additional and distantly related class.

2. The *afpB* gene is dispensable for the completion of the life cycle of the wild type *Penicillium digitatum* CECT 20796*,* but its constitutive expression under common gene expression regulators has a negative impact on fungal growth and virulence.

3. The presence and even the expression of an *afp* gene does not necessarily result in protein production, as it is the case of AfpB from *P. digitatum*, and AfpB and AfpC from *Penicillium expansum.*

4. The *P. expansum* CECT 20906 can naturally produce and secrete high amounts of AfpA under certain culture conditions that allow its easy one step purification with high yields above 125 mg/L.

5. The expression cassette comprising the promoter and terminator sequences from the *paf* gene of *Penicillium chrysogenum* enabled the homologous production of AfpB in *P. digitatum* and the heterologous production of AfpB and AfpC from *P. expansum* in *P. chrysogenum* for the first time.

6. The citrus pathogen *P. digitatum* works efficiently as biofactory for the production of AFPs in terms of yield and purity. However, the GRAS fungus *P. chrysogenum* would be a better biofactory for the heterologous production of AFPs in terms of protein yield, incubation time, and safety requirements.

7. The AfpB from *P. digitatum* shows extreme thermal stability, refolding capability and resistance to proteolysis. Spectroscopic data showed that the absence of the N-terminal leucine results in the incomplete refolding capability of the AfpB* variant, without affecting its antifungal activity after heat treatment. This latter result indicates that native folding is not required for antifungal activity.

8. The rational design of AfpB-derived cysteine-containing peptides has allowed the mapping and identification of two antifungal motifs

in AfpB, which correspond to the most cationic exposed-loops in the AfpB predicted tertiary structure.

9. The five AFPs used in this work show different profiles of antifungal activity, suggesting distinct applications. The AfpA from *P. expansum* and the AfpB from *P. digitatum*, in this order, were more active than the previously known PAF from *P. chrysogenum* towards a broad spectrum of fungi that includes plant and human pathogens. The AfpB and AfpC from *P. expansum* showed moderate and no antifungal activity, respectively.

10. Remarkably, AfpB from *P. digitatum* and AfpA from *P. expansum* are highly active against their self-fungus, a feature that had not been previously described and that deserves further studies.

11. All AFPs studied in this work can be preliminary regarded as safe since none showed haemolytic activity.

12. AfpA from *P. expansum* and AfpB from *P. digitatum* have been shown to successfully protect against fungal infections caused by phytopathogenic fungi *in vivo*. Therefore, the use of these two AFPs is proposed as a promising alternative for the treatment of diseases caused by filamentous fungi in agriculture.

GENERAL REFERENCES

Abdallah, N. A., Shah, D., Abbas, D., et al. (2010). Stable integration and expression of a plant defensin in tomato confers resistance to Fusarium wilt. GM crops 1(5): 344-350.

Aerts, A. M., François, I. E. J. A., Meert, E. M. K., et al. (2007). The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans.* Journal of Molecular Microbiology and Biotechnology 13(4): 243-247.

Agrawal, P., Bhalla, S., Chaudhary, K., et al. (2018). In Silico Approach for Prediction of Antifungal Peptides. Frontiers in Microbiology 9: 323.

Alem, D., Dellavalle, P., Leoni, C., et al. (2014). In search of topical agricultural biofungicides: properties of the recombinant antimicrobial peptide Trxaq-AMP obtained from *Amaranthus quitensis.* Journal of Microbial and Biochemical Technology 06(05).

Badosa, E., Ferre, R., Frances, J., et al. (2009). Sporicidal activity of synthetic antifungal undecapeptides and control of *Penicillium* rot of apples. Applied Environmental Microbiology 75(17): 5563-5569.

Ballester, A.-R., Lafuente, M. T., Forment, J., et al. (2011). Transcriptomic profiling of citrus fruit peel tissues reveals fundamental effects of phenylpropanoids and ethylene on induced resistance. Molecular Plant Pathology 12(9): 879-897.

Ballester, A.-R., Marcet-Houben, M., Levin, E., et al. (2015). Genome, transcriptome, and functional analyses of *Penicillium expansum* provide new insights into secondary metabolism and pathogenicity. Molecular Plant-Microbe Interactions 28(3): 232-248.

Barakat, H. (2014). Bio-control of *Alternaria alternata* during banana storage by purified AFP using isoelectric focusing technique. Food and Nutrition Sciences 05(15): 1482-1495.

Barakat, H., Spielvogel, A., Hassan, M., et al. (2010). The antifungal protein AFP from *Aspergillus giganteus* prevents secondary growth of different *Fusarium* species on barley. Applied Microbiology and Biotechnology 87(2): 617-624.

Barkai-Golan, R. (2001). Postharvest diseases of fruits and vegetables. Development and control. Amsterdam, Elsevier Science B.V.

Batta, G., Barna, T., Gaspari, Z., et al. (2009). Functional aspects of the solution structure and dynamics of PAF - A highly-stable antifungal protein from *Penicillium chrysogenum*. The FEBS Journal 276(10): 2875-2890.

Binder, U., Bencina, M., Eigentler, A., et al. (2011). The *Aspergillus giganteus* antifungal protein AFP_{NN5353} activates the cell wall integrity pathway and perturbs calcium homeostasis. BMC Microbiology 11:209.

