Aplicaciones biotecnológicas del gen *afp* (Antifungal Protein) de *Aspergillus giganteus* para la protección de plantas frente a infección por patógenos

" Antifungal mechanism of the *Aspergillus giganteus* AFP protein against the rice blast fungus *Magnaporthe grisea*"

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Resumen

Se ha descrito que la proteína antifúngica AFP de *Aspergillus giganteus* presenta actividad antifúngica frente a varios hongos patogénicos económicamente importantes. En este estudio se investiga el mecanismo de acción de la AFP frente a *Magnaporthe grisea*, el agente causador de la piriculariosis en arroz. Mediante un ensayo basado en la entrada del colorante sytox green en las células, se demuestra la capacidad de AFP para producir permeabilización de la membrana del hongo. Sin embargo, la AFP no provoca la permeabilización en membranas de células HeLa. Estudios de microscopía electrónica de transmisión revelaron importantes alteraciones estructurales y daños en la membrana plasmática de las células del hongo tratadas con AFP. A través de ensayos de colocalización con AFP marcada con el fluorocromo Alexa y sytox green, se ha visto que la proteína AFP entra en las células del hongo y se localiza en el núcleo. Además, ensayos de retardo en gel confirmaron que la AFP se une a ácidos nucleicos, incluyendo DNA genómico de *M. grisea*. En conjunto, estos resultados sugieren que la combinación de la permeabilización celular del hongo, la capacidad de penetración, el direccionamiento al núcleo y la capacidad de unirse a ácidos nucleicos determina su potente actividad antifúngica frente al hongo *M. grisea*, responsable de la piriculariosis en arroz.

Research Article

Antifungal mechanism of the *Aspergillus giganteus* AFP protein against the rice blast fungus *Magnaporthe grisea*.

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Summary.

The *Aspergillus giganteus* antifungal protein (AFP) has been reported to display antifungal activity against various economically important fungal pathogens. In this study the mechanism of action of AFP against *Magnaporthe grisea*, the causal agent of the rice blast disease, was investigated. Fungal membrane permeabilization induced by AFP was demonstrated by using an assay based on the uptake of SYTOX Green. AFP, however, failed to induce membrane permeabilization on rice or HeLa cells. Electron microscopy studies revealed severe cellular degradation and damage of plasma membranes in AFP-treated fungal cells. AFP was found to enter the fungal cell and target to the nucleus, as revealed by co-localization experiments of Alexa-labeled AFP with SYTOX Green. Moreover, gel-retardation experiments confirmed that AFP binds nucleic acids, including *M. grisea* genomic DNA. Together, our results suggest that the combination of fungal cell permeabilization, cell-penetrating ability and nucleic acid-binding activity of AFP determines its potent antifungal activity against the rice blast fungus *M. grisea.*

1. Introduction

Antimicrobial proteins and peptides are now recognized as an important component of nonspecific host defense systems (also termed innate immunity) in insects, amphibians, plants and mammals [1-4]. Several applications of natural occurring antimicrobial proteins have been proposed. Thus, they represent a source of clinically useful therapeutics, as well as an attractive alternative for crop protection through the use of transgenic plants [2, 5, 6].

Antimicrobial proteins are diverse in structure and display a broad spectrum of antibacterial and antifungal activities. Membrane permeabilization as a consequence of membrane interaction and pore-forming activities has been described for many antimicrobial proteins and peptides [3, 7]. Although membrane-acting proteins and peptides are extremely diverse as regards of their primary and secondary structure, they share a common feature, namely a positive net charge at physiological conditions that facilitates interaction with negatively charged microbial surfaces. Moreover, a broad range of small, membrane-acting, cationic proteins, adopt amphipathic structures which allow their incorporation into microbial membranes (i.e., insect cecropins). A different mode of action has been proposed for defensins, antimicrobial proteins isolated from various plant species [8, 9]. Thus, it has been shown that a defensin from dahlia, the Dm-AMP1 protein, interacts with a specific sphingolipid of the fungal plasma membrane [10].

Fungi are known to produce a variety of proteins with interesting biological actions. Fungal proteins that have been purified and characterized include ribosome inactivating proteins (RIPs), antifungal proteins, ribonucleases, lectins, cellulases, and xylanases, among others [11]. Most of these proteins are thought to play a role in the host defense against microorganisms from the environment. Antifungal proteins produced by fungi hold broad interest because of their potential use as defense factors for crop protection against fungal pathogens. The best known examples are represented by the fungal cell-wall degrading enzymes, i.e. chitinases and glucanases, produced by the biocontrol agent *Trichoderma* spp. [12]. When expressed in transgenic plants, these hydrolytic enzymes confer resistance to phytopathogens [13]. Moreover, RIP proteins, a family of proteins produced by many fungi, inhibit protein synthesis by inactivating ribosomes [14, 15]. RIP proteins have, for quite some time, been known for their selective killing of tumor cells compared to normal cells, and their potential for cancer immunotherapy using immunotoxins [16].

The mould *Aspergillus giganteus*, isolated from the soil of a farm in Michigan, WI, produces two major extracellular proteins. One of them, α-sarcin, is a RIP protein displaying ribonucleolytic activity [17]. α-sarcin is the most representative member of a protein family known as fungal ribotoxins [18, 19]. The other one is a highly basic and small protein (51 amino acids), which was reported to inhibit the growth of filamentous fungi, the antifungal

AFP protein [20, 21]. This protein was accidentally discovered during an anticancer screening program [20]. Further analysis of the antifungal properties of *Aspergillus* AFP revealed that this protein is a potent inhibitor of plant pathogens, including several agronomically important phytopathogens [22-24]. At present, the exact mechanism by which AFP exerts its antifungal activity against plant fungal pathogens is, however, unkwown.

The AFP protein has been throughly characterized from the structural point of view [21, 25]. Its three-dimensional structure, a small and compact β-barrel composed of five highly twisted antiparallel β-strands, highly resembles that of the plant defensins and γ-thionins. Additionally, the three-dimensional structure of AFP displays the characteristic features of the oligonucleotide/oligosaccharide binding (OB fold) structural motif. The observed similarities at the structural level between AFP and OB fold-containing proteins correlates well with its ability to bind nucleic acids, as judged by its *in vitro* interaction with DNA [26]. Binding of AFP promotes charge neutralization and condensation of DNA. In other studies, it was also reported that AFP interacts with phospholipid membranes [21].

In this work, we examined the mechanism by which AFP exerts its antifungal activity against *Magnaporthe grisea* (anamorph *Pyricularia grisea*). *M. grisea* is the causal agent of rice blast, one of the most devastating diseases of cultivated rice worldwide [27]. Fluorescent microscopy studies with a membrane impermeant dye indicated that fungal cytoplasmic membranes were compromised in AFP treated-*M. grisea* cultures. Whereas AFP causes permeabilization of fungal cells, neither rice nor human HeLa cells were affected by AFP. Electron microscopical observations revealed that treatment of fungal cells with AFP induced significant morphological and ultrastructural changes which were accompanied by damage of the cytoplasmic membrane. By labelling the protein with Alexa-568 we determined that AFP enters the fungal cells and targets to the nucleus. We also investigated the binding properties of AFP on nucleic acids, including binding to *M. grisea* DNA.

2. Materials and Methods

2.1. Antifungal activity assays

AFP was purified from the extracellular medium of *A. giganteus* MDP18894 culture as described by Martínez-Ruiz et al. [21]. Homogeneity of the protein preparation was confirmed by SDS-PAGE and amino acid composition analysis, as well as by its spectroscopical features fungal cultures in 96-well microtiter plates [22]. The microtiter well plate assay determines the AFP protein concentration required for inhibition of fungal growth. In microplate wells, 150 µl of PDB (potato dextrose broth; DIFCO, Detroit, MI) was mixed with 50 µl of the spore suspension (at the concentration of 1 x 10^6 spores/ml). M. grisea spores were allowed to pre-germinate for 6 h at 28ºC and the absorbance was determined (OD 595 nm). Purified AFP solutions were then added to the pre-germinated conidia to the desired final concentrations. The microtiter plates were incubated for 16 h at 28ºC and the absorbance was read. Controls with BSA (10 µM), or with nystatin (0.1µg/µl) were performed.

2.2. Hyphal membrane permeabilization assay

Membrane permeabilization was measured by uptake of SYTOX Green, a high affinity nuclear stain that penetrates cells with compromised membranes and fluoresces upon binding to nucleic acids [8, 29]. Fungal cultures were grown in the absence or in the presence of AFP as described for the antifungal bioassay. SYTOX green (Molecular Probes, www.probes.com) was then added to the fungal cultures (0.2 µM final concentration). After incubation for 10 min, fungal cells were analyzed by confocal laser scanning microscopy with a Leica TCS SP microscope (Heidelberg, Germany). For detection of SYTOX green uptake an excitation wavelength of 488 nm and an emission wavelengh of 500 to 554 nm was used. To obtain compromised membranes under control conditions, the fungal structures were incubated in 70% ethanol for 10 min at room temperature, washed in PDB and then stained with SYTOX [30].

2.3. Permeabilization assays with plant and mammalian cells

We examined the ability for AFP to permeabilize plant cells, by culturing rice protoplasts in the presence of increasing concentrations of AFP and studying the influx of SYTOX Green in AFP-treated protoplasts. Protoplasts were prepared from calli of the *japonica* rice (*Oryza sativa* L. cv. Senia) by overnight enzyme digestion following the protocol described by Marcotte et al. [31]. Protoplast density was adjusted to 1.6x10^6 protoplasts per ml per tube,

and the AFP was slowly added to the protoplast suspension to the required final concentration of AFP (4, 12 and 24 μM). Protoplasts were incubated with AFP at 28ºC in the dark for 18h before being used for the SYTOX green uptake assay. Controls with no AFP or with protoplasts that had been mechanically damaged by vigorous pipeting, frozen and then subjected to the SYTOX Green assay were also carried out. Three independent experiments were performed.

The SYTOX Green uptake assay was also used to test membrane integrity of cultured human cells that had been treated with AFP. HeLa cells (human cervix carcinoma cell line, 10^5 cells) were cultured in Dulbecco´s modified Eagle´s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin-streptomycin (10^4 units of penicillin per ml, 10 mg/ml of streptomycin) for 24 h in 24-well NUNC plates at 37ºC in 5% CO_2. AFP was added at different concentrations (4, 6, 8 and 12 μM) and the cells were incubated overnight. The cells were washed twice with 1xPBS buffer before addition of the SYTOX dye. The plates were incubated at room temperature for 15 min with slow stirring in the dark. Finally, cells were fixed with 4% paraformaldehyde in 1xPBS (0.05 M Na-phosphate, pH 7.5, 0.15 M NaCl), for 10 min at room temperature, washed twice with 1xPBS and allowed to dry in the dark before microscopical observations. As a positive control, HeLa cells were treated with 1%Triton X-100 for 15 min.

2.4. AFP localization studies

The interaction of AFP with *M. grisea* was studied by using fluorescently-labelled AFP protein. The protein was labeled with Alexa-568 (Molecular Probes, www.probes.com) according to the manufacturer´s instructions and stored at 4ºC in 0.1% NaN_3 until use. Fluorescent images of Alexa-labeled AFP fungal cultures were obtained with an excitation wavelength of 577 nm and an emission wavelength of 603 nm.

2.5. Congo red Staining

Congo red staining was used to allow visualization of chitin deposition at hyphal tips of *M. grisea.* Pregermination and treatment of fungal cultures with AFP was performed in 96-well microtiter plates as described above. Following incubation with AFP, Congo red was added to the fungal cultures to a final concentration of 1 mM. After 10 min, the fluorescence was viewed by confocal microscopy (excitation wavelength was 543 nm; emission wavelength 560 to 635). Growth at the tip was determined by lack of Congo red staining at the hyphal tip, whereas hyphae with arrested growth had Congo red staining across the series of optical sections at the hyphal tip [32].

2.6. Transmission electron microscopy

For transmission electron microscopy (TEM), the fungus was grown in 96-well plates as described above. AFP was then added to a final concentration of 50 nM. Controls without AFP were also made. Fungal cultures were fixed overnight at 4ºC in 1.5% glutaraldehyde and processed for TEM by using an agar bubble to enclose the sample [33]. After washing with cacodylate buffer (0.1 M sodium cacodylate, pH 7.5) 4 times, 10 min each, samples were post-fixed in osmium tetroxide in the same buffer (1% osmium tetroxide, 0.8% potassium ferrocyanide) for 3h at room temperature. They were washed three times in buffer and dehydrated in a standard acetone series, and embedded in Spurr resin. Sections (80 nm thick) were mounted on regular copper grids and stained with 2% uranyl acetate and lead citrate. Observations were carried out using a H600AB Hitachi transmission electron microscope (Hitachi, Tokio, Japan).

2.7. DNA and RNA binding assays

The ability of AFP to bind nucleic acids, DNA and RNA, was determined by analyzing the electrophoretic mobility of nucleic acids in the presence of AFP. Initially, the DNA binding properties of AFP were examined using *M. grisea* genomic DNA. Towards this end, fungal mycelium was collected from a liquid culture of *M. grisea* by filtration, lyophilized and pulverized with liquid nitrogen. For preparation of genomic DNA, the procedure described by Murray and Thompson [34] was used but with MATAB (0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG 6000, 0.5% sodium sulphite) as the extraction buffer.

