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Differential toxicity of antifungal protein AFP against mutants of *Fusarium oxysporum*

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Summary. Antifungal protein (AFP) from *Aspergillus giganteus* was assayed for toxicity against the *Fusarium oxysporum* wild-type strain and mutants in genes involved in cell signaling (Δ*pacC*, *pacC*^{c} Δ*fmk1*) or cell-wall biogenesis (Δ*chsV*, Δ*chs7*, Δ*gas1*). The mutants were classified into two groups according to their sensitivity to AFP: Δ*pacC*, Δ*gas1* and Δ*chs7*, which were significantly more resistant to AFP than the wild-type, and *pacC*^{C}, Δ*fmk1* and Δ*chsV*, which were more sensitive. Western blot analysis revealed increased binding of AFP to the three resistant mutants, Δ*pacC*, Δ*gas1* and Δ*chs7*, but also to Δ*chsV*, indicating that differential binding may not be a key determinant for sensitivity. Addition of Ca^{2+} or K^{+} dramatically reduced antifungal activity and binding of AFP, suggesting that these cations compete for the same targets as AFP at the surface of the fungal cell. [**Int Microbiol** 2009; 12(2):115-121]

Keywords: *Fusarium oxysporum* · antifungal protein · chitin synthase · cell wall

Introduction

The control of fungal infections is largely based on the use of chemicals with a more or less narrow spectrum of action. The emergence of resistant pathogenic strains upon prolonged application has triggered considerable interest in the isolation of new antifungal compounds that are more specific and with fewer side effects on human health and the environment. By exploiting the differential activity of a given antifungal compound towards a fungal wild-type strain and defined mutants, insight into its mechanism of action can be gained [8], allowing the development of even more targeted and efficient products.

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Antimicrobial peptides are host defense molecules that are ubiquitous in multicellular plants and animals, as well as in many single cell organisms [32]. Most antimicrobial peptides are cationic molecules, with a net positive charge, that bind to microbial membranes, which are generally negatively charged. The *Aspergillus giganteus* antifungal protein (AFP), a small polypeptide of 51 amino acids, is secreted as an inactive precursor containing six extra amino acid residues at the NH_2-terminal end, which are processed in the extracellular medium [16]. AFP has been assayed against a wide variety of microorganisms, including prokaryotes and eukaryotes, and its inhibitory effect on the growth of many filamentous fungi has been well documented [12,29,31]. Interestingly, AFP is not active against *Penicillium chrysogenum* and *Aspergillus niger*, two organisms producing antifungal proteins similar to AFP (PAF and Anapf, respectively) [29]. AFP binds to phospholipid membranes [12] and to nucleic acids [17]. The in vitro interaction with nucleic acids, resulting from the oligonucleotide/oligosaccharide (OB) fold structure of AFP, suggests that this property might be related to its biological activity [17]. Nonetheless, at present, the

antifungal mode of action of AFP remains largely unknown (for a recent review see [19]).

Fusarium oxysporum is a soil-borne plant pathogen that causes vascular wilt disease on many different plant species worldwide [6]. With the exception of grasses and most tree crops, few of the widely cultivated crops are not hosts to a pathogenic form of this species. Isolates from *F. oxysporum* have been classified into more than 120 different formae speciales according to their host range. More recently, the species has also been reported as an emerging human pathogen in immunocompromised individuals [23,25].

Successful infection of the host plant by *F. oxysporum* requires a series of highly regulated processes. Several groups, including our own, are studying this vascular wilt fungus in attempts to unravel key mechanisms of the fungal infection process by creating mutants in specific genes through targeted gene knockout. This approach has begun to shed light on the mechanisms underlying infection and the development of vascular wilt disease [6]. The targeted inactivation of *fmk1*, which encodes a mitogen-activated protein kinase (MAPK) orthologous to yeast Fus3/Kss1, produced mutants that were unable to penetrate the roots of tomato plants and failed to cause disease symptoms [5]. By contrast, mutants carrying loss-of-function (Δ*pacC*) or gain-of-function (*pacC*^{C}) alleles of the pH signal transcription factor PacC showed increased or reduced virulence, respectively, compared to the wild-type strain [3].