Binder, U., Bencina, M., Fizil, A., et al. (2015). Protein kinase A signaling and calcium ions are major players in PAF mediated toxicity against *Aspergillus niger*. FEBS letters 589(11): 1266-1271.

Binder, U., Chu, M., Read, N. D., et al. (2010). The antifungal activity of the *Penicillium chrysogenum* protein PAF disrupts calcium homeostasis in *Neurospora crassa.* Eukaryotic cell 9(9): 1374-1382.

Bleackley, M. R., Payne, J. A., Hayes, B. M., et al. (2016). *Nicotiana alata* defensin chimeras reveal differences in the mechanism of fungal and tumor cell killing and an enhanced antifungal variant. Antimicrobial Agents and Chemotherapy 60(10): 6302-6312.

Broekaert, W. F., Marien, W., Terras, F. R. G., et al. (1992). Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. Biochemistry 31(17): 4308-4314.

Brogden, K. A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nature Reviews Microbiology 3(3): 238-250.

Brogden, K. A., Guthmiller, J. M., Salzet, M., et al. (2005). The nervous system and innate immunity: the neuropeptide connection. Nature Immunology 6(6): 558-564.

Brown, G. D., Denning, D. W., Gow, N. A. R., et al. (2012). Hidden killers: human fungal infections. Science Translational Medicine 4(165).

Buron-Moles, G., López-Pérez, M., González-Candelas, L., et al. (2012). Use of GFP-tagged strains of *Penicillium digitatum* and *P. expansum* to study host-pathogen interactions in oranges and apples. International Journal of Food Microbiology 160(2): 162-170.

Campos-Olivas, R., Bruix, M., Santoro, J., et al. (1995). NMR solution structure of the antifungal protein from *Aspergillus giganteus*: evidence for cysteine pairing isomerism. Biochemistry 34(9): 3009-3021.

Campoy, S. and Adrio, J. L. (2017). Antifungals. Biochemical pharmacology 133: 86-96.

Carmona, L., Gandía, M., López-García, B., et al. (2012). Sensitivity of *Saccharomyces cerevisiae* to the cell-penetrating antifungal peptide PAF26 correlates with endogenous nitric oxide (NO) production. Biochemical and Biophysical Research Communications 417: 56-61.

Carvalho, A. O. and Gomes, V. M. (2009). Plant defensins-prospects for the biological functions and biotechnological properties. Peptides 30(5): 1007-1020.

Cary, J. W., Rajasekaran, K., Jaynes, J. M., et al. (2000). Transgenic expression of a gene encoding a synthetic antimicrobial peptide results in inhibition of fungal growth in vitro and in planta. Plant Science 154(2): 171-181.

Coca, M., Bortolotti, C., Rufat, M., et al. (2004). Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. Plant Molecular Biology 54(2): 245-259.

Coca, M., Peñas, G., Gómez, J., et al. (2006). Enhanced resistance to the rice blast fungus *Magnaporthe grisea* conferred by expression of a cecropin A gene in transgenic rice. Planta 223(3): 392-406.

Coca, M. A., Damsz, B., Yun, D.-J., et al. (2000). Heterotrimeric G-proteins of a filamentous fungus regulate cell wall composition and susceptibility to a plant PR-5 protein. The Plant Journal 22(1): 61-69.

Cotter, P. D., Hill, C. and Ross, R. P. (2005). Bacteriocins: Developing innate immunity for food. Nature Reviews Microbiology 3(10): 777-788.

Chan, Y. L., Prasad, V., Sanjaya, et al. (2005). Transgenic tomato plants expressing an *Arabidopsis* thionin driven by fruit-inactive promoter battle against phytopathogenic attack. Planta 221(3): 386-393.

Chen, Z., Ao, J., Yang, W., et al. (2013). Purification and characterization of a novel antifungal protein secreted by *Penicillium chrysogenum* from an Arctic sediment. Applied Microbiology and Biotechnology 97(24): 10381-10390.

De Lucca, A. J., Bland, J. M., Jacks, T. J., et al. (1997). Fungicidal activity of cecropin A. Antimicrobial Agents and Chemotherapy 41(2): 481-483.

Dean, R., Van Kan, J. A., Pretorius, Z. A., et al. (2012). The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology 13(4): 414-430.

Delgado, J., Acosta, R., Rodriguez-Martin, A., et al. (2015). Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products. International Journal of Food Microbiology 205: 23-29.

Denning, D. W., Park, S., Lass-Florl, C., et al. (2011). High-frequency triazole resistance found in nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. Clinical infectious diseases : an official publication of the Infectious. Diseases Society of America 52(9): 1123-1129.

Dos Reis Almeida, F. B., Pigosso, L. L., de Lima Damásio, A. R., et al. (2014). *α*-(1,4)-Amylase, but not *α*- and *β*-(1,3)-glucanases, may be responsible for the impaired growth and morphogenesis of *Paracoccidioides brasiliensis* induced by N-glycosylation inhibition. Yeast 31(1): 1-11.

Duncan, V. M. S. and O'Neil, D. A. (2013). Commercialization of antifungal peptides. Fungal Biology Reviews 26(4): 156-165.

Eigentler, A., Pocsi, I. and Marx, F. (2012). The anisin1 gene encodes a defensin-like protein and supports the fitness of *Aspergillus nidulans.* Archives of microbiology 194(6): 427-437.