To obtain the low molecular weight DNA fraction, *M. grisea* genomic DNA was digested to completion with restriction enzymes (PstI, EcoRI, Hind III and BamHI) and separated by electrophoresis on agarose gel. DNA fragments below 340 bp were purified from the gel (QIAquick gel extraction System, Qiagen). The size-fractionated *M. grisea* genomic DNA was used for binding assays.

These studies were also performed using short, randomly selected single-strand (ssDNA) and double-stranded (dsDNA) DNA fragments. The DNA oligonucleotides were chemically synthesized on an Applied Biosystems 394 DNA synthesizer. Oligonucleotides were prepared with the last dimethoxytrityl (DMT) group at the 5'-end in order to facilitate reverse-phase purification which was performed as described by Iacopino et al. [35]. The length and homogeneity of the purified oligonucleotides was checked by denaturing polyacrylamide gel electrophoresis. The oligonucleotide sequences were as follows: EL7, ssDNA (100-mer, 5´-TCCACTATTAAAGAACGTGAACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCAC TACGTGAACCATCACCCTAATCAAGTTTTTTGG-3´); EL3, ssDNA (100-mer, 5´ TGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGCCATCGCCCT

GATAGACGGTTTTTCGCCCTTTGACGTTGGAGT-3´); and EL3/EL7, dsDNA (EL3 and EL7 displayed complementary sequences and were used to obtain de double-stranded DNA by mixing an equimolar mixture of both oligonucleotides, boiling and then cooling down to room temperature). Another dsDNA, the EL2A fragment, was prepared. For this, two long complementary oligonucleotides, the EL2A-U (76-mer, 5´-GCGCATGCCAACTGTTGAACTTCGACCTTCTTAAGCTTGCGGGAGACGTCGAGTCCAACCCAGGCCC CGGATCCCG-3´) and the EL2A-L (5´-CGGGATCCGGGGCCTGGGTTGGACTCGACGTCTCCCGCAAGCTTAAGAAGGTCGAAGTTCAACAGT TGGCATGCGC-3´) were chemically synthesized and assembled as described above.

Gel retardation experiments were performed by mixing DNA with increasing amounts of AFP in Tris-acetate, pH 7.0, 1 mM EDTA in a total volume of 20 µl. The final concentration of each DNA in each assay was 24 ng/µl for each oligonucleotide, and 16 ng/µl for genomic DNA fragments). Assays to determine the DNA binding capacity of AFP were also carried out in the same binding buffer supplemented with either $MgCl_2$ or $MgSO_4$ (0.05 M each salt). Samples were incubated at room temperature for 10 min. Subsequently, 4 µl of native loading buffer was added (Tris-HCl 0.05M, pH 8.0, 0.5M EDTA containing 0.25% bromophenol blue, 0.25% xylen cyanol and 30% glycerol) and analyzed by electrophoresis on a 0.8% agarose gel in 0.5xTris borate-EDTA buffer.

Analysis of RNA binding activity was carried out by incubating tRNA from yeast (Roche) with various amounts of AFP followed by agarose gel electrophoresis as described above.

2.8. Study of specific RNase activity

The possible specific ribonuclease activity (ribosome inactivating activity) of AFP was investigated by using a cell-free rabbit reticulocyte lysate (Promega) as substrate [36]. α-Sarcin was used as a positive control. The lysate (20 µl) was treated with 1 µg of AFP, or α-Sarcin (Sigma), in 70 µl of buffer (40 mM Tris-HCl, pH 7.5, 40 mM KCl, 10 mM EDTA) for 30 min at 30ºC. The reaction was stopped by the addition of 130 µl of 0.5% SDS in 50 mM Tris-HCl, pH 7.5. Ribosomal RNA was then extracted from the reaction mixtures using 0.5% SDS and phenol, and precipitated with 2.5 volumes of ethanol. Then, this RNA fraction was incubated on ice for 30 min with freshly prepared 1M aniline acetate (pH 4.5, 20 µl), and precipitated with ethanol. The release of the 400-nt α-fragment from the 28S RNA of the eukaryotic ribosomes was monitored by electrophoresis on 1.5% formaldehyde-containing agarose gels and visualized by ethidium bromide staining.

3. Results

3.1. Fluorescence staining of AFP-treated M. grisea hyphae

The ability of AFP to inhibit the growth of the rice blast fungus *M. grisea* was previously reported by our group [22]. The concentrations required for 50% inhibition (IC_{50}) and for complete inhibition of fungal growth (MIC) were found to be 50 nM and 4 μM, respectively [22]. In the present work, the mechanism of inhibition of *M. grisea* mediated by AFP was investigated. First of all, we evaluated membrane damage or permeabilization of *M. grisea* cells exposed to AFP by using the SYTOX Green uptake assay and confocal microscopy. SYTOX Green is a cytotoxicity indicator that fluoresces upon interaction with nucleic acids and penetrates only cells with leaky plasma membranes. Results obtained in experiments of SYTOX Green uptake in *M. grisea* cultures are presented in Figure 1A. No fluorescence was observed in the fungal nuclear structures when *M. grisea* was grown in the absence of AFP and then incubated with SYTOX green (Fig. 1A, a - d). In fungal cultures grown in the presence of AFP at concentrations of 4μM (MIC value), however, the dye entered the nucleus and complexed with the DNA of fungal cells (Fig. 1A, e - f). Similar results were observed in fungal cultures treated with AFP at higher concentrations (up to 12 μM AFP; results not shown). In ethanol-treated cultures, SYTOX Green penetrated fungal cells and stained the nuclear structure (results not shown).

Next, the capacity for AFP to cause membrane permeabilization in plant and mammalian cells was investigated. Rice protoplasts were prepared from the commercial rice variety Senia and then incubated with AFP at concentrations lethal to *M. grisea* (4, 12 and 24 μM of AFP). SYTOX Green permeabilization was assessed in control, untreated and in the AFP-treated rice cells. As it is shown in Figure 1B (a, b), SYTOX Green did not penetrate control, untreated rice cells. Interestingly, no membrane permeabilization could be detected in rice cells that had been incubated with AFP up to a concentration of 24 μM (Fig. 1B, c, d). Protoplasts that had been mechanically damaged and then subjected to SYTOX Green staining depicted fluorescent nuclei, indicating that the plant cells had been compromised (Fig. 1B, e, f). These observations suggest that the plasma membrane of the rice protoplasts treated with AFP has not been damaged and the dye has not entered the cells.

AFP effect on membrane integrity of HeLa cells was also examined. Results are presented in Figure 1C. In the absence of AFP, about 1% of the cultured cells showed fluorescence. In the presence of AFP added at concentrations up to 12 μM, only 6% of the HeLa cells were affected, as judged by SYTOX Green staining. Maximal permeabilization was obtained by using 1% Triton X-100.

Together, results obtained in experiments using the SYTOX Green uptake assay and confocal microscopy allowed us to conclude that inhibition of fungal growth by AFP is related

to membrane permeabilization of fungal cells. Although AFP efficiently permeabilizes fungal cells, it has no significant effect on plant and mammalian cells (up to concentrations of 24 and 8 μM, respectively, the highest concentrations used in this work).

To get more insight into the mechanism of fungal growth inhibition we used the selective stain Congo red. Congo red, a dye which exhibits a strong affinity for β-glucans, binds to the chitin in fungal cell walls and allows to distinguish growing hyphae from non-growing hyphae [32]. Areas with active hyphal growth have little chitin deposition at their tips and accordingly have reduced Congo red staining. In contrast, Congo red staining increases at the non-growing hyphal tips, when cell growth is inhibited. Results obtained by Congo red staining of control and AFP-treated *M. grisea* cultures are shown in Figure 2. When compared with control cultures, *M. grisea* cultures treated with AFP exhibited strong Congo red staining at the tips of hyphae (fig. 2B). The Congo red staining patterns can be clearly seen in individual confocal sections (Fig. 2A and B, panels e). These results suggest that hyphal growth is arrested in fungal cultures that have been incubated with AFP.

3.2. Ultrastructural analysis of AFP-treated fungal cultures

A more detailed analysis of the effect of AFP on fungal cells was performed by transmission electron microscopy (Figure 3). Hyphae growing in PDB medium in the absence of AFP showed a dense cytoplasm adhering to the plasma membrane and cell wall (Fig. 3A, B). In fungal cultures that had been treated with AFP at low nanomolar concentrations (50 nM, IC_{50} value), hyphae with abnormal shapes were frequently observed. Fungal cells showed various degrees of cell alteration, including retraction or distortion of the plasma membranes (Fig. 3C-G). A pronounced disorganization of the cytoplasm, involution of the vacuole and invaginations of the plasma membranes, as well as the formation of polymorphic vesicles were detected. A close examination of the AFP-treated *M. grisea* cells allowed us to observe that the integrity of plasma membrane of AFP-treated cells was disrupted. Thus, specific sites of membrane damage and pore formation were observed in *M. grisea* cells treated with AFP (Fig. 3D ,G).

3.3. Intracellular target site of AFP

The observation of AFP-induced permeabilization and pore formation in the plasma membrane of fungal cells prompted us to investigate the capacity for AFP to enter fungal cells. Towards this end, the AFP protein was fluorescently labeled with Alexa-568 and subsequently used in antifungal assays. As labeling of AFP with Alexa could have a negative effect on the activity of AFP, the antifungal properties of the Alexa-labeled AFP protein were

tested and compared to that of AFP. The activity of the Alexa-labeled AFP was similar to that of the unlabeled AFP (results not shown). Next, confocal laser scanning microscopy was used to monitor the localization of fluorescence in *M. grisea* cultures grown in the presence of the Alexa-labeled AFP. Optical sectioning of fungal cells revealed intracellular fluorescence in cultures treated with Alexa-labeled AFP, indicating that the protein molecules were internalized (Fig. 4A). Fungal cultures incubated with only the Alexa-568 dye showed no fluorescence (results not shown). Equally, no fluorescence was detected in *M. grisea* cultures grown in the presence of unlabeled AFP and incubated with Alexa-568 dye.

Intracellular localization of the fluorescent AFP protein was further investigated by co-localization experiments. For this, SYTOX Green staining of the fungal cultures that had been treated with Alexa-labeled AFP was performed (Fig. 4B). Accumulation of Alexa-AFP in the nucleus of fungal cells (red), and its co-localization with the DNA-complexed SYTOX Green (green) is shown Figure 4 (C, E). In fungal cultures treated with Alexa-AFP, fluorescence that spread throughout at the cell surface along *M. grisea* hyphae could also be detected, suggesting AFP accumulation at the cell periphery (Fig. 4E). Thus, results obtained by co-localization experiments allow us to conclude that the Alexa-labeled AFP penetrated the fungal cell and targeted to the nucleus.

3.4. In vitro nucleic acid binding of AFP

The binding properties of AFP on nucleic acids were investigated. Towards this end, the effect of the protein on the electrophoretic behaviour of DNA was assayed. Results are presented in Figure 5. These studies were carried out using genomic DNA obtained from *M. grisea* cells. The *M. grisea* genomic DNA was digested to completion with restriction enzymes and separated by agarose gel electrophoresis (Fig. 5A, left panel). The DNA fraction selected for experiments of binding of AFP was that containing 340 bp and below. The size-fractionated fungal DNA was then incubated with increasing amounts of AFP and the mixtures run on agarose gel electrophoresis under non denaturing conditions (Figure 5A, right panel). Analysis of the electrophoretic mobility of *M. grisea* DNA at the various weight ratios of AFP to DNA revealed that the fraction of free DNA decreased as the amount of AFP increased. Charge neutralization caused by AFP binding to the DNA results in retention of DNA in the well (Fig 5A, lanes 4 to 7).

Binding assays of AFP to polydeoxynucleotides, either single-stranded (ssDNAs) or double-stranded (dsDNAs) sequences were also performed (Figure 5B, C). AFP reduced the electrophoretic of either ssDNAs (Fig. 5B, EL3 and EL7) or dsDNAs (EL2A) (Fig. 5C, lanes 1-7). These findings indicated that AFP binds to polydeoxynucleotides of random sequences under single strand and double-strand conformations. Finally, the binding properties of AFP

to DNA were compromised when salts were present in the binding assay buffer. In binding assays of AFP to the EL2A DNA with no salts added to the buffer, no free DNA was observed at an AFP/DNA molar ratio of 0.1 (Fig. 5C, lane 9). At the same AFP/DNA molar ratio, however, no complexes are formed when either $MgCl_2$ (Fig. 5C, lane 11), or $MgSO_4$ (Fig. 5C, lane 13) is present in the binding buffer. Free to total DNA ratios were calculated from the image analysis of agarose gel electrophoresis retardation experiments of AFP to ss- and ds-DNAs (Fig. 5D). AFP exhibited the highest affinity for the dsDNA, as no free DNA was observed when a AFP/dsDNA molar ratio of 0.1 was used. Together, results obtained in gel retardation experiments indicated that AFP efficiently binds to DNA, including *M. grisea* DNA. AFP exhibits binding properties towards both single-strand and double-strand DNAs.