A different mechanism of tolerance to plant defense compounds involves the fungal cell wall. The *F. oxysporum* wall contains an inner layer of chitin and glucan that acts as a support for an external layer of glycoproteins [27]. Chitin synthases catalyze the polymerization of β-1,4-linked *N*-acetyl-D-glucosamine to chitin, a major structural component of the fungal cell wall [26]. Through random insertional mutagenesis screening, a mutant showing complete loss of virulence on tomato was isolated, and the affected gene *chsV* was found to encode a class V chitin synthase [14]. A likely mechanism of ChsV in virulence is to prevent diffusion of plant antifungal compounds to their cellular targets. The *chs7* gene encodes a chaperone-like protein of *F. oxysporum* orthologous to *Saccharomyces cerevisiae* Chs7p [30] and is required for full virulence on tomato plants [15]. The *gas1* gene encodes a β-1,3-glucanosyltransferase of *F. oxysporum*, which is involved in the processing of β-1,3-glucan, another major component of the fungal wall. Mutants lacking Gas1 showed dramatically reduced virulence on tomato plants, both in a root infection assay and in a fruit invasion model [2].

In this study, we used a well-characterized collection of *Fusarium* mutants to test their sensitivity towards AFP. The observation that AFP is not inhibitory to yeasts and has differential effects on filamentous fungal species [29] strongly suggests that the compound has specific target(s) in the fungal cell. *Fusarium* belongs to the group of fungi that are susceptible to AFP [11,12,31]. The goal of this study was to gain further insight into the mode of action of AFP by comparing its inhibitory effect against the *F. oxysporum* wild-type strain and a collection of defined gene knockout mutants. The antifungal activity of AFP was previously reported to be sensitive to cations [29]. Therefore, its inhibitory effect against resistant and sensitive *F. oxysporum* mutants was tested in the presence of 0.1 M $CaCl_2$ or 0.2 M KCl.

Materials and methods

Protein purification. AFP was purified to homogeneity from *Aspergillus giganteus* cultures as described previously [12,16,17,24]. Protein concentration was determined from absorbance measurements based on an $E^{0.1\%}$ (1-cm optical path) at 278 nm of 1.76 [12], using a Nanodrop ND-1000 spectrophotometer.

Fungal strains and culture conditions. Fungal strains used in this work are listed in Table 1. *Fusarium oxysporum* f. sp. *lycopersici* wild-type strain 4287 and mutants derived thereof were stored as microconidial suspensions with 30% glycerol at –80ºC. Fungal strains were grown in potato dextrose broth (PDB) on a rotary shaker at 170 rpm and 28ºC as described [7].

AFP susceptibility assays. Microconidia were collected from PDB cultures by filtration and centrifugation, washed in sterile water, counted, and transferred to fresh PDB or YPG medium (pH 4.5) [11]. The susceptibility of the six *Fusarium oxysporum* f. sp. *lycopersici* mutants to AFP was compared to that of the wild-type. Aliquots containing 5×10^5 or 10^3 microconidia were added to 200 µl PDB or YPG, respectively. When indicated, the media contained AFP at concentrations ranging from 0 to 4 µg/ml in PDB or from 0 to 200 µg/ml in YPG. The inhibitory effect of AFP on *Fusarium* growth in YPG was evaluated after 42 h at 28ºC by determining the optical density at 600 nm (OD_{600}) in a Tecan SpectraFluor Plus spectrophotometer. Experiments were carried out in triplicate in PDB or in duplicate in YPG and repeated at least twice, with similar results. Growth was expressed as percentage of the control without AFP (100%) for each strain.

Table 1. *Fusarium oxysporum* strains used in this study

Strain	Genotype	Source/reference
4287 (FGSC 9935)	*F. o.* f. sp. *lycopersici*, wild type	Fungal Genetics Stock Center
Δ*fmk1*	*fmk1*::*Phleo*^{R}	[5]
Δ*chsV*	*chsV*::*Hyg*^{R}	[14]
Δ*pacC*	*pacC*::*Hyg*^{R}	[3]
pacC^{c}	*pacC*^{c}, *Hyg*^{R}	[3]
Δ*chs7*	*chs7*::*Hyg*^{R}	[2]
Δ*gas1*	*gas1*::*Hyg*^{R}	[15]

Assays for the effect of cations on AFP. The effect of cations on the inhibitory activity of AFP towards *F. oxysporum* was tested in PDB supplemented with 0.1 M $CaCl_2$ or 0.2 M KCl and containing 0–4 μg AFP/ml. Fungal growth was determined after 36 h as described above and expressed as percentage of the control (PDB with 0.1 M $CaCl_2$ or 0.2 M KCl and without AFP). Experiments were performed in duplicate and repeated at least twice with similar results.