Errampalli, D. (2014). *Penicillium expansum* (Blue Mold). Postharvest decay. Elsevier Academic Press. Eastbourne, UK**:** 189-231.

Errampalli, D., Brubacher, N. R. and DeEll, J. R. (2006). Sensitivity of *Penicillium expansum* to diphenylamine and thiabendazole and postharvest control of

blue mold with fludioxonil in 'McIntosh' apples. Postharvest Biology and Technology 39(1): 101-107.

Fisher, M. C., Henk, D. A., Briggs, C. J., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. Nature 484: 186-194.

Galgóczy, L., Kovács, L., Karacsony, Z., et al. (2013a). Investigation of the antimicrobial effect of *Neosartorya fischeri* antifungal protein (NFAP) after heterologous expression in *Aspergillus nidulans*. Microbiology 159(2): 411-419.

Galgóczy, L., Kovács, L. and Vágvölgyi, C. (2010). Defensin-like antifungal proteins secreted by filamentous fungi. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology: 550-559.

Galgóczy, L., Viragh, M., Kovacs, L., et al. (2013b). Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among *Fusaria.* Peptides 39: 131-137.

Ganz, T. (2003). Defensins: Antimicrobial peptides of innate immunity. Nature Reviews Immunology 3(9): 710-720.

García-Olmedo, F., Molina, A., Alamillo, J. M., et al. (1998). Plant defense peptides. Peptide Science 47(6): 479-491.

Garrigues, S., Gandia, M., Borics, A., et al. (2017). Mapping and identification of antifungal peptides in the putative antifungal protein AfpB from the filamentous fungus *Penicillium digitatum*. Frontiers in Microbiology 8: 592.

Garrigues, S., Gandía, M. and Marcos, J. F. (2016). Occurrence and function of fungal antifungal proteins: a case study of the citrus postharvest pathogen *Penicillium digitatum*. Applied Microbiology and Biotechnology 100(5): 2243-2256.

Geoghegan, I., Steinberg, G. and Gurr, S. (2017). The role of the fungal cell wall in the infection of plants. Trends in Microbiology 25(12): 957-967.

Gesell, J., Zasloff, M. and Opella, S. J. (1997). Two-dimensional 1 H NMR experiments show that the 23-residue magainin antibiotic peptide is an à-helix in dodecylphosphocholine micelles, sodium dodecylsulfate micelles, and trifluoroethanol/water solution. Journal of Biomolecular NMR 9(2): 127-135.

Ghag, S. B., Shekhawat, U. K. S. and Ganapathi, T. R. (2012). Petunia floral defensins with unique prodomains as novel candidates for development of Fusarium wilt resistance in transgenic banana plants. PLoS ONE 7(6).

Guidotti, G., Brambilla, L. and Rossi, D. (2017). Cell-Penetrating Peptides: from basic research to clinics. Trends in Pharmacological Sciences.

Guilhelmelli, F., Vilela, N., Albuquerque, P., et al. (2013). Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. Frontiers in Microbiology 4: 353.

Gun, L. D., Shin, S. Y., Maeng, C. Y., et al. (1999). Isolation and characterization of a novel antifungal peptide from *Aspergillus niger.* Biochemical and Biophysical Research Communications 263(3): 646-651.

Hajji, M., Jellouli, K., Hmidet, N., et al. (2010). A highly thermostable antimicrobial peptide from Aspergillus clavatus ES1: biochemical and molecular characterization. Journal of Industrial Microbiology & Biotechnology 37(8): 805-813.

Hancock, R. E. W. and Sahl, H. G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nature Biotechnology 24(12): 1551-1557.

Haney, E. F. and Hancock R. E. W., (2013). Peptide design for antimicrobial and immunomodulatory applications. Peptide Science 100(6): 572-583.

Harries, E., Carmona, L., Munoz, A., et al. (2013). Genes involved in protein glycosylation determine the activity and cell internalization of the antifungal peptide PAF26 in *Saccharomyces cerevisiae.* Fungal Genetics and Biology 58-59: 105-115.

Harries, E., Gandia, M., Carmona, L., et al. (2015). The *Penicillium digitatum* protein O-mannosyltransferase Pmt2 is required for cell wall integrity, conidiogenesis, virulence and sensitivity to the antifungal peptide PAF26. Molecular Plant Pathology 16(7): 748-761.

Hegedüs, N., Leiter, E., Kovács, B., et al. (2011a). The small molecular mass antifungal protein of *Penicillium chrysogenum*--a mechanism of action oriented review. Journal of Basic Microbiology 51(6): 561-571.

Hegedüs, N. and Marx, F. (2013). Antifungal proteins: More than antimicrobials? Fungal Biology Reviews 26(4): 132-145.

Hegedüs, N., Sigl, C., Zadra, I., et al. (2011b). The *paf* gene product modulates asexual development in *Penicillium chrysogenum*. Journal of Basic Microbiology 51(3): 253-262.

Henriques, S. T., Melo, M. N. and Castanho, M. A. R. B. (2006). Cell-penetrating peptides and antimicrobial peptides: How different are they? Biochemical Journal 399(1): 1-7.

Hernanz-Koers, M., Gandía, M., Garrigues, S., et al. (2018). FungalBraid: a GoldenBraid-based modular cloning platform for the assembly and exchange of DNA elements tailored to fungal synthetic biology. Fungal Genetics and Biology 116: 51-61.