Gel retardation assays were also carried out to examine the RNA binding ability of AFP. As it is shown in Figure 6A, retardation of the electrophoretic mobility of tRNAs was observed indicating that in addition to DNA, AFP also binds to RNA. Knowing that AFP binds to RNA, it was of interest to investigate whether AFP displays its toxic action by exerting ribonucleolytic activity on ribosomal RNA as it is the case for the α-sarcin protein, a RIP protein which is also produced by *A. giganteus* [37]. α-sarcin hydrolyses a single phophodiester bond in the 3´ portion of the rRNA in the large ribosomal subunit, resulting in the characteristic α-fragment of approx. 400 nucleotides. In this work, a cell-free rabbit reticulocyte lysate was incubated with α-sarcin and subsequently analyzed on agarose gels. The expected α-fragment was generated in the α-sarcin-treated samples (Fig. 6B, lane 4). The size of 28 S rRNA decreased due to the release of the α-fragment. Under the same experimental conditions, however, ribosomal RNA was not affected by AFP as the production of the α-fragment was not detected (Fig. 6B, lane 3). These results suggest that AFP has no ribonucleolytic activity on ribosomal RNA.

4. Discussion

The purpose of this study was to investigate the mode of action of the *Aspergillus* AFP protein against the rice blast fungus *M. grisea*, as well as to determine the susceptibility of plant and human cells to AFP. Results here presented suggest that AFP induces membrane permeabilization of fungal cells, as judged by the SYTOX Green uptake assays. Definitive prove of the AFP-induced cellular damage and membrane pore formation came by our electron microscopical observations of AFP-treated fungal cultures. Together, these studies suggest that AFP exerts its antifungal activity against *M. grisea* by mechanisms involving initial disruption of plasma membrane and membrane permeabilization of the fungal cells. Moreover, examination of AFP-treated fungal cultures by Congo red staining revealed a

significant chitin deposition at the hyphal tips, reflecting no hyphal growth. Thus, membrane permeabilization activity correlated with growth inhibition, suggesting a link of mechanism of action for permeabilization of fungal cells and hyphal growth inhibition.

The use of AFP as antifungal agent for protection of plants against fungal pathogens, either by direct application of AFP or by expression of the *afp* gene in transgenic plants, raises the issue of its potential toxicity to plant cells. It is of interest to note that AFP was not able to permeabilize rice cells, not even at concentrations sixfold higher than the MIC value for *M. grisea* cells. Since the IC_{50} of AFP against the rice blast fungus *M. grisea* was found to be 50 nM, there is a wide range of concentrations at which AFP would kill the pathogen with no harm to the plant cells. A direct evidence that AFP does not harm rice cells comes from results obtained by transgenic expression of the *afp* gene in rice plants. No effect on plant morphology was observed in the *afp*-expressing rice lines [38, 39].

On the other hand, some antifungal proteins that were initially described as membrane-interacting proteins actually exhibit their antifungal activity upon interaction with intracellular targets. For example, the antimicrobial peptide from the Asian toad *Bufo bufo garagriozans* buforin II has been reported to kill microorganisms by penetrating the cell membrane and binding to DNA and RNA of the cells [40]. The target site of histatin 5, an antifungal protein from human saliva has been shown to be the mitochondrion [41]. In this work, the AFP protein was labelled with Alexa 568 and the target site of AFP in the fungal cell was examined by confocal microscopy. Labeled AFP distributed uniformly in the periphery of *M. grisea* hyphae supporting that the initial interaction with the fungal cell takes place at the outer layers. But most importantly, labelled AFP was found to enter the fungal cell and to localize to the nucleus. Together, results here presented demonstrated that AFP displays antifungal activity against the pathogen *M. grisea* through a mechanism that involves first permeabilization and pore formation at the plasma membrane followed by internalization of the AFP protein into the fungal cell.

The observation that Alexa-labeled AFP protein accumulated in the nucleus of fungal cells, prompted us to investigate the binding properties of AFP on nucleic acids. In this respect, Martínez del Pozo et al. [26] reported that the AFP protein is an oligonucleotide/oligosaccharide binding (OB) fold-containing protein. By using different experimental approaches (ethidium bromide displacement, protein fluorescence emission and circular dichroism), these authors demonstrated that interaction of AFP with DNA promotes charge neutralization and condensation of DNA (calf thymus DNA, bacteriophage F1 ssDNA, and 27-mer deoxyoligonucleotides were used in these studies). Results here presented further support that AFP interacts with DNA, more particularly with *M. grisea* DNA. Thus, we show that AFP binds either to both double-stranded and single-stranded DNAs. We also show that the nucleotide sequence itself is not significant for the interaction of AFP to

DNA, no specific nucleotide sequence being required for this interaction. When the effect of high salt concentrations in AFP-DNA binding experiments was evaluated, its DNA binding activity was notably affected. This observation points to the involvement of ionic interactions in AFP-DNA binding. It is worth to recall that, the AFP protein is a highly basic protein (pI 10.65) with high positive net charge at neutral pH. In addition to its three-dimensional structure, the cationic character of AFP might well explain the non-specific, binding properties of AFP to nucleic acids. These findings also indicate that cell permeabilization properties and nucleic acid binding activity can coexist within a single peptide. In this respect, other acidic phospholipids-interacting proteins such as the Dna A protein or even α-sarcin, have been reported to bind nucleic acids [42-43].

AFP is synthesized by the producing *A. giganteus* simultaneously with the ribotoxin α-sarcin, the cytotoxicity of α-sarcin arising from its ribonuclease activity on 28S ribosomal RNA [43]. Taking into account that AFP was found to bind not only DNA but also RNA, the possibility that ribosomes could be an intracellular target of AFP was considered. However, when the possible specific ribosome-inactivating activity (RIP activity) of AFP was investigated using a cell-free rabbit reticulocyte lysate, AFP did not display it, under the same experimental conditions as α-sarcin did.

In other studies, fluorescein isothiocyanate-labeled AFP protein was found to localize at the plasma membrane of the AFP-sensitive fungus *Aspergillus niger*, whereas it was mainly detected inside the cells of the AFP-resistant fungus *Penicillium chrysogenum* [45]. These authors also reported the accumulation of AFP at distinct areas within the cell wall of the AFP-sensitive fungus *A. niger* while some AFP was observed in the cytoplasm of *P. chrysogenum*, as judged by immunoelectron microscopy. However, no nuclear localization of AFP was detected in the AFP-treated *A. niger* and *P. chrysogenum* cells [45]. During the course of the work herein presented, immunoelectron microscopy experiments were also performed in AFP-treated fungal cultures. In this case, intense immunolabeling with the anti-AFP antiserum could also be observed in the fungal cell wall (results not shown). Immunolabeling of the AFP-treated fungal cells, however, did not significantly differ from the non specific immunodecoration that was observed when the non-immune serum was used in sections prepared from AFP-treated fungal cultures. The already reported affinity of AFP for binding to chitin present in the fungal cell wall might explain these results [46]. Binding of plant thionins, proteins which are structurally related to AFP, to polysaccharides components of the fungal cell walls has been also described [47]. This study provides evidence that AFP, once internalized into the cells of *M. grisea*, is targeted to the nucleus. Further studies are, however, needed to elucidate the mechanisms involved in AFP translocation into the fungal cells.

In summary, we have shown that the combination of fungal cell permeabilization, cell-penetrating ability and nucleic acid-binding activities of AFP determines its potent antifungal activity against the blast fungus *M. grisea*, an important pathogen of cultivated rice. These findings are important for functional purposes. Thus, these results suggest that AFP, after penetrating the cell, could inhibit cellular functions by binding to fungal DNA and/or RNA. Binding of AFP to nucleic acids will cause neutralization and condensation of the nucleic acid, and finally cell death.

An important aspect which has to be taken into account for prospective application of the AFP protein is the lack of cytotoxicity on mammalian cells. Results here presented indicated that AFP does not induce membrane permeabilization in HeLa cells, as judged by the lack of intracellular SYTOX Green uptake, at least at dosis twofold higher than those that are lethal for *M. grisea* cells. It is generally assumed that differences in membrane composition and structure provide a basis for antimicrobial activities of membrane-acting proteins and peptides which preferentially damage microbes but not animal or plant cells [48]. Whether or not AFP would kill a cell through an intracellular mechanism would be then dependent on its membrane penetrating ability. If so, AFP being unable to affect the membrane integrity of plant and HeLa cells would not penetrate into these cells and will not be able to interfere with their cellular functions. In this context, there is an ongoing discussion about the possible structure/biological function relationship of antimicrobial proteins. As previously mentioned, AFP shares similarities with plant defensins in terms of three-dimensional structure and basic character. Interestingly, the structural patterns of plant and insect defensins is also found in some scorpion neurotoxins [49, 50]. When comparing the three-dimensional structure of various antifungal proteins, proteins that all act as defense molecules or neurotoxins, it is concluded that a high level of structural similarity between these proteins does not necessarily imply a similar mode of action [51]. It appears that differences in the surface topology may entail totally different mechanisms of action and biological activity.

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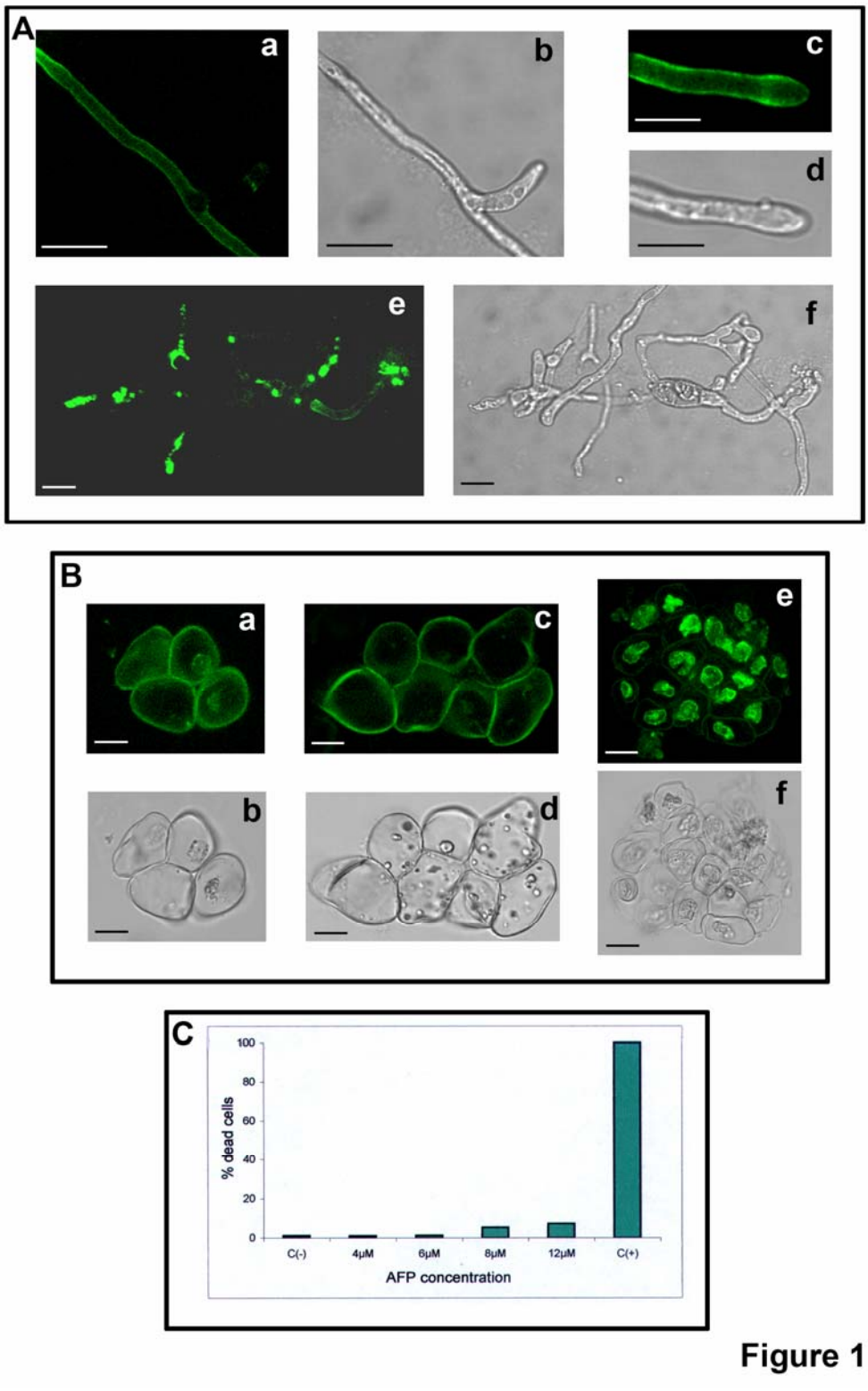
A
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b
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a
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d
f
C
% dead cells
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60
40
20
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C(-)
4µM
6µM
8µM
12µM
C(+)
AFP concentration