Western blot analyses. To determine the binding capacity of AFP to different *F. oxysporum* strains, 2.5×10^6 microconidia/ml of the wild-type strain were pre-germinated in PDB at 28ºC, 170 rpm during 4 h. After this time, different concentrations of AFP (0.02–0.5 μg/ml) were added to 100 μl-aliquots of the germling suspensions, and samples were incubated at 170 rpm, 28ºC, for 2 h. After centrifugation of the samples, the supernatants were removed carefully and concentrated to 20 μl in a Speed-Vac SVC100 (Savant). To quantify unbound AFP, the samples were separated by SDS-PAGE. As a control, 20 μl of the different AFP concentrations without microconidia were loaded.

AFP was detected by Western blot hybridization using a primary rabbit polyclonal anti-AFP antibody [16] at a dilution of 1/3000. After a 1-h hybridization at room temperature, the blots were washed and IgG-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA, USA) was added at a 1/2000 dilution, according to the instructions of the manufacturer.

The effect of Ca^{2+} on AFP binding to microconidia of the different strains was determined by pre-germinating 2.5×10^6 microconidia/ml in PDB at 2ºC and 170 rpm for 4 h, followed by the addition of 0.1 M $CaCl_2$ (final concentration) and a 5-min incubation. The pre-germinated microconidia were then washed with water and incubated with 0.25 μg AFP/ml at 170 rpm, 28ºC, for 2 h. After centrifugation of the samples, supernatants were removed, concentrated to 20 μl in a Speed-Vac, and subjected to SDS-PAGE to determine the unbound AFP fraction by Western blot as described above.

Results

Different sensitivities to AFP of *Fusarium oxysporum* mutants. The mutants could be divided into two groups according to their AFP sensitivity (Fig. 1). The first group consisted of mutants Δ*pacC*, Δ*gas1*, and Δ*chs7*, which were significantly more resistant to AFP than the wild-type strain. For example, growth of the wild-type strain in the presence of 0.75 μg AFP/ml was inhibited by 90% compared to the untreated control, whereas in the resistant mutants inhibition was only between 30 and 50% (Fig. 1A). Relative differences within the group of resistant mutants were also observed. The growth of Δ*pacC* was almost totally inhibited by 100 μg AFP/ml, whereas Δ*gas1* and Δ*chs7* were less affected, even at concentrations of 200 μg/ml (Fig. 2). The second group of mutants consisted of *pacC*^{c}, Δ*fmk1*, and Δ*chsV*, which showed higher sensitivity to AFP than the wild-type strain (Fig. 1B). Within this group, Δ*chsV* was the most sensitive strain, followed by Δ*fmk1* and *pacC*^{c} (Fig. 2). Results similar to those in YPG were also obtained in PDB medium. Resistant mutants Δ*pacC*, Δ*gas1*, and Δ*chs7* showed no decrease in growth at 0.5 μg AFP/ml, whereas sensitive strains *pacC*^{c}, Δ*fmk1* and Δ*chsV* hardly grew at all at this concentration (results not shown).