Holmes, G. J. and Eckert, J. W. (1999). Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. Phytopathology 89(9): 716-721.

Huber, A., Hajdu, D., Bratschun-Khan, D., et al. (2018). New antimicrobial potential and structural properties of PAFB: A cationic, cysteine-cich protein from *Penicillium chrysogenum* Q176. Scientific Reports 8(1).

Ibeas, J. I., Yun, D. J., Damsz, B., et al. (2001). Resistance to the plant PR-5 protein osmotin in the model fungus *Saccharomyces cerevisiae* is mediated by the regulatory effects of SSD1 on cell wall composition. The Plant Journal 25(3): 271-280.

Janisiewicz, W. J., Pereira, I. B., Almeida, M. S., et al. (2008). Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing Psd1 antifungal peptide. Postharvest Biology and Technology 47(2): 218-225.

Jha, S., Tank, H. G., Prasad, B. D., et al. (2009). Expression of Dm-AMP1 in rice confers resistance to *Magnaporthe oryzae* and *Rhizoctonia solani.* Transgenic Research 18(1): 59-69.

Jones, R. W. and Prusky, D. (2002). Expression of an antifungal peptide in *Saccharomyces*: A new approach for biological control of the postharvest disease caused by *Colletotrichum coccodes.* Phytopathology 92(1): 33-37.

Kader, J. C. (1996). Lipid-transfer proteins in plants. Annual Review in Plant Physiology and Plant Molecular Biology. 47(1): 627-654.

Kaewklom, S., Euanorasetr, J., Intra, B., et al. (2016). Antimicrobial activities of novel peptides derived from defensin genes of *Brassica hybrid* cv Pule. International Journal of Peptide Research and Therapeutics 22(1): 93-100.

Kaiserer, L., Oberparleiter, C., Weiler-Gorz, R., et al. (2003). Characterization of the *Penicillium chrysogenum* antifungal protein PAF. Archives of Microbiology 180(3): 204-210.

Kant, P., Liu, W. Z. and Pauls, K. P. (2009). PDC1, a corn defensin peptide expressed in *Escherichia coli* and *Pichia pastoris* inhibits growth of *Fusarium graminearum*. Peptides 30(9): 1593-1599.

Kinay, P., Mansour, M. F., Mlikota Gabler, F., et al. (2007). Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Protection 26(4): 647-656.

Ko, K. S., Norelli, J. L., Reynoird, J. P., et al. (2002). T4 lysozyme and attacin genes enhance resistance of transgenic 'Galaxy' apple against *Erwinia amylovora.* Journal of the American Society for Horticultural Science 127(4): 515-519.

Kong, Q., Liang, Z., Xiong, J., et al. (2016). Overexpression of the bivalent antibacterial peptide genes in *Pichia pastoris* delays sour rot in citrus ruit and induces *Geotrichum citri-aurantii* cell apoptosis. Food Biotechnology 30(2): 79-97.

Kovacs, L., Viragh, M., Tako, M., et al. (2011). Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). Peptides 32(8): 1724-1731.

Lacadena, J., Martínez del Pozo, A., Gasset, M., et al. (1995). Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus.* Archives of biochemistry and biophysics 324(2): 273-281.

Lee, D. G., Kim, D. H., Park, Y., et al. (2001). Fungicidal effect of antimicrobial peptide, PMAP-23, isolated from porcine myeloid against *Candida albicans*. Biochemical and Biophysical Research Communications 282(2): 570-574.

Leiter, E., Szappanos, H., Oberparleiter, C., et al. (2005). Antifungal protein PAF severely affects the integrity of the plasma membrane of *Aspergillus nidulans* and induces an apoptosis-like phenotype. Antimicrobial Agents and Chemotherapy 49(6): 2445-2453.

Li, K., Ouyang, H., Lü, Y., et al. (2011). Repression of N-glycosylation triggers the unfolded protein response (UPR) and overexpression of cell wall protein and chitin in *Aspergillus fumigatus*. Microbiology 157(7): 1968-1979.

Liu, R. S., Huang, H., Yang, Q., et al. (2002). Purification of alpha-sarcin and an antifungal protein from mold (*Aspergillus giganteus*) by chitin affinity chromatography. Protein expression and purification 25(1): 50-58.

Lobo, D. S., Pereira, I. B., Fragel-Madeira, L., et al. (2007). Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle. Biochemistry 46(4): 987-996.

López-García, B., Gandía, M., Muñoz, A., et al. (2010a). A genomic approach highlights common and diverse effects and determinants of susceptibility on the yeast *Saccharomyces cerevisiae* exposed to distinct antimicrobial peptides. BMC Microbiology 10(1): 289.

López-García, B., González-Candelas, L., Pérez-Payá, E., et al. (2000). Identification and characterization of a hexapeptide with activity against phytopathogenic fungi that cause postharvest decay in fruits. Molecular Plant-Microbe Interactions 13(8): 837-846.

López-García, B., Harries, E., Carmona, L., et al. (2015). Concatemerization increases the inhibitory activity of short, cell-penetrating, cationic and tryptophan-rich antifungal peptides. Applied Microbiology and Biotechnology.

López-García, B., Moreno, A. B., San Segundo, B., et al. (2010b). Production of the biotechnologically relevant AFP from *Aspergillus giganteus* in the yeast *Pichia pastoris*. Protein Expression and Purification 70(2): 206-210.