Figure 1

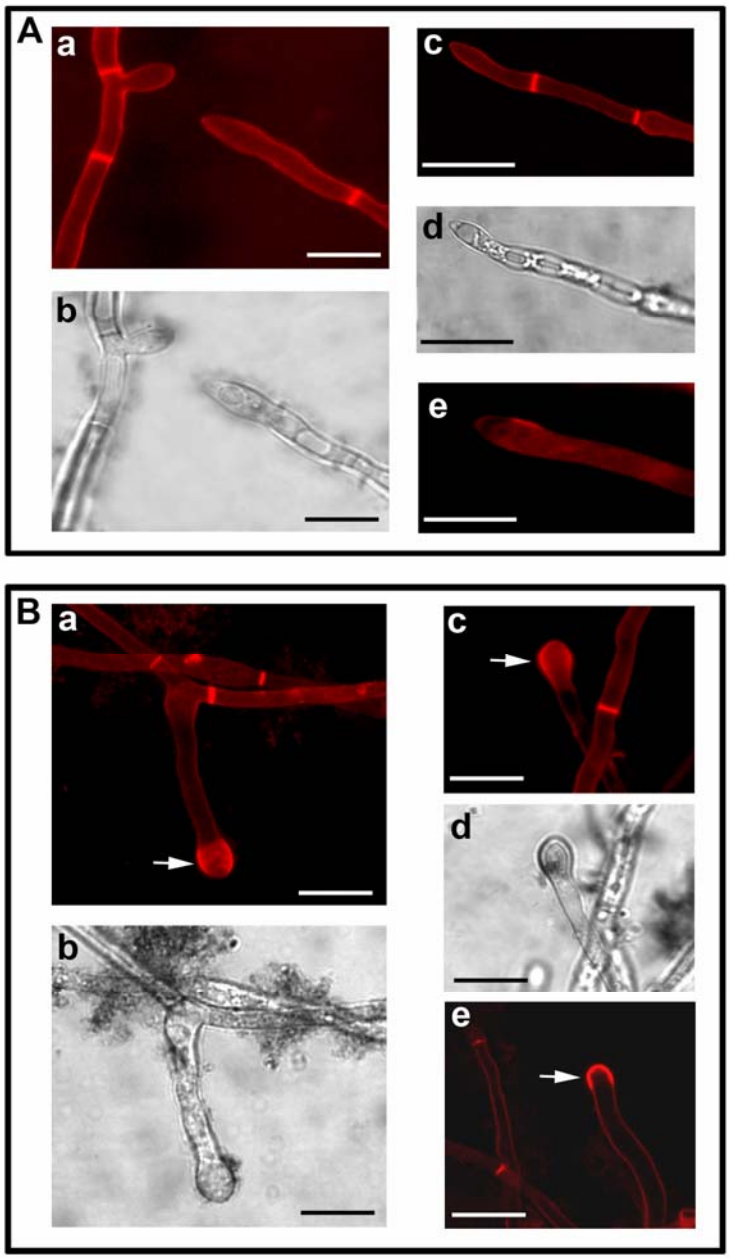


Figure 2

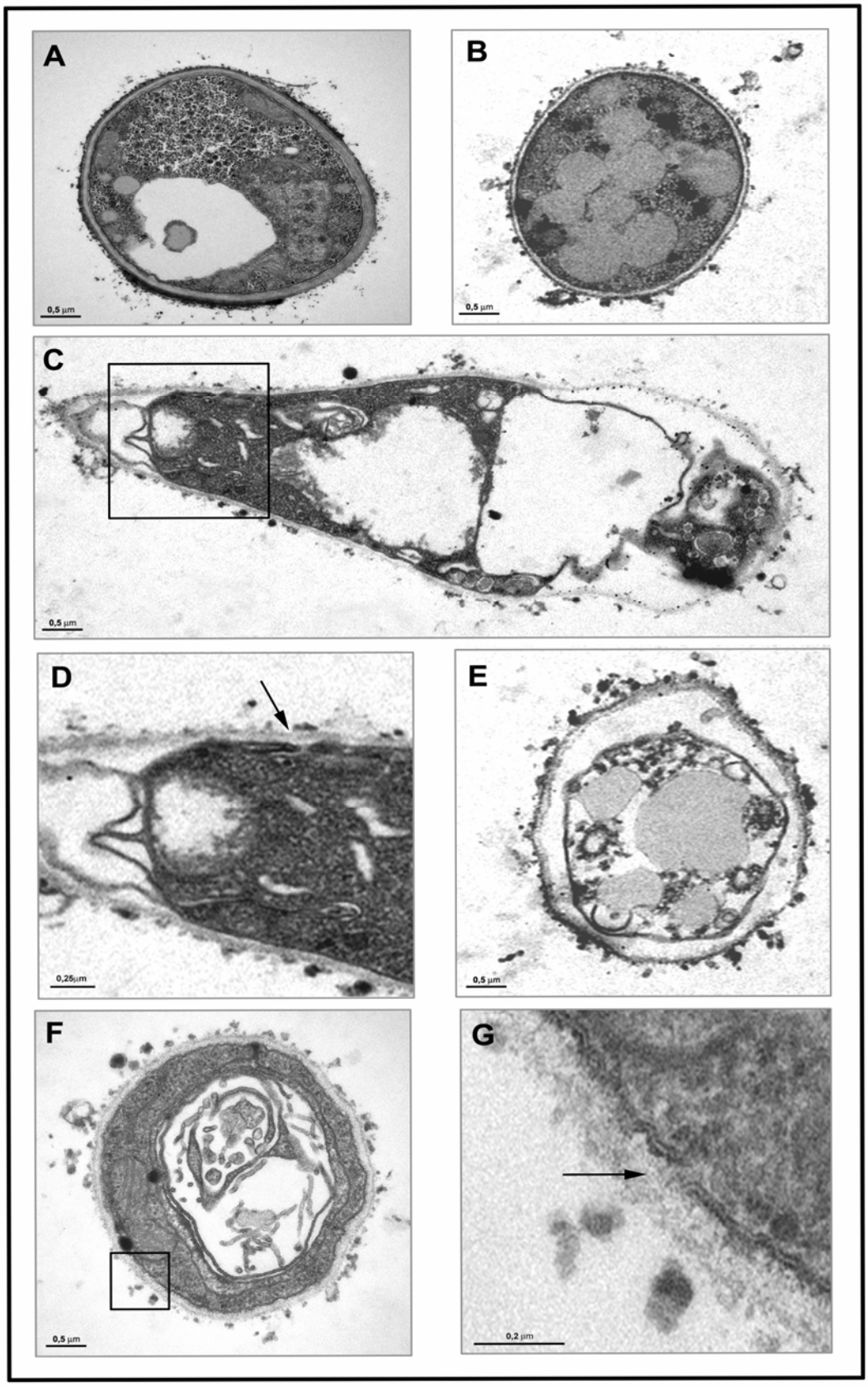


Figure 3

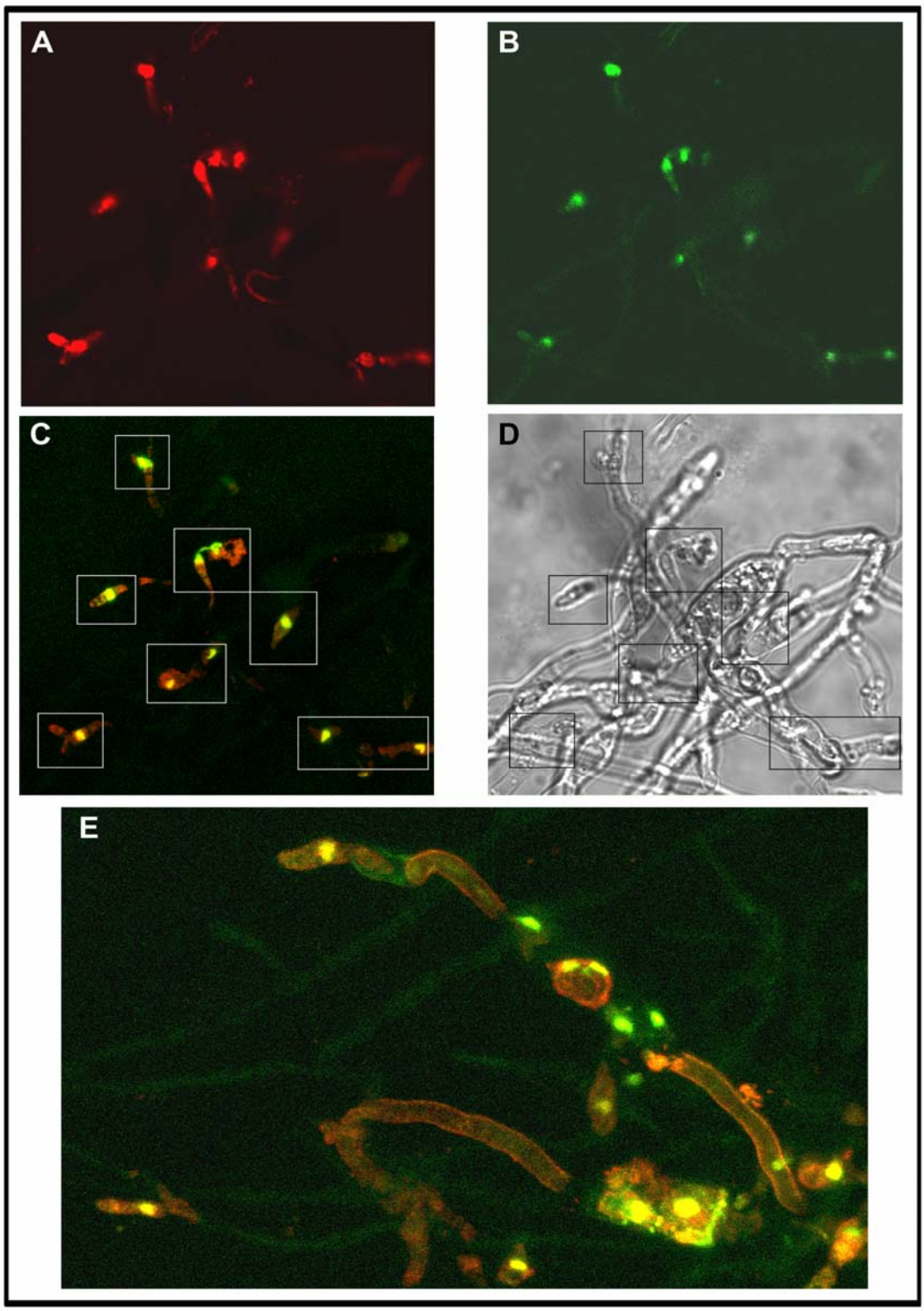
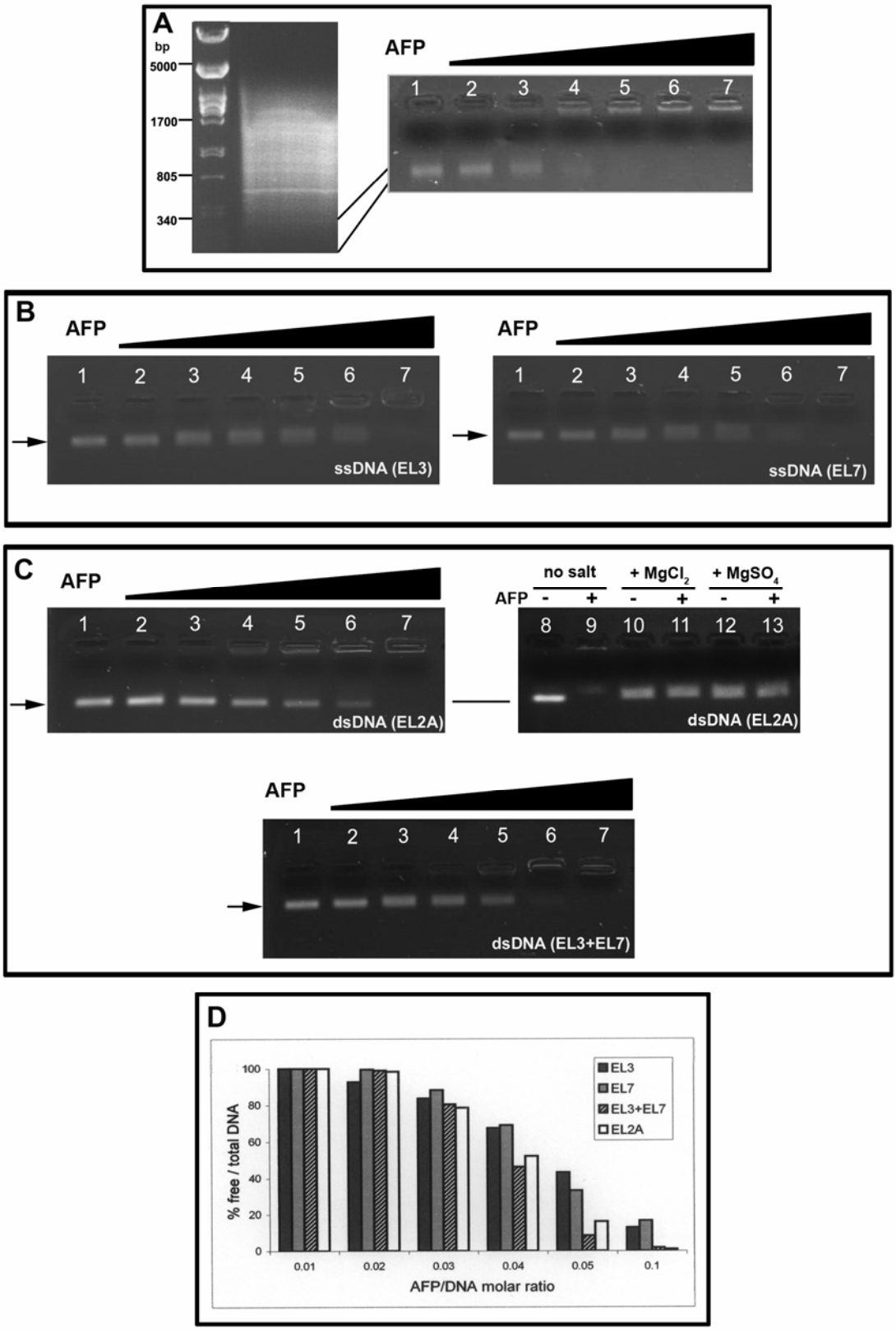


