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Characterization of a novel cysteine-rich antifungal protein from *Fusarium graminearum* with activity against maize fungal pathogens

Belén Patiño[a], Covadonga Vázquez[a], James M. Manning[b], María Isabel G. Roncero[c], Dolores Córdoba-Cañero[c], Antonio Di Pietro[c], Álvaro Martínez-del-Pozo[d,*]

[a] Department of Microbiology III, Faculty of Biology, Complutense University of Madrid Jose Antonio Novais 12, 28040 Madrid, Spain
[b] Department of Biology, Northeastern University, 134 Mugar Life Sciences, 360 Huntington Avenue, Boston, MA 02115, USA
[c] Departmento de Genética, Universidad de Córdoba and Campus de Excelencia Agroalimentario (ceiA3), E-14071 Córdoba, Spain
[d] Department of Biochemistry and Molecular Biology, Faculty of Chemistry, Complutense University, 28040 Madrid, Spain

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ABSTRACT

Filamentous fungi are an invaluable source for biocontrol strategies and for production and development of different antifungal polypeptides. Within this context, cysteine-rich antifungal AFP-like peptides stand out among many different antimicrobial compounds given their production easiness, stability, versatility, and efficacy. AFP from *Aspergillus giganteus* represents the hallmark of this still increasing family of antifungal polypeptides. Close *in silico* analyses of the *Fusarium graminearum* genome revealed the presence of an AFP-like peptide, here designated as FgAFP. This new peptide was cloned, produced in the yeast *Pichia pastoris*, and characterized. The results obtained showed its strong and specific antifungal activity against several well-recognized maize pathogens, but inefficacy against *F. oxysporum*, which has not been described as a natural biological competitor of other fungal pathogens assayed. All results together suggest that this small peptide is an important factor for the fungal interplays involved in maize infection and reveals unforeseen potential biotechnological applications for FgAFP in maize production and storage.

1. Introduction

The biological role of antimicrobial peptides and proteins in defense strategies against challenging microbes is being increasingly recognized as a promising tool for the development of new antifungal approaches in agriculture and food storage (Marcos et al., 2008; Montesinos, 2007; Rautenbach et al., 2016). Filamentous fungi are in fact valuable sources for the adoption of biocontrol strategies and the production and development of different antifungal polypeptides (Hegedus and Marx, 2013; Leiter et al., 2017). In this regard, one of the most promising possibilities is based in the use of a family of small, usually basic, highly stable, and cysteine-rich peptides (Garrigues et al., 2015; Marx et al., 2008; Meyer, 2008), secreted by ascomycetes, and best represented by the so-called antifungal protein from *Aspergillus giganteus* or AFP (Jennings et al., 1965; Olson and Goerner, 1965). It is a low molecular weight (51 residues long) polypeptide showing *in vitro* antifungal properties against important plant pathogens, including *Magnaporthe oryzae*, *Fusarium verticillioides*, *Phytophthora infestans*, *Botrytis cinerea*, and *Fusarium oxysporum* (Barakat et al., 2010; Lacadena et al., 1995; Martín-Urdiroz et al., 2009; Martínez-Ruiz et al., 1997; Moreno et al., 2003; Moreno et al., 2006; Vila et al., 2001). Furthermore, transgenic expression of the *afp* gene, either alone or in combination with other antimicrobial genes, has been shown to confer plant disease resistance (Coca et al., 2004; Girgi et al., 2006; Moreno et al., 2005; Narvaez et al., 2018). Other well characterized members of the same family are PAF (Marx et al., 2008) and PgAFP (Rodríguez-Martín et al., 2009) from *Penicillium chrysogenum*, AfpB from *Penicillium digitatum* (Garrigues et al., 2017), AcAFP from *Aspergillus clavatus* (Skouri-Gargouri et al., 2009), AnAFP from *Aspergillus niger* (Paege et al., 2016), and NFAP from *Neosartorya fischeri* (Kovacs et al., 2011; Toth et al., 2016). Several other very similar peptides have been already detected but are still pending of a more detailed characterization (Chen et al., 2013; Wen et al., 2014), including PAF-like orthologues which have been reported to exist in > 10 different *Fusarium* species (Galgoczy et al., 2013). Interestingly, these antifungal proteins do not seem to produce deleterious toxic effects when assayed against mammalian cells (Szappanos et al., 2006; Szappanos et al., 2005). Although all members of the family are closely related in terms of structural features, they show