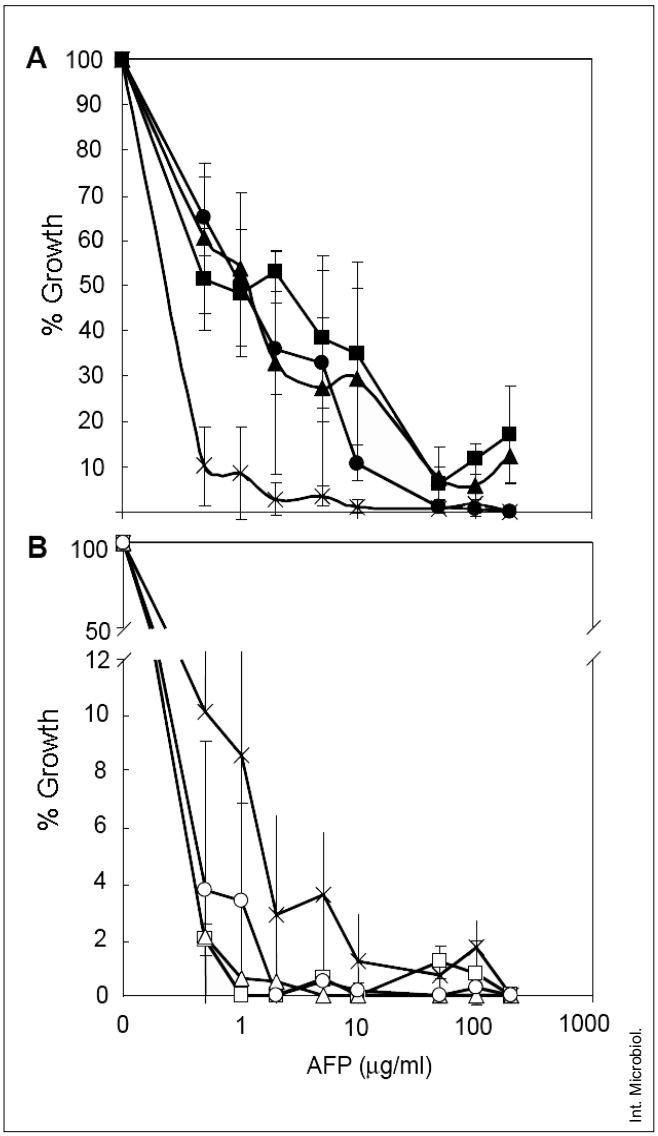

Fig. 1. Antifungal activity of AFP against *Fusarium oxysporum* strains. Results for each strain are expressed as percentage of A_{600} in the control without AFP. (**A**) Wild-type strain (×) and the resistant mutants Δ*pacC* (closed circles), Δ*gas1* (closed squares) and Δ*chs7* (closed triangles). (**B**) Wild-type strain (×) and the sensitive mutants *pacC*c (open circles), Δ*fmk1* (open triangles) and Δ*chsV* (open squares). Mean values and standard deviations were calculated from two independent experiments.

All mutants except D*fmk1* had higher membrane permeability than the wild-type strain when incubated with 0.2 μM Sytox Green. Fluorescence microscopy analysis did not show detectable differences in chitin distribution between the different mutants and the wild-type strain. Similarly, the distribution of cell-wall mannoproteins, as revealed by Concanavalin A staining, did not differ significantly between mutant and wild-type strains (data not shown).

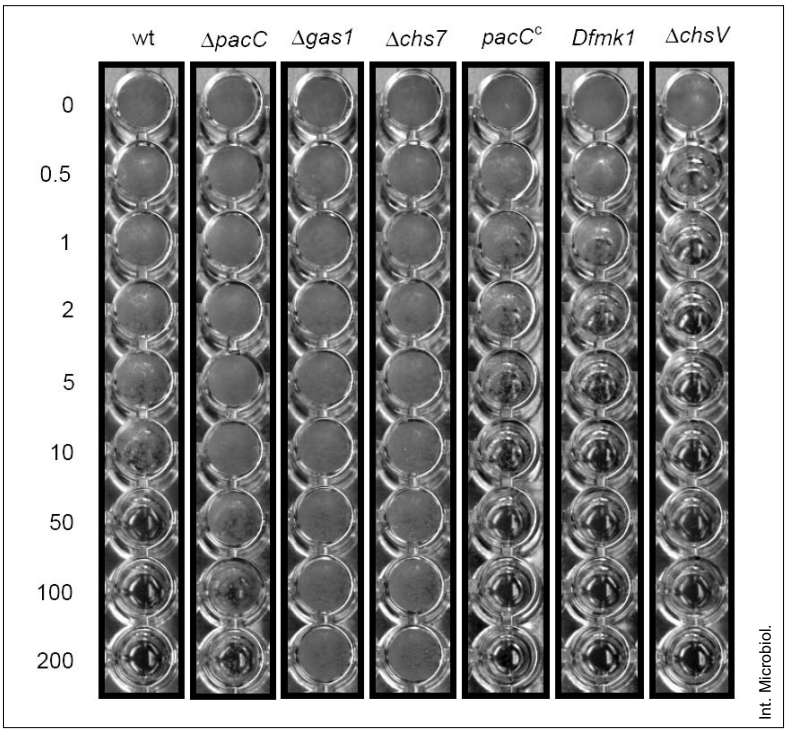

Fig. 2. Growth of *Fusarium oxysporum* strains in the presence of increasing concentrations of AFP (μg/ml). Lack of turbidity in the medium indicates the absence of hyphal growth.