Figure 4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dsDNA (EL3+EL7)
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100
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AFP/DNA molar ratio
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EL3+EL7
EL2A

Figure 5

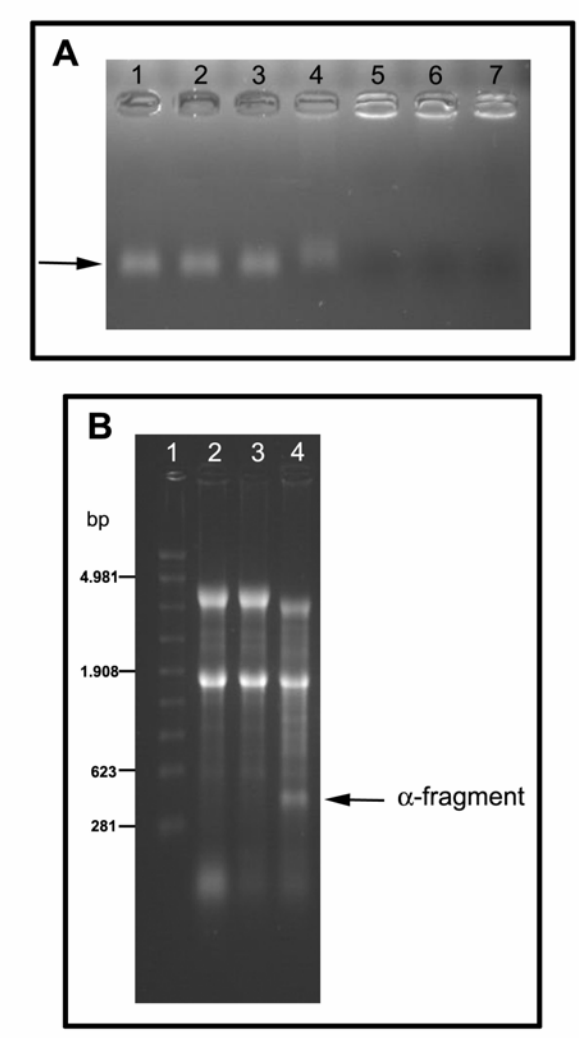

Figure 6

Figure Legends

Figure 1. Membrane permeabilization induced by AFP in *M. grisea* cells. Permeabilization measured by SYTOX Green fluorescence of *M. grisea* (A), rice (B) and HeLa (C) cells are shown. *(A)* Confocal fluorescence microscopy (a, c, and e) and transmission image (b, d, and f) of *M. grisea* grown in the absence (a to d) or in the presence of 4 μM AFP (e, f). (*B)* Confocal fluorescence microscopy (a, c and e) and transmission image (b, d and f) of rice cells grown in cell culture medium without (a, b) or with AFP at a concentration of 24 μM of AFP (c and d). As a control, rice cells were mechanically damaged by vigorous pipetting, frozen and then subjected to SYTOX Green staining (e and f). (*C*) HeLa cells grown in culture medium without added AFP, C(-), or treated with AFP at concentrations of 4, 6, 8 and 12 μM. As a control, HeLa cells were treated with 1% Triton, C(+). The percentage of dead cells for each treatment is presented (100% represents dead cells in treatment with 1% Triton). Bars = 2 μm (A) and 10 μm (B)

Figure 2. Congo red staining of *M. grisea* cells grown in the absence *(A)* or in the presence of AFP at a concentration of 4 μM *(B).* Fungal cultures were stained with Congo red and examined by confocal microscopy by taking sequential pictures at 0.1 μm intervals. Projections (*A* and *B*, panels a and c) and individual sections (*A* and *B*, panels e) of *M. grisea* hyphae stained with Congo red are presented. Transmission images of the *M. grisea* hyphae are also shown (*A* and *B*, panels b and d). Growth inhibition at the hyphal tips was observed in AFP-treated fungal cultures, as revealed by chitin deposition at the hyphal tips (white arrows in Fig. B). Bars = 2 μm.

Figure 3. Transmission electron micrographs of *M. grisea* cells exposed to AFP. *M. grisea* grown in PDB medium in the absence (*A* and *B*) or in the presence of AFP at a concentration of 50 nM (*C* to *G*). Fungal cells treated with AFP exhibited significant morphological and ultrastructural changes, such as increased vacuolation, invagination distorsion and retraction of the plasma membranes, and formation of polymorphic vesicles. Loss of plasma membrane integrity and pore formation (*D* and *G*, arrows) was observed.

Figure 4. Distribution of Alexa-labeled AFP in *M. grisea* cells. Confocal fluorescence microscopy (*A* to *C*, and *E*) and transmission image (*D*) of *M. grisea* cultures grown in the presence of Alexa-568-labeled AFP at a concentration of 4 μM *(A).* Fungal cells were also stained with SYTOX Green (*B)*. Red shows fluorescence of Alexa 568. Green shows fluorescence of the nuclear-staining dye SYTOX Green. The co-localization of Alexa-AFP and SYTOX Green can been seen in yellow (*C* and *E*). Bars = 2 μm.

Figure 5. Binding properties exerted by AFP on DNA. Binding was monitored by retardation of the electrophoretic mobility of DNAs in the presence of increasing amounts of AFP. DNA is retained in the well upon complex formation. *(A)* Binding assay using *M. grisea* genomic DNA. Left panel, 0.8% agarose electrophoresis of the restriction enzyme-digested *M. grisea* genomic DNA. Right panel, 3% agarose gel electrophoresis of the incubation mixtures of *M. grisea* genomic DNA (0. 32 :g of DNA, each line): lane 1, no AFP; lane 2, 0.01 μg; lane 3, 0.04 μg; lane 4, 0.07 μg; lane 5, 0.1 μg; lane 6, 0.13 μg; and lane 7, 0.14 μg of AFP. *(B)* Binding assay using the single stranded DNAs, the EL3 (left panel, lanes 1 to 7), and EL7 (right panel, lanes 1 to 7) DNAs (0.48 μg of each DNA). The AFP/molar ratios used were as follows: lane 1, no AFP ; lane 2, 0.01; lane 3, 0.02; lane 4, 0.03; lane 5, 0.04; lane 6, 0.05; and lane 7, 0.1 AFP/DNA molar ratio. *(C)* Binging assay using the double stranded EL2A and EL3+EL7 DNAs (upper and lower panels, respectively. Lanes 1 to 7, as indicated in B. AFP binding to EL2A DNA using a high-ionic streng binding buffer is shown (lanes 8 to 13). Binding assays with no salts added to the buffer, EL2A DNA without (lane 8) or with AFP (lane 9, AFP/DNA molar ratio, 0.1). EL2A DNA in buffer containing 0.05M $MgCl_2$, with no AFP (lane 10) or with AFP (lane 11, AFP/DNA molar ratio, 0.1). EL2A DNA in buffer containing 0.05M $MgSO_4$, with no AFP (lane 12) or with AFP (lane 13, AFP/DNA molar ratio, 0.1). *(D)* Percentage of free to total DNA was calculated from digitalized images of the agarose gels (Quantity One Program from Bio-Rad). For each agarose gel, the intensity of the DNA band at lane 1 was taken as 100%. Values correspond to one representative experiment of three.

Figure 6. (*A)* RNA binding activity of AFP. Yeast tRNA (0.32 μg) was incubated with various amounts of AFP at room temperature for 10 min and the reaction mixtures were run on a 1.5% formaldehyde-containing agarose gel electrophoresis. Lane 1, no AFP; lane 2, 0.13 μg; lane 3, 0.14 μg; lane 4, 0.28 μg; lane 5, 0.43 μg; lane 6, 0.57 μg; and lane 7, 0.72 μg of AFP. *(B)* Agarose gel electrophoresis of rabbit reticulocyte lysates (20 μl) incubated with H_2O (lane 2), AFP (1 μg, lane 3), α-sarcin (1 μg, lane 4). Lane 1, molecular weight markers (RNA markers 0.28-6.58 kb, Promega). The ribonuclease activity of α-sarcin is revealed by the release of the α-fragment (indicated by an arrow) from the 28S RNA. AFP failed to release any RNA fragment. The results are representative for three separate experiments.

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La protección de cultivos de interés agronómico frente a enfermedades ha venido realizándose mediante diferentes estrategias, tales como el entrecruzamiento de especies, el empleo de prácticas de cultivo organizadas (p.e. rotación de los cultivos, selección de suelos con menor incidencia de patógenos concretos, etc) y sobre todo mediante la aplicación de compuestos químicos. Sin embargo, el uso indiscriminado y masivo de dichos agentes químicos ejerce una fuerte presión de selección sobre los patógenos, de manera que éstos pueden sobrepasar los efectos de protección observados al inicio del tratamiento. Por otra parte, las prácticas introducidas en la agricultura moderna basadas en la existencia de grandes extensiones de monocultivos hacen que la presencia de un patógeno tenga efectos destructivos muy importantes. Dentro del amplio rango de organismos que pueden ser patogénicos para las plantas, destacan los hongos debido a su elevado número y a las importantes pérdidas que originan tanto en los cultivos de campo como en las plantas cultivadas en invernaderos. En determinados casos, el control biológico de patógenos ha resultado ser efectivo para el control de enfermedades. Se basa en la utilización de microorganismos antagonistas de los agentes fitopatógenos (Rey *et al*, 2000). Las especies del género *Trichoderma* son las más utilizadas en el control biológico, debido a la facilidad para ser aisladas y mantenidas en cultivo, a su crecimiento rápido y en una gran variedad de sustratos, y a su ubicuidad. Desplazan al hongo fitopatógeno por competición directa por el espacio o los nutrientes, por parasitismo directo, o mediante la producción de compuestos antifúngicos.

La creciente demanda de producción y la necesidad cada vez mayor de limitar el uso de productos químicos, hacen necesaria la búsqueda de nuevas estrategias para el control de enfermedades. La biotecnología puede ayudar en gran manera a la protección frente a enfermedades de especies cultivadas, principalmente en aquellos cultivos en los que los métodos más tradicionales no resultan efectivos (European Plant Science Organization, EPSO, 2005). Por otra parte, la mejora biotecnológica de plantas no sufre las limitaciones que impone la existencia de barreras sexuales, un factor limitante para la mejora genética clásica, y es aplicable a las diferentes especies vegetales para las cuales existen protocolos de transformación. Permite además seleccionar y utilizar genes específicos, genes que sean más adecuados para cada tipo de interacción planta-patógeno (Biezen, 2001; Altpeter *et al*, 2005; Gurr y Rushton, 2005a).

El éxito o fracaso que se pueda alcanzar en el tema de resistencia a patógenos en plantas transgénicas depende, entre otros, de los siguientes aspectos: a) del tipo de transgén que se utiliza y la efectividad de la proteína codificada por el transgén para inhibir el crecimiento del patógeno; b) de la correcta expresión y estabilidad del producto del transgén en los tejidos de la planta; c) la utilización de un promotor adecuado para dirigir su expresión, en niveles suficientemente elevados, tejidos de la planta y momentos en los que

interese que actúe el producto del transgén. Asimismo, se necesita que el transgén se transmita a la descendencia y mantenga su nivel de expresión en las sucesivas generaciones. Estos factores son fundamentales y además deben ser considerados individualmente para cada interacción planta-patógeno.

En los últimos años se ha recogido una gran cantidad de información acerca de los mecanismos de defensa de las plantas, habiéndose identificado una colección importante de genes que participan en dichos procesos. Muchos de estos genes de defensa vegetales han sido ya utilizados como transgenes para la protección de plantas frente a enfermedades. Sin embargo, los resultados obtenidos hasta la fecha en las estrategias basadas en la utilización de genes de origen vegetal como transgenes no han sido totalmente satisfactorios. Muy probablemente, la defensa natural de las plantas conlleva la expresión simultánea de grupos concretos de genes que participan conjuntamente para contrarrestar el proceso de infección por un determinado patógeno, pudiendo ser necesarias diferentes combinaciones de genes de defensa para los diferentes tipos de patógenos. La falta de información acerca de cual es la combinación más adecuada para cada patógeno puede ser la causa de los bajos niveles de protección que se observan en estrategias basadas en la utilización de genes de defensa vegetales como transgenes. Ello viene apoyado por los resultados observados tras la expresión simultánea de dos o más genes de defensa vegetales en plantas transgénicas en cuyo caso se alcanzan niveles de protección superiores a los que se obtienen en la expresión individual de cada uno de ellos (Datta *et al*, 1999).