Abbreviations: AFP, antifungal protein; FgAFP, antifungal protein from *Fusarium graminearum*; GRAS, generally regarded as safe; BMGH, buffered minimal glycerol medium supplemented with histidine; BMMH, buffered minimal methanol medium supplemented with histidine; DTT, D,L-dithiothreitol

* Corresponding author at: Departamento de Bioquímica y Biología Molecular, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain.
E-mail address: alvaromp@quim.ucm.es (Á. Martínez-del-Pozo).

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different functionality and antifungal specificity (Barna et al., 2008; Binder et al., 2011; Binder et al., 2015; Binder et al., 2010a; Binder et al., 2010b; Delgado et al., 2015; Delgado et al., 2016; Delgado et al., 2017; Galgoczy et al., 2017; Galgoczy et al., 2008; Hagen et al., 2007; Leiter et al., 2005; Marcos and Gandia, 2009; Ouedraogo et al., 2011; Szappanos et al., 2006; Szappanos et al., 2005; Viragh et al., 2015). In fact, the mechanism by which most of them exert their activity has been only partially solved and still waits to be completely determined.

The genome of *Fusarium graminearum* was completely sequenced > 10 years ago (Cuomo et al., 2007). Close *in silico* analyses of this sequence revealed the presence of an AFP-like peptide, here designated as FgAFP. This new peptide has been cloned, produced in the GRAS yeast *Pichia pastoris*, and characterized. The results presented show how this FgAFP displays antifungal activity against several well-recognized maize pathogens, but it is ineffective against *F. oxysporum* when assayed in identical conditions, revealing unforeseen potential biotechnological applications in maize production and storage.

2. Materials and methods

2.1. Cloning procedures

The *F. graminearum* genomic fragment containing the complete preproFgAFP sequence (Cuomo et al., 2007), and two introns (385 nucleotides) (Fig. 1), was used as template for PCR amplification of the cDNA coding for the sequence of the mature FgAFP protein (55 amino acids and the stop codon), using an already established methodology previously described for cloning and production of AFP from *A giganteus* (Fig. 2) (López-García et al., 2010; Martínez-Ruiz et al., 1997; Martínez-Ruiz et al., 1998). This amplification was performed in two different steps using four different primers (two of them overlapping and the other two containing extensions for *Xho*I and *Xba*I restriction sites). The final amplified cDNA fragment, already lacking the introns, was in-frame fused to the yeast α-factor signal sequence, for secretion, using the corresponding XhoI and XbaI restriction sites of the *P. pastoris* pPICZαA plasmid. The resulting construction (pPICZαFgAFP), which integrity was verified by nucleotide sequencing (DNA sequencing facility from the Universidad Complutense), was capable of producing a mature FgAFP version, which was secreted to the yeast culture extracellular medium (see next section).