Ca^{2+} and K^+ cations abolishion of the antifungal activity of AFP. The addition of either cation strongly reduced or almost abolished antifungal activity of AFP against the wild-type strain and the sensitive mutants Δ*fmk1* and Δ*chsV* (Fig. 3). $CaCl_2$ was more effective than KCl in reducing AFP antifungal activity. No effect of the two cations was detected in any of the resistant mutants because even the highest concentration of AFP used in the experiment (4 μg/ml) had no inhibitory activity (the Δ*chs7* mutant is shown in Fig. 3 as a representative example).

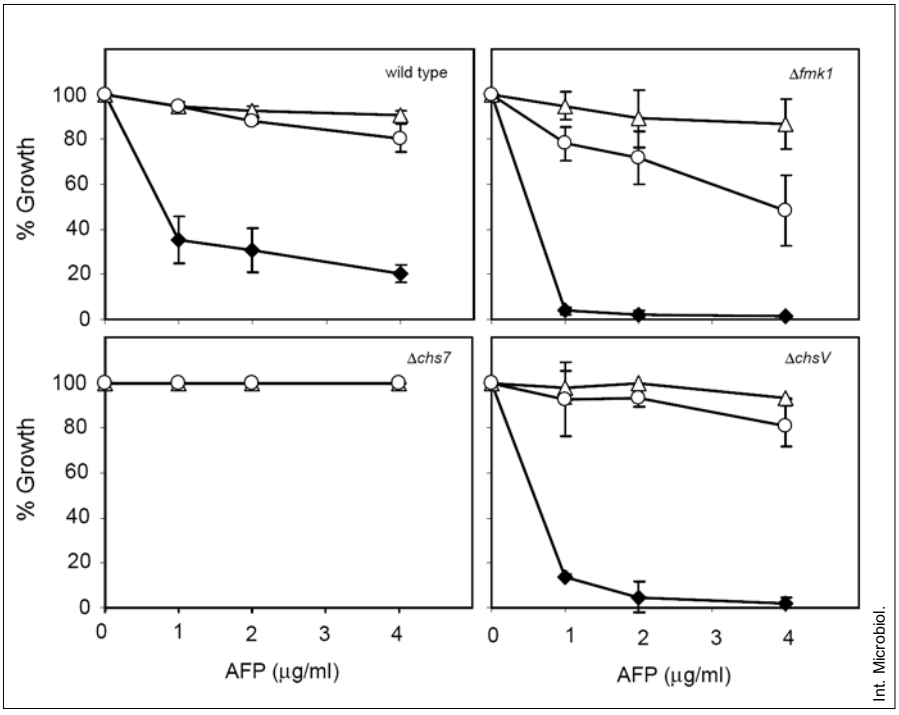

Fig. 3. Suppression of the antifungal activity of AFP on *Fusarium oxysporum* by cations. Aliquots of 5×10^5 microconidia of the indicated strains were added to 96-well microtiter plates containing PDB medium supplemented with the indicated concentrations of AFP in the absence (closed diamonds) or presence of 0.1 M CaCl2 (open triangles) or 0.2 M KCl (open circles), and incubated for 36 h at 28ºC. Hyphal growth was measured by determining optical density at 600 nm. Results for each strain are expressed as percentage of A_{600} in the control without AFP. Mean values and standard deviations were calculated from two independent experiments.

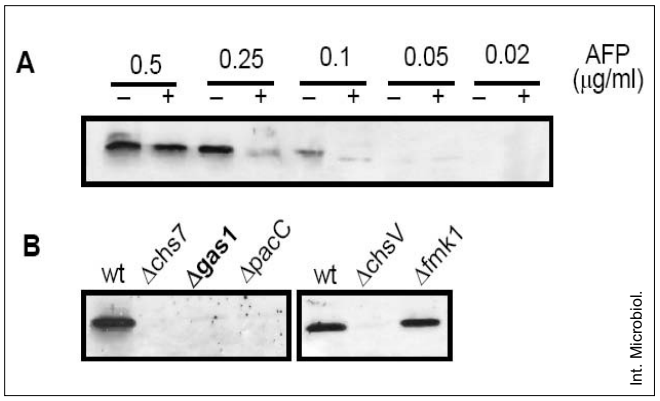

Fig. 4. Binding of AFP does not correlate strictly with sensitivity. (**A**) Solutions containing the indicated concentrations of AFP were incubated for 2 h in the absence (–) or presence (+) of 2.5×10^6 microconidia of the *F. oxysporum* wild type strain/ml. Conidia were removed by centrifugation and supernatants fractionated by SDS PAGE, blotted to membranes, and hybridized with anti-AFP antibody. (**B**) Solutions containing 0.25 mg AFP/ml AFP were incubated for 2 h with 2.5×10^6 microconidia of the indicated strains/ml and analyzed by Western blot as indicated in (A), but using a longer exposure time. The absence of a hybridizing band in the supernatant indicates increased binding of AFP to the fungal surface.