La utilización de genes de defensa de origen no vegetal para la protección de plantas ha permitido obtener mejores resultados, observándose niveles de protección más importantes y protección frente a un mayor espectro de patógenos. Se han descrito genes que participan en las respuestas de defensa de organismos tan variados como pueden ser bacterias, hongos, insectos y animales (incluyendo el hombre). En eucariotas superiores, los productos génicos de dichos genes representan la primera línea de defensa contra microorganismos invasores, mientras que en procariotas y eucariotas inferiores, pueden conferir una ventaja competitiva a los organismos que los producen frente a otros microorganismos de su mismo hábitat. El ejemplo que ilustra mejor el interés de la utilización de genes de origen no vegetal para la protección de plantas es el caso de los genes de la bacteria *Bacillus thuringiensis* (genes *Bt.*), los cuales han sido ampliamente utilizados para obtener resistencias a insectos en diferentes variedades comerciales (maíz, patata, algodón).

Los mecanismos por los cuales las proteínas/péptidos ejercen su actividad antimicrobiana son muy diversos e incluyen la desestructuración y formación de poros en la membrana plasmática. En algunos casos, la proteína/péptido antimicrobiano penetra al interior de la célula e interacciona con dianas intracelulares muy diferentes como pueden ser

ribosomas, mitocondrias, DNA y RNA. En este sentido, cabe mencionar que la posibilidad de adaptación de un patógeno a un compuesto antimicrobiano, o lo que es lo mismo, la durabilidad y permanencia de su eficacia como agente antimicrobiano, está estrechamente ligada al mecanismo de acción del compuesto antimicrobiano. Así, un mecanismo de acción de tipo general, como puede ser la formación de poros en la membrana plasmática, es más difícil de ser anulado que un mecanismo basado en un reconocimiento específico (p.e. el reconocimiento por receptores específicos de la membrana plasmática de moléculas específicas provenientes del patógeno). En este último caso, una mutación en cualquiera de las partes, receptor del patógeno o proteína antifúngica, es suficiente para anular el mecanismo de acción.

Los hongos micoparásitos o antagonistas del suelo son productores de numerosas proteínas con actividad antimicrobiana, incluyendo proteínas antifúngicas que son efectivas para combatir a otros hongos pero no a las plantas con las cuales conviven. Estos organismos representan por lo tanto una buena fuente para la identificación y aislamiento de genes antimicrobianos. Además, estas proteínas antimicrobianas suelen actuar frente a un amplio espectro de patógenos, lo cual las hace particularmente interesantes para el diseño de estrategias dirigidas a la protección de las plantas frente a fitopatógenos. Por citar un ejemplo, la obtención de plantas transgénicas de tabaco y patata que expresan una quitinasa de *Trichoderma harzianum*, presentan niveles de resistencia muy elevados y frente a diferentes fitopatógenos (Lorito *et al*, 1998).

En este trabajo se ha estudiado una proteína producida por el hongo del suelo *Aspergillus giganteus,* la proteína AFP que se secreta al medio exterior. Se trata de una proteína muy estable para la cual se había descrito una actividad antifúngica elevada frente a hongos filamentosos, incluidos los hongos fitopatógenos *Fusarium verticillioides* y *Magnaporthe grisea*, así como también el oomiceto *Phytophthora infestans* (Lacadena *et al*, 1995; Vila *et al*, 2001).

En el primer capítulo de esta tesis, se presenta el estudio realizado para determinar la capacidad de la proteína AFP para actuar como agente antifúngico frente a *Botrytis cinerea*. El objetivo final de este estudio es la utilización del gen *afp* en la protección de las plantas de geranio (*Pelargonium hortorum*) frente a este patógeno (estudios actualmente en curso en el laboratorio). Ello hacía necesario realizar una evaluación previa, mediante ensayos *in vitro*, de la actividad de esta proteína frente a los aislados de *B. cinerea* que infectan de forma natural las plantas de geranio. Estos aislados fueros obtenidos de plantas mantenidas en los invernaderos del vivero de producción de plantas ornamentales de la Fundación Promiva (Madrid). El ambiente húmedo de los invernaderos dónde se producen las plantas ornamentales favorece el rápido crecimiento y los elevados niveles de

esporulación de *B. cinerea.* Este es uno de los principales motivos por los cuales los daños producidos por este hongo (la podredumbre gris) siguen siendo una de las principales causas de pérdidas económicas en todos los estadios de la producción de plantas ornamentales. Además del geranio, *Botrytis cinerea* infecta otros muchos cultivos de interés ornamental, entre los cuales se encuentran el crisantemo, la rosa y la flor de nochebuena. Todos ellos son propagados vegetativamente por esquejes. Es precisamente este modo de propagación lo que favorece la proliferación del hongo, ya que las zonas heridas por el corte son especialmente susceptibles a la infección. Otra característica que facilita la diseminación de la enfermedad es la capacidad de los conidios de permanecer en un estado latente por largos periodos de tiempo (más de 3 semanas) antes de la germinación.

Los ensayos realizados para determinar la actividad antifúngica de la proteína AFP frente a *B. cinerea* permitieron comprobar que esta proteína es muy efectiva para inhibir el crecimiento del hongo, ya que se obtuvieron unos valores de IC_{50} de entre 0,5 y 5 μM dependiendo del aislado utilizado (siendo el valor IC_{50} la concentración necesaria para inhibir el 50% del crecimiento del hongo). Estos valores de IC_{50} son similares o incluso más bajos a los que se observan en ensayos similares descritos para otras proteínas antifúngicas, siendo indicativos de una fuerte actividad de la proteína AFP frente a este patógeno. La observación microscópica del hongo crecido en presencia de AFP reveló profundos cambios en la morfología del hongo, con hifas más cortas y menos ramificadas, al tiempo que se producía la agregación y condensación del micelio.

Desde el punto de vista de la protección frente a hongos, es interesante estudiar el efecto de la combinación de diferentes proteínas antifúngicas con el fin de obtener un mejor control de las enfermedades y de reducir así la probabilidad de que se pierda la resistencia por adaptación del patógeno. Por esta razón, una vez comprobada la actividad antifúngica de la proteína AFP se estudió el efecto de la combinación de esta con otra proteína antifúngica, la cecropina A, sobre *B. cinerea*. En cuanto al efecto que cabe esperar de la combinación de dos compuestos antifúngicos, se puede encontrar un efecto aditivo, sinérgico, o puede que no se observe ninguna ventaja al combinar ambos compuestos con respecto a cada uno de ellos por separado (Westerhoff *et al*, 1995; Gisi, 1996; Segura *et al*, 1999). En este trabajo se llevaron a cabo ensayos de actividad antifúngica frente a la cepa CC1 del hongo *B. cinerea* combinando las proteínas AFP y cecropina A. Previamente al uso combinado de ambas proteínas, se hicieron ensayos de actividad antifúngica con la proteína cecropina A sobre *Botrytis cinerea*. Este estudio mostró que la cecropina A también ejerce un efecto inhibidor sobre el crecimiento de este hongo, si bien en menor grado que el que se obtiene en el caso de la proteína AFP (valor IC_{50} de 20 μM para la cecropina A). Cuando se ensayó el efecto sobre *B. cinerea* de la combinación de las dos proteínas, AFP y cecropina A, se pudo concluir que existe un efecto aditivo entre ambas proteínas frente a *B. cinerea*. Esta

información resultará particularmente útil para la obtención de plantas transgénicas resistentes a la infección por este hongo que expresen simultáneamente los genes *afp* y *cecropina A*.

A priori, cabe esperar que la combinación de dos proteínas antifúngicas con diferentes mecanismos de acción pueda ser más efectiva para producir un efecto aditivo o sinérgico como agentes antifúngicos, con respecto a la combinación de dos proteínas con igual mecanismo de acción (competencia entre ambas por las mismas dianas en la célula vegetal). Aunque en este trabajo no se ha estudiado el mecanismo por el cual la cecropina A inhibe el crecimiento de *B. cinerea*, es bien sabido que las cecropinas ejercen su actividad a través de la desestructuración de la membrana plasmática de microorganismos con consiguiente formación de poros en la misma. Tal y como se presenta en el capítulo III de la presente tesis, el mecanismo de acción de la proteína AFP implica una doble actividad, la permeabilización y formación de poros en la membrana del hongo y la interacción con dianas intracelulares, DNA y/o RNA. El efecto aditivo observado en la combinación de AFP y cecropina A podría pues reflejar un mayor efecto desestructurador sobre la membrana de la célula del hongo por la acción de ambas proteínas, lo cual permitiría una mayor entrada de la proteína AFP en la célula del hongo.

Otro aspecto estudiado en este trabajo ha sido la determinación del efecto de la proteína AFP en la germinación de conidios de *B. cinerea*. En estos ensayos se pudo demostrar que la AFP es capaz de inhibir la germinación de los conidios de *B. cinerea* a una concentración de 5 μM (la más baja utilizada en este estudio). Además, también se pudo determinar que al eliminar la proteína AFP del medio de crecimiento del hongo, éste no progresa en su crecimiento, lo que es indicativo de que la actividad antifúngica de la proteína AFP frente a este hongo es de carácter fungicida y no fungistática. Este aspecto, junto con la capacidad de inhibir la germinación de los conidios, es particularmente interesante ya que determina una acción más efectiva para combatir la propagación del hongo.

La proteína AFP es producida en cantidades importantes y se secreta al medio extracelular en cultivos líquidos de *A. giganteus*, a partir de los cuales es posteriormente purificada. Así pues, además de la posibilidad de producir esta proteína por la planta transgénica, cabe considerar también la posibilidad de utilizar la proteína AFP como agente antifúngico mediante aplicación directa sobre las plantas. Por lo tanto, se analizó el efecto que tenía la aplicación directa de la proteína AFP en hojas de geranio, para determinar si se observaba un efecto inhibidor del crecimiento *B. cinerea* en estos tejidos, es decir, para determinar si el efecto antifúngico observado en los ensayos *in vitro* se reproducía en una situación *in vivo*. Para eso se inocularon localmente hojas de plantas de geranio con una suspensión de conidios de *B. cinerea*. Inmediatamente, se añadía sobre los sitios inoculados

o bien la proteína AFP (a una concentración de 10 μM), o bien agua estéril. Se hizo luego un seguimiento de la aparición de los síntomas de infección. Después de 6 días de infección, se observaron síntomas claros en los puntos infectados y tratados con agua, pero no en los sitios infectados y tratados con la proteína AFP. Este resultado corroboraba el efecto inhibitorio previamente observado *in vitro* sobre la germinación de los conidios de *B. cinerea.* Cuando se utilizaron diferentes concentraciones de AFP (10, 1,0 y 0,1 μM), se pudo comprobar que las concentraciones inhibitorias encontradas en los ensayos *in vitro,* también eran efectivas en estos ensayos *in vivo*. En otros experimentos, se dejó transcurrir un periodo de tiempo (6 horas) desde que se realizó la inoculación con esporas hasta que se hizo el tratamiento con la AFP. Se pretendía así permitir la germinación de las esporas antes del tratamiento con la proteína AFP. También en este ensayo se pudo ver como la proteína AFP evita la aparición de síntomas de infección.

Por último, se adelantó el momento de aplicación de la proteína AFP con respecto al momento de la infección con *B. cinerea.* En este caso, se aplicó la proteína AFP sobre puntos concretos de las hojas de geranio y transcurridos 3 o 14 días se procedió a la infección de esas mismas regiones. En estas condiciones la proteína AFP mantuvo su actividad y demostró ser estable y funcional para contrarrestar el crecimiento del hongo (incluso cuando la aplicación de la proteína se realizaba 14 días antes de la inoculación con el hongo). Muy probablemente, la estructura compacta de esta proteína y su gran estabilidad a la degradación proteolítica pueden explicar este efecto protector a tan largo plazo. Ello es de interés si se pretende utilizar la proteína AFP como agente antifúngico, mediante rociado de plantas.

En definitiva, estos estudios han permitido demostrar la efectividad de la proteína AFP para inhibir el crecimiento del hongo *Botrytis cinerea*, tanto el crecimiento del micelio como la germinación de los conidios. La aplicación exógena de AFP también es capaz de prevenir la infección por *B. cinerea* en hojas de geranio infectadas. Esta información resulta de interés ya que justifica la utilización del gen *afp* en plantas transgénicas y de la proteína AFP por aplicación directa, para el control de este patógeno en geranio.

Tradicionalmente, la industria biotecnológica se ha centrado en plantas de interés agronómico, mientras que las plantas ornamentales han sido en un principio marginadas por su menor volumen de negocio. La mayoría de los trabajos realizados hasta la fecha para la mejora biotecnológica de plantas ornamentales ha estado dirigida a la modificación de las características ornamentales, tales como el color de los pétalos, el tamaño y la producción de flores, o el porte de la planta. Se han dedicado muy pocos esfuerzos para la obtención de resistencias frente a patógenos en plantas ornamentales. Esta situación tiende a cambiar debido en parte al incremento que se observa a nivel mundial en el mercado de las plantas ornamentales, en especial de geranios, y sobre todo a la creciente demanda en reducir el

uso de compuestos químicos en el control de enfermedades. Además de las ventajas directas sobre la producción, el desarrollo y comercialización, las plantas transgénicas ornamentales, puesto que no están destinadas al consumo humano o animal, no presentan los problemas asociados a la falta de aceptación por parte de la opinión pública de las plantas transgénicas, u organismos modificados genéticamente en general.