López-García, B., Pérez-Payá, E. and Marcos, J. F. (2002). Identification of novel hexapeptides bioactive against phytopathogenic fungi through screening of a synthetic peptide combinatorial library. Applied and Environmental Microbiology 68(5): 2453-2460.

López-García, B., Veyrat, A., Pérez-Payá, E., et al. (2003). Comparison of the activity of antifungal hexapeptides and the fungicides thiabendazole and imazalil against postharvest fungal pathogens. International Journal of Food Microbiology. 89**:** 163-170.

Mandal, S. M., Silva, O. N. and Franco, O. L. (2014). Recombinant probiotics with antimicrobial peptides: a dual strategy to improve immune response in immunocompromised patients. Drug Discovery Today 19(8): 1045-1050.

Manzanares, P., Garrigues, S., Gandia, M., et al. (2018a). Proteína antifúngica y usos de la misma. Oficina Española de Patentes y Marcas. CSIC. Spain. P201830546

Manzanares, P., Garrigues, S., Marcos, J. F., et al. (2018b). Métodos para el control biológico de infecciones producidas por fitopatógenos en plantas y cultivos. Oficina Española de Patentes y Marcas. CSIC. Spain. P201830609.

Marcet-Houben, M., Ballester, A. R., de la Fuente, B., et al. (2012). Genome sequence of the necrotrophic fungus *Penicillium digitatum*, the main postharvest pathogen of citrus. BMC Genomics 13(1).

Marcos, J. F. and Gandía, M. (2009). Antimicrobial peptides: to membranes and beyond. Expert Opinion on Drug Discovery 4(6): 659-671.

Marcos, J. F., Gandía, M., Harries, E., et al. (2012). Antifungal peptides: Exploiting non-lytic mechanisms and cell penetration properties. Small Wonders: Peptides for Disease Control. K. Rajasekaran, J. W. Cary, J. Jaynes and E. Montesinos. Washington, DC, American Chemical Society. 1095**:** 337-357.

Marcos, J. F., Muñoz, A., Pérez-Payá, E., et al. (2008). Identification and rational design of novel antimicrobial peptides for plant protection. Annual Review of Phytopathology 46: 273-301.

Marmiroli, N. and Maestri, E. (2014). Plant peptides in defense and signaling. Peptides 56: 30-44.

Martínez-Ruiz, A., Martínez, d. P., Lacadena, J., et al. (1997). Characterization of a natural larger form of the antifungal protein (AFP) from *Aspergillus giganteus*. Biochimica et Biophysica Acta 1340(1): 81-87.

Marx, F., Haas, H., Reindl, M., et al. (1995). Cloning, structural organization and regulation of expression of the *Penicillium chrysogenum paf* gene encoding an abundantly secreted protein with antifungal activity. Gene 167(1-2): 167-171.

Meyer, V. (2008). A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. Applied Microbiology and Biotechnology 78(1): 17-28.

Meyer, V., Andersen, M. R., Brakhage, A. A., et al. (2016). Current challenges of research on filamentous fungi in relation to human welfare and a sustainable bio-economy: a white paper. Fungal Biology and Biotechnology 3(1).

Meyer, V. and Jung, S. (2018). Antifungal peptides of the AFP family revisited: are these cannibal toxins? Microorganisms 6(2).

Meyer, V. and Stahl, U. (2002). New insights in the regulation of the afp gene encoding the antifungal protein of *Aspergillus giganteus*. Current Genetics 42(1): 36-42.

Michielse, C. B. and Rep, M. (2009). Pathogen profile update: *Fusarium oxysporum*. Molecular Plant Pathology 10(3): 311-324.

Monk, B. C., Niimi, K., Lin, S., et al. (2005). Surface-active fungicidal D-peptide inhibitors of the plasma membrane proton pump that block azole resistance. Antimicrobial Agents and Chemotherapy 49(1): 57-70.

Montesinos, E. (2007). Antimicrobial peptides and plant disease control. FEMS Microbiology Letters 270(1): 1-11.

Montesinos, L., Bundo, M., Badosa, E., et al. (2017). Production of BP178, a derivative of the synthetic antibacterial peptide BP100, in the rice seed endosperm. BMC Plant Biology 17(1): 63.

Montesinos, L., Bundo, M., Izquierdo, E., et al. (2016). Production of biologically active cecropin A peptide in rice seed oil bodies. PLoS ONE 11(1).

Morales, H., Marín, S., Ramos, A. J., et al. (2010). Influence of post-harvest technologies applied during cold storage of apples in *Penicillium expansum* growth and patulin accumulation: A review. Food Control 21(7): 953-962.

Moreno, A. B., Del Pozo, A. M., Borja, M., et al. (2003). Activity of the antifungal protein from *Aspergillus giganteus* against *Botrytis cinerea.* Phytopathology 93(11): 1344-1353.

Moreno, A. B., Martinez Del Pozo, A. and San Segundo, B. (2006). Biotechnologically relevant enzymes and proteins. Antifungal mechanism of the *Aspergillus giganteus* AFP against the rice blast fungus *Magnaporthe grisea*. Applied Microbiology and Biotechnology 72(5): 883-895.