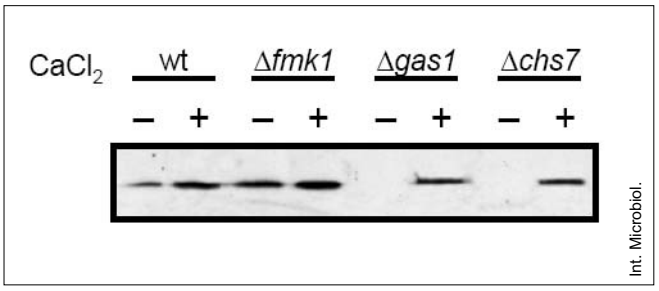

Fig. 5. Ca2+ inhibits AFP binding to Fusarium oxysporum microconidia. Solutions containing 0.25 mg AFP/ml were incubated for 2 h with 2.5×10^6 microconidia the indicated strains /ml in the absence (–) or presence (+) of 0.1 M $CaCl_2$ and analyzed by Western blot as indicated in Fig. 4.

Ability of AFP to bind to *F. oxysporum*, and inhibition of binding by Ca^{2+}. When decreasing concentrations of AFP were added to pre-germinated microconidia of the wild-type strain, depletion of AFP from the supernatant, due to binding of the protein to fungal cells, was clearly detected at concentrations of 0.25 μg/ml and 0.1 μg/ml (Fig. 4A). Accordingly, 0.25 μg/ml was then used to determine AFP binding to the different mutant strains. As seen in Fig. 4B, all resistant mutants showed significantly increased binding to AFP compared to binding by the wild-type strain whereas binding by the two sensitive mutants Δ*chsV* and Δ*fmk* differed. Specifically, the Δ*chsV* strain bound more avidly to AFP, while binding of Δ*fmk* was comparable to that observed for the wild-type. These results suggest that binding of AFP to the fungal spore is not a key determinant of sensitivity or resistance to this antifungal peptide.

As shown above, the antifungal activity of AFP was abolished in the presence of cations such as Ca^{2+} and K^+. When 0.25 μg AFP/ml was added after incubation of pre-germinated microconidia with 0.1 M $CaCl_2$, significantly more unbound AFP was detected in both classes of mutants as well as in the wild-type strain (Fig. 5), suggesting that cations interfere with AFP binding to *F. oxysporum*.

Discussion

AFP is a naturally derived molecule that efficiently inhibits the growth of a wide range of filamentous fungi, including agronomically important plant pathogens such as *Magnaporthe grisea* [21], *Botrytis cinerea* [20], *F. oxysporum* [11, this work] and others [31], as well as the human pathogen *A. fumigatus* [29]. Its fungitoxic properties and apparent lack of cytotoxicity towards mammalian cells [28] make AFP a promising candidate for antifungal intervention. AFP expression in transgenic rice plants, either from a constitutive or a pathogen-inducible promoter, has been shown to confer enhanced resistance to rice blast disease [4,22]. Besides AFP, other small fungal proteins with similar characteristics have been identified, including PAF from *Penicillium chrysogenum* [18] and Anafp from *A. niger* [10]. This family of AFP-like proteins represents a promising and largely untapped resource for agronomical and medical applications [19].

An essential prerequisite for exploiting the biotechnological potential of AFP is the precise elucidation of its cellular targets and mode of action. A number of mechanisms have been proposed for AFP's antifungal activity, including binding and destabilization of the plasma membrane [21,29], inhibition of chitin synthesis [11], nuclear localization and binding to nucleic acids [17,21], or a combination thereof. So far, none of these mechanisms has been conclusively linked to antifungal activity [19], and other modes of action therefore cannot be ruled out.