Son muy pocos los trabajos descritos en la literatura relacionados con la obtención de plantas ornamentales resistentes a hongos, particularmente a *B. cinerea* (a pesar de que este patógeno es responsable de pérdidas muy importantes en varias especies ornamentales). Marchant y colaboradores (1998) obtuvieron plantas de rosa (*Rosa hybrida*) resistentes a la infección por el hongo *Diplocarpon rosae* mediante la expresión de un gen que codifica una quitinasa. También en rosa, se obtuvieron plantas transgénicas que expresan el gen de una defensina de cebolla (Ace-AMP1) y que son resistentes a *Sphaerotheca pannosa* var. *Rosae* (Li *et al*, 2003). El gen que codifica esta misma proteína, la Ace-AMP1 de cebolla, también se ha expresado en plantas de geranio bajo control del promotor constitutivo 35S CaMV obteniéndose resistencia frente a *Botrytis cinerea* (Bi *et al*, 1999).

En el segundo capítulo de esta tesis se han obtenido plantas transgénicas de arroz que expresan el gen *afp* de una manera regulada e inducible por la presencia del patógeno (*M. grisea*). Plantas de arroz que expresan el gen *afp* bajo control del promotor constitutivo del gen de la *ubiquitina* obtenidas en nuestro laboratorio habían mostrado resistencia frente a *M. grisea* (Coca *et al*, 2004). El objetivo planteado en esta tesis fue demostrar que el gen *afp* cuando es expresado bajo control de un promotor inducible por infección protege la planta de arroz frente a la piriculariosis.

El problema que se presenta en el momento de plantearse una estrategia de utilización de promotores inducibles, es la limitada disponibilidad de este tipo de promotores para plantas de arroz (Gurr y Rushton, 2005). De hecho, son pocos los promotores para los cuales se haya demostrado inducibilidad por infección en plantas transgénicas de especies monocotiledóneas. Por este motivo, se llevó a cabo un análisis funcional de los promotores de 3 genes *PR* de maíz en plantas trangénicas de arroz, con el objeto de disponer de un promotor adecuado para la posterior expresión inducible del gen *afp*. Se han ensayado los promotores de los genes *PRms* (Pathogenesis-Related maize seed), *mpi* (maize proteínase inhibitor) y *ZmPR4* (*Zea mays* Pathogenesis-Related 4). La expresión de estos genes se induce en respuesta a la infección fúngica en plantas de maíz (Casacuberta *et al*, 1991; Cordero *et al*, 1994; Bravo *et al*, 2003).

El análisis detallado de la secuencia nucleotídica de estos promotores reveló la presencia de determinados elementos reguladores de la expresión de genes de defensa

vegetales. Así, en los tres promotores se encuentra localizado el elemento o caja-W (*W-box*) C/TTTGACT/C. Esta secuencia se encuentra presente en los promotores de muchos genes de defensa y está asociada a su inducibilidad por patógenos o elicitores (Euglem *et al*, 1999; Hara *et al*, 2000; Euglem *et al*, 2000; Kirsch *et al*, 2001; Vila Ujaldón, 2003). Esta secuencia es el sitio de unión de factores de transcripción del tipo dedos de zinc, pertenecientes a la superfamilia WRKY de factores de transcripción de plantas (Foster et al, 1994). La región central de esta secuencia, TGAC, es fundamental para la unión de dichos factores de trancripción (Euglem *et al*, 2000).

El elemento ERE (Elicitor Responsive Element, ATTGACC), se encuentra en los promotores de los genes *PRms* y *ZmPR4*. La funcionalidad de este elemento como regulador de la transcripción del gen *PRms* en respuesta a elicitores fúngicos había sido descrita anteriormente por el grupo (Raventós *et al*, 1995). Con posterioridad, se ha visto que esta secuencia se encuentra presente en varios promotores de genes relacionados con las respuestas de defensa (Rushton *et al*, 1996; Schubert *et al*, 1997; Chao *et al*, 2000; Chiron *et al*, 2000). La existencia de varios elementos, estructuralmente distintos pero funcionalmente similares, de respuesta a elicitores basados en el consenso TGAC está de acuerdo con la existencia de diferentes isoformas de proteínas WRKY, que presentan diferentes afinidades por estos elementos (Kirsch *et al*, 2001).

Por último, en los promotores de los genes *ZmPR4* y *mpi*, se identificaron varios elementos *cis* asociados a la inducción por metil jasmonato o a herida en otros genes de plantas, como es el caso del gen que codifica una aminopeptidasa (Boter *et al*, 2004), o el gen de una lipoxigenasa de cebada (Rouster *et al*, 1997). Estas secuencias reguladoras (GAGTA, TGACG, CGTCA) se pueden encontrar en posición directa o inversa y en una u otra cadena del DNA del promotor.

Los resultados obtenidos en el estudio de promotores, mostraron que el promotor del gen *PRms* no es funcional en plantas de arroz. No se detectó actividad de este promotor en ningún tejido de la planta de arroz transgénica (plantas transformadas con el gen *gusA* bajo control del promotor *PRms*) ni tampoco en respuesta a ninguno de los tratamientos realizados (infección por esporas, tratamiento con elicitores, herida).

En el caso de los promotores *mpi* y *ZmPR4* los resultados fueron más satisfactorios. En ambos casos, el patrón de expresión que muestran estos genes en la planta de maíz se reproduce en las plantas transgénicas de arroz. Ambos promotores muestran una clara respuesta a la infección por el hongo y a la herida mecánica. Sin embargo, mientras que el promotor *ZmPR4* es inducible por elicitores fúngicos, no es este el caso del promotor *mpi*.

Es de destacar el hecho de que el promotor *ZmPR4* no sea activo en el endospermo de la semilla de arroz, órgano de la planta que se destina al consumo humano. Este dato, juntamente con su rápida y fuerte inducción frente a la infección por el patógeno *M. grisea*

(infección con esporas y tratamiento con elicitores) fueron determinantes para la elección del promotor del gen *ZmPR4* de maíz como el promotor más adecuado para dirigir la expresión del gen *afp* en plantas transgénicas de arroz.

El gen *afp* utilizado para la transformación había sido sintetizado químicamente en nuestro laboratorio mediante el uso de oligonucleótidos largos ensamblados mediante PCR. La secuencia del gen se adaptó al uso de codones en plantas monocotiledóneas (Liu *et al*, 2004). Además, en el proceso de ensamblaje para la síntesis química del gen *afp* se incorporó la secuencia nucleotídica que codifica un péptido señal N-terminal de internalización al retículo endoplasmático y entrada en la vía de secreción (péptido señal de la proteína AP24 de tabaco). Los ensayos de resistencia realizados con las plantas transgénicas que expresan el gen *afp* bajo control del promotor *ZmPR4* frente a la infección por *M. grisea*, ya sean ensayos en hoja cortada o en planta entera, demostraron la eficacia de la estrategia empleada. Las plantas transgénicas se mostraron más resistentes que las controles frente a la piriculariosis.

El último objetivo que se planteó para la realización de la presente tesis, cuyos resultados se muestran en el capítulo III, fue determinar el mecanismo por el cual la proteína AFP ejerce su actividad antifúngica, utilizándose como modelo para este estudio el hongo *M. grisea.*

Tal y como se ha comentado anteriormente, se han descrito mecanismos de acción muy diferentes para la gran diversidad de proteínas o péptidos que se han encontrado en diferentes organismos. Por citar algunos ejemplos, se sabe que las proteínas de plantas de tipo taumatina (familia PR-5) pueden tener múltiples actividades, desestabilizando la membrana de hongos e incluso estimulando una vía de transducción de señal mediada por una proteína quinasa que resulta en alteraciones en la pared celular. El caso más estudiado es el de una osmotina de tabaco (Yun *et al*, 1998; Grenier *et al*, 1999). Las quitinasas y las β-1,3 glucanasas hidrolizan respectivamente la quitina (un polímero de N-acetilglicosaminas) y los β-1,3 glucanos, que representan los principales componentes de la pared celular de hongos (Datta *et al*, 1999; Kasprzewska, 2003). Sin embargo, el modo de acción más generalizado entre las proteínas de bajo peso molecular y los péptidos antimicrobianos, tanto de plantas como de otros organismos, se basa en interacciones con la membrana plasmática de los organismos diana. Los péptidos y proteínas que actúan de esta manera, son moléculas muy compactas y frecuentemente adoptan estructuras anfipáticas, que presentan una región cargada positivamente y otra neutra o hidrofóbica. Algunas ejercen su actividad antimicrobiana por interacción con la membrana, provocando su desestabilización e incluso pueden llegar a formar poros en las mismas. Otras son capaces de penetrar al

interior de la célula e interaccionar con dianas intracelulares (de Lucca y Walsh, 1999; Marshall y Arenas, 2003; Theis y Stahl, 2004).

El hongo del suelo *Aspergillus giganteus* produce y secreta al espacio extracelular dos proteínas mayoritarias, la α-sarcina, una proteína de tipo RIP (ribosomal inactivating protein) que presenta una fuerte actividad ribonucleásica, y la proteína AFP (Lacadena *et al*, 1995). El hecho de que estas dos proteínas con actividades antimicrobianas se secreten al espacio extracelular sugiere que ambas pueden estar involucradas en la defensa de *Aspergillus giganteus* frente a otros microorganismos antagonistas del suelo. Sin embargo, el mecanismo de acción de la proteína AFP hasta la fecha era desconocido.

Estudios anteriores realizados en el grupo del Dr. A. Martínez del Pozo (Universidad Complutense de Madrid) pusieron de manifiesto la gran similitud que la estructura tridimensional de la proteína AFP tiene con un dominio estructural de unión a oligonucleotidos/oligosacáridos (OB *fold*) presente en algunas proteínas. Esta característica llevó a la realización de ensayos *in vitro* en los que se pudo comprobar que la proteína AFP se une a DNA a través de interacciones electrostáticas, induciendo la condensación del DNA por la neutralización de su carga (Martínez del Pozo *et al*, 2002). En otros estudios realizados en este grupo, se había también observado la capacidad de esta proteína para interaccionar con fosfolípidos ácidos de membranas. Un sitio catiónico y una región adyacente hidrofóbica podrían ser los responsables de promover interacción de esta proteína con residuos cargados negativamente y con fosfolípidos hidrofóbicos de la membrana plasmática (Nakaya *et al*, 1990; Campos-Olivas *et al*, 1995; Lacadena *et al*, 1995). Por otra parte, el hecho de que la proteína AFP posea una fuerte actividad frente a diferentes hongos filamentosos, pero no frente a bacterias ni levaduras (Lacadena *et al*, 1995; Vila *et al*, 2001; Moreno *et al*, 2003), sugeriría la existencia de receptores de membrana implicados en el reconocimiento de AFP, tal y como se ha observado en el caso de algunas defensinas de plantas (Thevissen *et al*, 1997). En definitiva, y a pesar de los años transcurridos desde que se aisló esta proteína, el mecanismo por el cual actúa frente a microorganismos era desconocido.

El primer estudio realizado para determinar el mecanismo de acción de la proteína AFP en el hongo fitopatógeno *M. grisea* se realizó con el indicador fluorescente sytox green. Este compuesto fluorescente tiene la capacidad de unirse a DNA, sin embargo solo es capaz de atravesar la membrana plasmática si ésta se encuentra dañada. De esta forma, la localización nuclear de este compuesto es un indicador de daños en la estructura de la membrana capaces de permitir la entrada del colorante al interior de la célula. En cultivos de *M. grisea* crecidos en presencia de AFP se pudo observar de forma muy clara la fluorescencia del colorante sytox green localizada en el núcleo de las células del hongo, mientras que no era este el caso en cultivos no tratados con AFP. Estos resultados indicaban que la proteína

AFP daña la membrana plasmática del hongo e induce permeabilización. Ensayos de microscopía electrónica de transmisión de cultivos fúngicos tratados con AFP confirmaron este efecto y además pusieron de manifiesto la formación de poros en la membrana plasmática de las células de *M. grisea*.

Se realizaron asimismo experimentos de tinción de las hifas del hongo con el colorante congo red. Este colorante tiene afinidad por la quitina, componente estructural de la pared celular de los hongos. Las hifas en proceso normal de crecimiento muestran un menor contenido de quitina en sus extremos (zona de elongación de las hifas), por lo que en esta región se observa una menor intensidad de marcaje con el colorante congo red. Un elevado marcaje de estas zonas con este colorante evidencia que la hifa no se encuentra en fase de elongación. En hifas de *M. grisea* tratadas con AFP y teñidas con el colorante congo red, se pudo apreciar una importante deposición de quitina en sus extremidades, indicativo de que las hifas no se encuentran en proceso de crecimiento activo. Así, la proteína AFP, además de afectar las propiedades de la membrana de la célula fúngica e inducir permeabilización en la misma, también determina una parada del crecimiento y desarrollo de las hifas.