Moreno, A. B., Peñas, G., Rufat, M., et al. (2005). Pathogen-induced production of the antifungal AFP protein from *Aspergillus giganteus* confers resistance to the blast fungus *Magnaporthe grisea* in transgenic rice. Molecular Plant-Microbe Interactions 18(9): 960-972.

Munro, C. A. (2013). Chapter four - Chitin and glucan, the yin and yang of the fungal cell wall, implications for antifungal drug discovery and therapy. Advances in Applied Microbiology. S. Sariaslani and G. M. Gadd, Academic Press. 83**:** 145-172.

Muñoz, A., Gandía, M., Harries, E., et al. (2013a). Understanding the mechanism of action of cell-penetrating antifungal peptides using the rationally designed hexapeptide PAF26 as a model. Fungal Biology Reviews 26(4): 146-155.

Muñoz, A., Harries, E., Contreras-Valenzuela, A., et al. (2013b). Two Functional Motifs Define the Interaction, Internalization and Toxicity of the Cell-Penetrating Antifungal Peptide PAF26 on Fungal Cells. PLoS ONE 8(1).

Muñoz, A., López-García, B. and Marcos, J. F. (2006). Studies on the mode of action of the antifungal hexapeptide PAF26. Antimicrobial Agents and Chemotherapy 50(11): 3847-3855.

Muñoz, A., López-García, B. and Marcos, J. F. (2007a). Comparative study of antimicrobial peptides to control citrus postharvest decay caused by *Penicillium digitatum.* Journal of Agricultural and Food Chemistry 55(20): 8170-8176.

Muñoz, A., López-García, B., Pérez-Payá, E., et al. (2007b). Antimicrobial properties of derivatives of the cationic tryptophan-rich hexapeptide PAF26. Biochemical and Biophysical Research Communications 354(1): 172-177.

Muñoz, A. and Marcos, J. F. (2006). Activity and mode of action against fungal phytopathogens of bovine lactoferricin-derived peptides. Journal of Applied Microbiology 101(6): 1199-1207.

Muñoz, A., Marcos, J. F. and Read, N. D. (2012). Concentration-dependent mechanisms of cell penetration and killing by the de novo designed antifungal hexapeptide PAF26. Molecular Microbiology 85(1): 89-106.

Mygind, P. H., Fischer, R. L., Schnorr, K. M., et al. (2005). Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature 437(7061): 975-980.

Nakaya, K., Omata, K., Okahashi, I., et al. (1990). Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus*. European Journal of Biochemistry 193(1): 31-38.

Ng-Choi, I., Soler, M., Güell, I., et al. (2014). Antimicrobial peptides incorporating non-natural amino acids as agents for plant protection. Protein and Peptide Letters 21(4): 357-367.

Nguyen, L. T., Haney, E. F. and Vogel, H. J. (2011). The expanding scope of antimicrobial peptide structures and their modes of action. Trends in Biotechnology 29(9): 464-472.

Nicolas, P. (2009). Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. The FEBS Journal 276(22): 6483-6496.

Nucci, M. and Anaissie, E. (2007). *Fusarium* infections in immunocompromised patients. Clinical Microbiology Reviews 20(4): 695-704.

Nuttall, S. and Walsh, R. (2008). Display scaffolds: protein engineering for novel therapeutics. Current Opinion in Pharmacology 8(5): 609-615.

O'Donnell, K., Sutton, D. A., Rinaldi, M. G., et al. (2004). Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. Journal of Clinical Microbiology 42(11): 5109-5120.

Oberparleiter, C., Kaiserer, L., Haas, H., et al. (2003). Active internalization of the *Penicillium chrysogenum* antifungal protein PAF in sensitive *Aspergilli.* Antimicrobial Agents and Chemotherapy 47(11): 3598-3601.

Oldach, K. H., Becker, D. and Lorz, H. (2001). Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. Molecular Plant-Microbe Interactions 14(7): 832-838.

Olombrada, M., Lazaro-Gorines, R., Lopez-Rodriguez, J. C., et al. (2017). Fungal ribotoxins: a review of potential biotechnological applications. Toxins 9(2).

Olsen, J. G., Flensburg, C., Olsen, O., et al. (2004). Solving the structure of the bubble protein using the anomalous sulfur signal from single-crystal in-house Cu K[alpha] diffraction data only. Acta Crystallographica Section D 60(2): 250-255.

Olson, B. H. and Goerner, G. L. (1965). Alpha sarcin, a new antitumor agent. Applied Microbiology 13(3):3 22–326.

Ouedraogo, J. P., Hagen, S., Spielvogel, A., et al. (2011). Survival strategies of yeast and filamentous fungi against the antifungal protein AFP. The Journal of biological chemistry 286(16): 13859-13868.

Paege, N., Jung, S., Schape, P., et al. (2016). A transcriptome meta-analysis proposes novel biological roles for the antifungal protein AnAFP in *Aspergillus niger*. PLoS ONE 11(11).

Palou, L. (2014). *Penicillium digitatum*, *Penicillium italicum* (Green Mold, Blue Mold). Postharvest Decay: Control Strategies: 45-102.

Palou, L., Ali, A., Fallik, E., et al. (2016). GRAS, plant- and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. Postharvest Biology and Technology 122: 41-52.

Parachin, N. S., Mulder, K. C., Viana, A. A., et al. (2012). Expression systems for heterologous production of antimicrobial peptides. Peptides 38(2): 446-456.