In the present study, significant differences in strain susceptibility were noted. For instance, *F. oxysporum* mutants lacking the β-1,3-glucanosyltransferase Gas1 or the chaperone-like protein Chs7 were resistant to AFP concentrations as high as 200 μg/ml, while growth of the wild-type was almost completely inhibited by 10 μg/ml (see Fig. 2). By contrast, susceptible mutants lacking the MAPK Fmk1 or the chitin synthase ChsV were inhibited by 2 and 1 μg AFP/ml, respectively. The increased susceptibility of the Δ*chsV* mutant was surprising, since a previous study had reported enhanced resistance of this strain to AFP [11]. The reason for this discrepancy is unclear, but we consider it unlikely that it is due to differences in methodology, because the experimental con-

ditions (microtiter plates, OD_{600}) and the medium (YPG) used in the present work were comparable to those of the previous study. To confirm the results obtained in YPG, the experiments were repeated in a different medium (PDB), yielding essentially the same results. Thus the higher sensitivity of the *Fusarium* Δ*chsV* mutant to AFP appears to be significant. Note that Δ*chsV* was previously shown to have increased sensitivity to the structurally unrelated plant saponin α-tomatine [14].

Our results demonstrate that mutations in a single gene can dramatically affect the interaction between AFP and its unknown cellular target(s). However, since the mutants tested in this study have mostly pleiotropic phenotypes, at present we can only speculate on the mechanisms underlying the differences in AFP susceptibility/resistance. A feature common to these strains is the altered structure of the cell wall. Some of the mutations (Δ*chsV,* Δ*gas1,* Δ*chs7*) directly affect genes involved in cell wall biogenesis [2,14,15], while others (Δ*fmk1,* Δ*pacC*) target signaling components that regulate a wide array of cellular functions, including cell-wall structure [1,5]. The results from Western analysis, showing that AFP binds differentially to the surface of these strains, support the idea that differences in sensitivity between the mutants are related to cell-wall alterations. The fact that no clear correlation between sensitivity and binding affinity was detected—both resistant (Δ*gas1,* Δ*chs7*) and sensitive (Δ*chsV*) mutants showed increased binding to AFP—suggests that binding itself is not a major determinant for AFP activity, although it may be a prerequisite for inhibition.

Why mutations in certain cell-wall genes (*gas1, chs7*) lead to enhanced AFP resistance while others (*chsV*) increase susceptibility is currently an open question. One possible explanation is that some of these genes carry out divergent functions in cell-wall architecture and maintenance. In the Δ*gas1* mutant, which is highly resistant to AFP, transcript levels of the *chsV* gene are much higher than in wild-type cells [2]. If increased expression of *chsV* were related to AFP resistance, then a mutant lacking *chsV* should be more sensitive to the antifungal peptide. In this context, it is worth noting that the Δ*gas1* mutant is also highly resistant to protoplasting enzymes [2], whereas Δ*fmk1* and Δ*chsV* are more sensitive than the wild-type strain (Martínez-Rocha et al., unpublished data). The structural bases for these differences are currently unknown, but sensitivity to cell-wall-degrading enzymes and to AFP might be functionally related. In fact, AFP can bind chitin in vitro and, similar to certain chitinases, harbors a putative chitin-binding domain at its N-terminus [11,13].

To determine whether cations inhibited the binding of AFP to the different fungal strains, the amount of non-bound AFP was assayed by Western blot analysis in the absence and presence of 0.1 M $CaCl_2$ in the wild-type, two resistant strains (Δ*gas1* and Δ*chs7*), and one sensitive (Δ*fmk1*) strain. The results showed that the antifungal activity of AFP against the wild-type strain and the susceptible mutants was essentially abolished in the presence of cations (Ca^{2+}, K^+). This finding confirms those of a previous report showing that the growth inhibitory activity of AFP, as well as its membrane permeabilization effect, was sensitive to cations [29]. Moreover, our Western blot data demonstrate that cations interfere with AFP binding to the fungal surface. Since AFP itself also has a positive net charge, cations might exert their inhibitory effect on AFP activity by competing for putative binding sites at the fungal cell surface.

In summary, the present study shows that mutations in single genes have significant effects on the sensitivity and binding of fungal strains to the antifungal protein AFP. Pinpointing the molecular bases underlying these differences should provide further insight into the antifungal mode of action of AFP and extend our understanding of the biological role of this conserved family of proteins in filamentous fungi.

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