ATG CAG TTC TCA ACT ATC ATT CCT CTC TTT GTT GCC 36

GCC ATG GGC GTC GTA GCT ACC CCC GTC AAC TCT CCA 72

GCT CAA GAG CTT GAT GCC AGA GGC AAC CTA TTC CCT 108

CGT CTG GAA TAC TGG GGA 126

GTAAGTTCAATCTCTAATGTCTTGAGGGAATATAAACTAAC

167 CACATTCTAG AAA TGC ACC AAG GCC GAG AAC CGA 201

TGC AGG TAC AAG AAC GAT AAG GGT AAA GAC GTT CTT 237

CAG AAT TGC CCC AAG TTT GAC AAC AAA AAG 267

GTACAGCTTCACAAGGTTCCGAAAAAGTACCCATGCTAACT

308 ACTGTTTTCTATAG TGT ACC AAG GAT GGT AAC AGC

TGC 346 AAG TGG GAC AGT GCT TCA AAA GCG CTC ACC TGC

TAC 382 TAA 385

Fig. 1. Genomic sequence of *F. graminearum fgafp*. Intron regions appear in red while the segment corresponding to the pre- and pro-sequences are black. The sequence corresponding to the mature polypeptide cloned and characterized along this work is shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MQFSTIPLFVAMGVATPVNSPAQELDARGNLFPRLEYWG

KCTKAENRCRYKNDKGKDVLQNCPKFDNKCTKDGNSCK

WDSASKALTCY

PREPRO-AFP

MKFVSLASLGFALVALGAVATPVEADSLTAGLDARDES

AVLATYNGKCYKDNICKYKAQSGKTAICKCYVKCPRDG

AKCEFDSYKGKCYC

Fig. 2. Amino acid sequences of prepro-FgAFP and prepro-AFP (Martínez-Ruiz et al., 1997) polypeptides. The sequences of the final mature active proteins appear in blue. The four amino acid residues in red correspond to a very well-known fungal motif for signal peptide proteolytic cleavage, by a putative Kex2p-like endopeptidase (Martínez-Ruiz et al., 1998), in order to process the prepro-proteins to their pro-forms. Cys residues appear underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Protein production and purification

In order to produce the recombinant version of FgAFP in *P. pastoris*, plasmid (1 μg) pPICZαFgAFP was digested with *Sac* I and used to transform KM71 cells by electroporation on a Bio-Rad Gene-Pulser apparatus as described (López-García et al., 2010; Martínez-Ruiz et al., 1997; Martínez-Ruiz et al., 1998). The pPICZαA plasmid contains a gene that confers resistance to the antibiotic zeocin. Thus, after the pulse cells were immediately diluted with 1 ml of cold 1 M sorbitol and plated on YPD (1% yeast extract, 2% peptone and 2% dextrose) medium containing 1 M sorbitol and different amounts of zeocin (100–1500 μg/ml). Incubation at 30 °C was performed until colonies appeared (4 days). Then, the best producing colony and the optimum incubation time were selected by small-scale production experiments of ten individual zeocin resistant colonies and SDS–PAGE analysis of the extracellular media at different times of culture (up to 96 h). This best producing colony was cultured for 2 days in buffered minimal glycerol medium supplemented with histidine (BMGH). The resulting cells were pelleted and used for large-scale production of recombinant FgAFP in 200 ml of buffered minimal methanol medium supplemented with histidine (BMMH). Glycerol acts as a repressor of the AOX1 gene while methanol, which is replenished every 12 h during the incubation, is a strong inducer of its expression. This final incubation in BMMH was carried out at 30 °C for 3 days (optimum time selected) with strong aeration. Then, the extracellular medium was obtained by centrifugation and used to purify the recombinant proteins essentially as described before for AFP (Álvarez-García et al., 2009b; Lacadena et al., 1995; López-García et al., 2010; Martínez-Ruiz et al., 1998). After dialysis of the extracellular medium against 50 mM sodium phosphate, pH 7.0, the protein was retained on a cation exchange column of Amberlite IRC 50, equilibrated in the same buffer. The column was washed with 0.2 M NaCl and the highly basic recombinant antifungal peptide was eluted with a 0.6 M solution of the same salt. These fractions, containing only, FgAFP were thoroughly dialyzed against water and lyophilized.

2.3. Characterization of the purified protein

SDS–PAGE, protein hydrolysis and amino acid analyses were carried out as previously described (Álvarez-García et al., 2009a; García-Ortega et al., 2005; Lacadena et al., 1995; Martínez-Ruiz et al., 1997; Martínez-Ruiz et al., 1998). Mass-spectrometry analysis was made as described before (Álvarez-García et al., 2009a, 2009b; García-Ortega et al., 2005;