Patiño, B., Vázquez, C., Manning, J. M., et al. (2018). Characterization of a novel cysteine-rich antifungal protein from *Fusarium graminearum* with activity against maize fungal pathogens. International Journal of Food Microbiology.

Pérez-Nadales, E. and di Pietro, A. (2011). The membrane mucin Msb2 regulates invasive growth and plant infection in *Fusarium oxysporum*. Plant Cell 23(3): 1171-1185.

Petit, A. N., Fontaine, F., Vatsa, P., et al. (2012). Fungicide impacts on photosynthesis in crop plants. Photosynthesis Research 111(3): 315-326.

Polli, F., Meijrink, B., Bovenberg, R. A., et al. (2016). New promoters for strain engineering of *Penicillium chrysogenum*. Fungal Genetics and Biology 89: 62-71.

Puig, M., Moragrega, C., Ruz, L., et al. (2016). Interaction of antifungal peptide BP15 with *Stemphylium vesicarium*, the causal agent of brown spot of pear. Fungal Biology 120(1): 61-71.

Rahnamaeian, M., Langen, G., Imani, J., et al. (2009). Insect peptide metchnikowin confers on barley a selective capacity for resistance to fungal ascomycetes pathogens. Journal of Experimental Botany 60(14): 4105-4114.

Rao, A. G. (1995). Antimicrobial peptides. Molecular Plant-Microbe Interactions 8(1): 6-13.

Rautenbach, M., Troskie, A. M. and Vosloo, J. A. (2016). Antifungal peptides: To be or not to be membrane active. Biochimie 130: 132-145.

Regente, M. C. and de la Canal, L. (2000). Purification, characterization and antifungal properties of a lipid-transfer protein from sunflower (*Helianthus annuus*) seeds. Physiologia Plantarum 110(2): 158-163.

Ren, X., Kong, Q., Wang, H., et al. (2012a). Control of apple blue mold by *Pichia pastoris* recombinant strains expressing cecropin A. Bioprocess and Biosystems Engineering 35(5): 761-767.

Ren, X., Kong, Q., Wang, H., et al. (2012b). Biocontrol of fungal decay of citrus fruit by *Pichia pastoris* recombinant strains expressing cecropin A. Food Chemistry 131(3): 796-801.

Rodriguez-Martín, A., Acosta, R., Liddell, S., et al. (2010). Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. Peptides 31(4): 541-547.

Rothstein, D. M., Spacciapoli, P., Tran, L. T., et al. (2001). Anticandida activity is retained in P-113, a 12-amino-acid fragment of histatin 5. Antimicrobial Agents and Chemotherapy 45(5): 1367-1373.

Sagaram, U. S., El-Mounadi, K., Buchko, G. W., et al. (2013). Structural and functional studies of a phosphatidic acid-binding antifungal plant defensin MtDef4: Identification of an RGFRRR motif governing fungal cell entry. PLoS ONE 8(12).

Sagaram, U. S., Pandurangi, R., Kaur, J., et al. (2011). Structure-activity determinants in antifungal plant defensins MsDef1 and MtDef4 with different modes of action against *Fusarium graminearum.* PLoS ONE 6(4).

Schreiber, C., Muller, H., Birrenbach, O., et al. (2017). A high-throughput expression screening platform to optimize the production of antimicrobial peptides. Microbial Cell Factories 16(1): 29.

Schuster, E., Dunn-Coleman, N., Frisvad, J., et al. (2002). On the safety of *Aspergillus niger* - a review. Applied microbiology and biotechnology 59(4): 426-435.

Seibold, M., Wolschann, P., Bodevin, S., et al. (2011). Properties of the bubble protein, a defensin and an abundant component of a fungal exudate. Peptides 32(10): 1989-1995.

Skouri-Gargouri, H., Ben Ali, M. and Gargouri, A. (2009). Molecular cloning, structural analysis and modelling of the AcAFP antifungal peptide from *Aspergillus clavatus*. Peptides 30(10): 1798-1804.

Skouri-Gargouri, H. and Gargouri, A. (2008). First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*. Peptides 29(11): 1871-1877.

Sonderegger, C., Fizil, A., Burtscher, L., et al. (2017). D19S mutation of the cationic, cysteine-rich protein PAF: novel insights into its structural dynamics, thermal unfolding and antifungal function. PLoS ONE 12(1).

Sonderegger, C., Galgoczy, L., Garrigues, S., et al. (2016). A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. Microbial Cell Factories 15(1): 192.

Sun, X., Ruan, R., Lin, L., et al. (2013). Genomewide investigation into DNA elements and ABC transporters involved in imazalil resistance in *Penicillium digitatum*. FEMS Microbiology Letters 348(1): 11-18.

Szappanos, H., Szigeti, G. P., Pal, B., et al. (2006). The antifungal protein AFP secreted by Aspergillus giganteus does not cause detrimental effects on certain mammalian cells. Peptides 27(7): 1717-1725.

Terras, F. R. G., Eggermont, K., Kovaleva, V., et al. (1995). Small cysteine-rich antifungal proteins from radish: their role in host defense. The Plant Cell 7(May): 573-588.

Terras, F. R. G., Torrekens, S., Van Leuven, F., et al. (1993). A new family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* species. FEBS Letters 316(3): 233-240.