La siguiente etapa fue estudiar si la propia proteína AFP era capaz de penetrar en la célula del hongo. Para ello la proteína AFP fue marcada con el fluorocromo Alexa 568. Previamente, se realizaron ensayos para comprobar que el marcaje no afectaba a las propiedades antifúngicas de la proteína AFP frente a *M. grisea*. Mediante microscopía confocal, se observó un intenso marcaje en el interior de las células. A continuación se llevaron a cabo experimentos de doble marcaje, de tal manera que las hifas tratadas con AFP-Alexa 568 eran posteriormente teñidas con sytox green. La colocalización de las fluorescencias debidas a la proteína AFP marcada con Alexa (rojo) y del sytox green (verde) demostró que la proteína AFP muestra una localización nuclear en las células *de M. grisea*.

Tal y como se ha indicado anteriormente, la estructura tridimensional de la proteína AFP continene un dominio tipo OB fold que se encuentra presente en otras proteínas que se unen a oligonucleótidos y oligosacáridos (Martínez del Pozo *et al*, 2002). La observación de la localización nuclear de la proteína AFP hizo considerar la posibilidad de que la AFP pudiera interaccionar con el DNA del hongo. Mediante ensayos de retardo de movilidad electroforética de DNA en geles de agarosa, se pudo verificar que la proteína AFP tiene capacidad de unirse al DNA genómico de *M. grisea*. Además, también mediante ensayos de unión *in vitro*, se demostró que la proteína AFP puede unirse tanto a moléculas de DNA de cadena simple como de cadena doble, y que esta unión no es dependiente de la secuencia de nucleótidos. Esta afinidad de AFP por el DNA se podría explicar por la naturaleza básica de la proteína AFP (pI 10,65) a pH neutro, lo cual posibilitaría una unión estable e inespecífica con las cargas negativas del DNA. Además en este estudio de unión AFP-DNA,

se comprobó que la interacción se ve muy afectada por la presencia de sales, corroborando un importante papel de las interacciones iónicas. Además de esta propiedad de interaccionar con el DNA, se observó que la proteína AFP también interacciona con RNA, en este caso tRNA de levadura.

Por otra parte, la proteína AFP y la proteína α-sarcina son las proteínas mayoritarias secretadas por el hongo *Aspergillus giganteus*. Para la α-sarcina se ha descrito una actividad ribonucleásica del tipo RIP (Ribosome Inactivating Protein). Dada la interacción observada entre AFP y RNA, se consideró la posibilidad de que la AFP pudiera tener una actividad ribonucleásica como es el caso de la α-sarcina. En ensayos con reticulocitos de conejo, tal y como era esperable, la proteína α-sarcina hidroliza específicamente el RNA ribosomal de la subunidad 28S, liberando un fragmento de aproximadamente 400nt conocido como fragmento α (Wool *et al*, 1992; Glück y Wool, 1996). En estas mismas condiciones experimentales, sin embargo, no se pudo detectar ninguna actividad ribonucleásica por parte de la proteína AFP (no se observó la producción del fragmento α).

Los resultados presentados en este capitulo permiten concluir que la proteína AFP ejerce su actividad antifúngica frente al fitopatógeno *Magnaporthe grisea*, mediante una combinación de actividades: por un lado induciendo la formación de poros en la membrana plasmática, y por otro, interaccionando con ácidos nucleicos, ya sea DNA o RNA. Existen varios ejemplos en la literatura donde se describe la interacción de proteínas/péptidos antimicrobianos con dianas intracelulares, como es el caso de la proteína buforina, aislada del sapo asiático *Bufo bufo garagriozans*. Esta proteína se une tanto al DNA como al RNA de las células del organismo invasor (Park *et al*, 1998). Otros ejemplos son las proteínas histatina 5 de la saliva humana y la magainina de anfibios, que tienen su diana de acción en la mitocondria (Westerhoff *et al*, 1989; Helmerhorst *et al*, 1999). Tal y como se ha comentado anteriormente, la proteína α-sarcina, también secretada por el hongo *Aspergillus giganteus*, ejerce su función inhibiendo la síntesis de proteínas por hidrólisis específica del rRNA 28S (Olmo *et al*, 2001). Una proteína homóloga a la AFP, la proteína PAF secretada por el hongo *Penicillium chrysogenum* (42,6% de identidad entre ambas), también se localiza en el interior de la célula de hongos susceptibles (Oberparleiter *et al*, 2003). La internalización de esta proteína mostró ser dependiente de un metabolismo activo, de la disponibilidad de ATP y de la presencia de filamentos de actina intactos en la célula. Ello sugiere la existencia de un mecanismo de endocitosis mediado por receptores para la internalización de la proteína PAF. Los mecanismos involucrados en el proceso de internalización de la proteína AFP a través de la membrana y de su transporte hasta el núcleo están todavía por determinar.

Por último, en este trabajo se realizaron ensayos para verificar si la proteína AFP mostraba algún efecto nocivo sobre células vegetales o humanas. Para eso, se utilizó

también el ensayo con el colorante sytox green o bien con protoplastos de arroz, o bien con células humanas HeLa. Los resultados de este estudio indicaron que la presencia de la proteína AFP (incluso en concentraciones elevadas, 24μM), no induce permeabilización en membranas de protoplastos de arroz. Este resultado era previsible ya que plantas de arroz que expresan constitutivamente (Coca *et al*., 2004) o bajo el control de un promotor inducible (Moreno *et al*, 2005) el gen *afp*, muestran un fenotipo y desarrollo normales. Se ha propuesto que las diferencias en la composición y topología de las membranas citoplasmáticas puede condicionar la actividad biológica y los mecanismos de acción de las proteínas y péptidos antimicrobianos (Liang y Kim, 1999). Ello explicaría las diferencias de susceptibilidad que se observan entre microorganismos y células vegetales o animales.

En el caso de las células HeLa, cuando se utilizan concentraciones de AFP superiores a 8 μM (doble de la concentración necesaria para una inhibición total del crecimiento del hongo *M. grisea*, valor MIC) se observa una cierta sensibilización de la membrana plasmática. Aunque el efecto observado sobre células HeLa no es elevado y difícilmente se podrían alcanzar niveles de acumulación de esta proteína en los tejidos de plantas transgénicas tan elevados, hay que tener en consideración este resultado desde el punto de vista de la producción de plantas transgénicas que produzcan esta proteína. De aquí el interés que representa el hecho de poder expresar la proteína AFP, y en general cualquier proteína antifúngica, en plantas transgénicas en régimen de inducibilidad (bajo control de un promotor inducible en tejidos susceptibles de ser infectados por el hongo) y que además no se exprese en tejidos u órganos de la planta que se utilicen para el consumo humano o animal (en este caso, el endospermo de la semilla de arroz).

A modo de resumen, los resultados obtenidos en la presente tesis indican que la proteína AFP es un buen agente antifúngico frente a *M. grisea* y *B. cinerea*, patógenos muy importantes para las plantas de arroz y geranio, respectivamente. La eficacia de esta proteína antifúngica en la protección de arroz frente a piriculariosis ha sido demostrada en plantas transgénicas. En el caso de plantas de geranio, se encuentran en curso los trabajos para la obtención de plantas transgénicas. Asimismo, se ha demostrado la utilidad del promotor del gen *ZmPR4* de maíz para dirigir la expresión del gen *afp* en plantas de arroz de una manera inducible por la infección de *M. grisea* y en condiciones en las que el transgén no se expresa en la semilla de arroz. El promotor *ZmPR4* puede resultar de gran utilidad para la expresión de otros genes antimicrobianos en plantas transgénicas de arroz. Finalmente, se ha avanzado en el conocimiento de los mecanismos por los cuales la proteína AFP ejerce su actividad antifúngica sobre *M. grisea*.

Resultados anteriores del grupo mostraron que la proteína AFP es efectiva para inhibir el crecimiento de muy diferentes fitopatógenos que causan enfermedades en diversos cultivos de interés agronómico (cereales, tomate, patata, etc). Así, además de los hongos

utilizados en este trabajo, son también susceptibles de inhibición por AFP los hongos *Fusarium verticilloides*, *F. lateritium*, *F. proliferatum*, *F. oxysporum* f. sp. *radicis lycopersici*, *Microdochium nivale*, *Magnaporthe salvinii* (Lacadena *et al*, 1995; Vila *et al*, 2001). El oomiceto *Phytophtora infestans* también es inhibido por AFP (Vila *et al*, 2001). Ello hace pensar que las aplicaciones del gen *afp* para la transformación de plantas pueden ir más allá de las contempladas en este trabajo, las cuales están dirigidas a la mejora genética de arroz y en un futuro próximo de geranio. Una de las ventajas de la biotecnología es, precisamente el hecho de que un gen determinado puede ser introducido en diferentes especies vegetales para las cuales se encuentren desarrollados los sistemas de transformación. Lamentablemente, las aplicaciones de la biotecnología en la agricultura se encuentran bastante limitadas debido el rechazo que se observa por parte de la sociedad, sobre todo en algunos países europeos, que definen a la biotecnología como algo no natural y potencialmente peligroso para la salud. Este clima de desconfianza, respaldado en parte por una falta de información, está contribuyendo a la limitación del desarrollo e implantación de las nuevas tecnologías para la mejora genética de plantas cultivadas (Strange y Scott, 2005).

CAPÍTULO I

1) La proteína AFP ejerce actividad antifúngica sobre diferentes cepas del hongo *Botrytis cinerea*, siendo más efectiva frente a la cepa CC1. La concentración de AFP que inhibe el 50% del crecimiento del hongo (valores de IC_{50}) se encuentra en el rango de 0,4 y 1 μM para las cepas ensayadas. La inhibición total (valor MIC) se observa con una concentración de AFP de 10 μM.
2) La proteína cecropina A ejerce actividad antifúngica frente a *B. cinerea* (cepa CC1). Los valores de concentración IC_{50} y MIC encontrados fueron de 20 y 80 μM, respectivamente.
3) Las proteínas AFP y cecropina A cuando se utilizan conjuntamente presentan un efecto antifúngico aditivo frente a la cepa CC1 de *B. cinerea*.
4) La observación microscópica de las diferentes cepas de *B. cinerea* tratadas con la proteína AFP reveló alteraciones morfológicas en el micelio, tales como condensación del micelio, hifas más cortas y extremos abultados.
5) La proteína AFP inhibe la germinación de esporas de las 4 cepas de *B. cinerea* utilizadas.
6) La actividad antifúngica de la proteína AFP es de naturaleza fungicida frente a *B. cinerea*.
7) La aplicación exógena de la proteína AFP sobre hojas de geranio, confiere protección frente a *B. cinerea*.

CAPÍTULO II

1) Se ha llevado a cabo una caracterización funcional de los promotores de tres genes *PR* de maíz, los genes *PRms, mpi y ZmPR4,* en plantas transgénicas de arroz.
2) El promotor *ZmPR4* responde a infección por esporas, tratamiento con elicitores del hongo *Magnaporthe grisea* y a herida mecánica en plantas de arroz.
3) No se observa actividad del promotor *ZmPR4* en el endospermo de semillas de arroz. En flores de plantas expresando el gen *gusA* bajo control del promotor *ZmPR4* se observó actividad en el lema, palea y polen, y no se observó actividad en el pedicelo, ovario, estigma, estilete y anteras.
4) El promotor *mpi* responde a herida mecánica en hojas y tallo, y tiene una expresión constitutiva en raíz. El promotor *mpi* también mostró inducción frente a infección por esporas del hongo *M. grisea*, pero no en respuesta al tratamiento con elicitores de este hongo.
5) No se observa actividad del promotor *mpi* en el endospermo de la semilla ni en el polen de la flor.

6) No se pudo detectar actividad del promotor *PRms* en plantas de arroz ni tampoco en respuesta a los tratamientos utilizados en este trabajo (infección con esporas, tratamiento con elicitores o herida mecánica).
7) Las plantas transgénicas de arroz que expresan el gen *afp* bajo el control del promotor *ZmPR4* son resistentes a la infección por el hongo *M. grisea.*

CAPÍTULO III

1) La proteína AFP induce permeabilización de la membrana plasmática de las células de *M. grisea*. Mediante microscopía electrónica de transmisión se pudo comprobar la presencia de poros en la membrana plasmática de *M. grisea.* Se observan asimismo importantes alteraciones en la morfología y estructura de la célula de *M. grisea* en respuesta al tratamiento con la proteína AFP.
2) El crecimiento o elongación de las hifas de *M. grisea* se detiene en presencia de la proteína AFP.
3) En protoplastos de arroz no se observa daño en la membrana plasmática, cuando se utiliza una concentración de AFP de hasta 24 μM.
4) En células HeLa, la proteína AFP no ejerce efecto sobre la membrana plasmática cuando se utilizan concentraciones de hasta de 6 μM.
5) La proteína AFP penetra en la célula del hongo y se acumula en el núcleo.
6) La proteína AFP es capaz de interaccionar *in vitro* con DNA genómico de *M. grisea*, y también con oligonucleótidos de simple y doble cadena.
7) La proteína AFP se une a tRNA de levadura y no presenta actividad ribonucleásica sobre el RNA ribosomal de reticulocitos de conejo.

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