Theis, T., Marx, F., Salvenmoser, W., et al. (2005). New insights into the target site and mode of action of the antifungal protein of *Aspergillus giganteus.* Research in Microbiology 156(1): 47-56.

Theis, T., Wedde, M., Meyer, V., et al. (2003). The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization. Antimicrobial Agents and Chemotherapy 47(2): 588-593.

Toth, L., Kele, Z., Borics, A., et al. (2016). NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization. AMB Express 6(1): 75.

Toth, L., Varadi, G., Borics, A., et al. (2018). Anti-candidal activity and functional mapping of recombinant and synthetic *Neosartorya fischeri* Antifungal Protein 2 (NFAP2). Frontiers in Microbiology 9: 393.

Tu, C. Y., Chen, Y. P., Yu, M. C., et al. (2016). Characterization and expression of the antifungal protein from *Monascus pilosus* and its distribution among various *Monascus* species. Journal of Bioscience and Bioengineering.

Turrà, D., Segorbe, D. and Di Pietro, A. (2014). Protein kinases in plant-pathogenic Fungi: Conserved regulators of infection. Annual Review of Phytopathology. 52**:** 267-288**.**

Tuset, J. J. (1987). Podredumbres de los frutos cítricos. Valencia, Generalitat Valenciana, Conselleria d'Agricultura i Pesca.

van der Weerden, N., Bleackley, M. and Anderson, M. (2013). Properties and mechanisms of action of naturally occurring antifungal peptides. Cellular and Molecular Life Sciences: 1-26.

van der Weerden, N. L., Hancock, R. E. W. and Anderson, M. A. (2010). Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process. Journal of Biological Chemistry 285(48): 37513-37520.

van der Weerden, N. L., Lay, F. T. and Anderson, M. A. (2008). The plant defensin, NaD1, enters the cytoplasm of *Fusarium oxysporum* hyphae. Journal of Biological Chemistry 283(21): 14445-14452.

Varadi, G., Toth, G. K., Kele, Z., et al. (2013). Synthesis of PAF, an antifungal protein from P. chrysogenum, by native chemical ligation: native disulfide pattern and fold obtained upon oxidative refolding. Chemistry 19(38): 12684-12692.

Vila, L., Lacadena, V., Fontanet, P., et al. (2001). A protein from the mold *Aspergillus giganteus* is a potent inhibitor of fungal plant pathogens. Molecular Plant-Microbe Interactions 14(11): 1327-1331.

Viñas, I., Usall, J., Teixidó, N., et al. (1998). Biological control of major postharvest pathogens on apple with *Candida sake*. International Journal of Food Microbiology 40(1-2): 9-16.

Viragh, M., Voros, D., Kele, Z., et al. (2014). Production of a defensin-like antifungal protein NFAP from *Neosartorya fischeri* in *Pichia pastoris* and its antifungal activity against filamentous fungal isolates from human infections. Protein Expression and Purification 94: 79-84.

Vriens, K., Cammue, B. P. and Thevissen, K. (2014). Antifungal plant defensins: mechanisms of action and production. Molecules 19(8): 12280-12303.

Wang, J. Y., Sun, X. P., Lin, L. Y., et al. (2012). *PdMfs1*, a major facilitator superfamily transporter from *Penicillium digitatum*, is partially involved in the imazalil-resistance and pathogenicity. African Journal of Microbiology Research 6(1): 95-105.

Wayne, M. J., Wojciech, J. J., Saftner, R. A., et al. (2011). Identification of wild apple germplasm (*Malus spp*.) accessions with resistance to the postharvest decay pathogens *Penicillium expansum* and *Colletotrichum acutatum.* Plant Breeding 130(4): 481-486.

Wilmes, M., Cammue, B. P. A., Sahl, H. G., et al. (2011). Antibiotic activities of host defense peptides: More to it than lipid bilayer perturbation. Natural Product Reports 28(8): 1350-1358.

Williamson, B., Tudzynski, B., Tudzynski, P., et al. (2007). *Botrytis cinerea*: the cause of grey mould disease. Molecular plant pathology 8(5): 561-580.

Wnendt, S., Ulbrich, N. and Stahl, U. (1994). Molecular cloning, sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*. Current Genetics 25(6): 519-523.

Yeung, A. T. Y., Gellatly, S. L. and Hancock, R. E. W. (2011). Multifunctional cationic host defence peptides and their clinical applications. Cellular and Molecular Life Sciences 68(13): 2161.

Yount, N. Y. and Yeaman, M. R. (2004). Multidimensional signatures in antimicrobial peptides. Proceedings of the National Academy of Sciences of the USA 101(19): 7363-7368.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature 415(6870): 389-395.

Zhang, T., Sun, X., Xu, Q., et al. (2013a). The pH signaling transcription factor PacC is required for full virulence in *Penicillium digitatum*. Applied Microbiology and Biotechnology 97: 9087-9098.

Zhang, T., Sun, X., Xu, Q., et al. (2013b). PdSNF1, a sucrose non-fermenting protein kinase gene, is required for *Penicillium digitatum* conidiation and virulence. Applied microbiology and biotechnology 97: 5433-5445.

Zhao, W., Lü, Y., Ouyang, H., et al. (2013). N-Glycosylation of Gel1 or Gel2 is vital for cell wall β-glucan synthesis in *Aspergillus fumigatus*. Glycobiology 23(8): 955-968.