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New insights into the target site and mode of action of the antifungal protein of *Aspergillus giganteus*

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Abstract

The antifungal protein (AFP) secreted by *Aspergillus giganteus* exerts growth inhibitory effects on various filamentous fungi. In order to obtain more information on the mode of action of AFP, we used transmission electron microscopy in this study to compare the cellular ultrastructure of the AFP-sensitive *Aspergillus niger* and of the AFP-resistant *Penicillium chrysogenum* upon AFP treatment. Furthermore, AFP was localized by immunogold staining in both fungi. Severe membrane alterations in *A. niger* were observed, whereas the membrane of *P. chrysogenum* was not affected after treatment with AFP. The protein localized predominantly to a cell wall attached outer layer which is probably composed of glycoproteins, as well as to the cell wall of *A. niger*. It was found to accumulate within defined areas of the cell wall, pointing towards a specific interaction of AFP with cell wall components. In contrast, very little protein was bound to the outer layer and cell wall of *P. chrysogenum*. For future applications of AFP as an antimycotic drug, the mode of action of the protein was further characterized. The protein was found to act in a dose-dependent manner: it was fungistatic when applied at concentrations below the minimal inhibitory concentration, but fungicidal at higher concentrations. Using an in vivo model system, we were able to finally show that AFP indeed prevented the infection of tomato roots (*Lycopersicon esculentum*) by the plant-pathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici*.
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Keywords: *Aspergillus giganteus*; Antifungal protein; Localization; Fungistatic/fungicidal; Infection by *Fusarium oxysporum*

1. Introduction

Proteins with antifungal activity have been isolated from various organisms ranging from bacteria, plants, insects and amphibians to human beings. Both their fungal target site and their mode of action are extremely diverse (for review see [23]). However, despite the ubiquitous occurrence of antifungal proteins, none of these proteins are currently being utilized in either food preservation or in treating clinically relevant pathogenic fungi. One reason for this discrepancy might be that data on the mode of action of these proteins is sparse. In order for it to be applied, an antifungal protein needs to fulfill several prerequisites such as antifungal activity in vivo and lack of effects on the host cells. Furthermore, resistance mechanisms need to be excluded as far as possible. Therefore, investigation of the target site and the mode of action of an antifungal protein should reveal whether the protein is suitable for an application.

The antifungal protein (AFP) which is secreted by the imperfect ascomycete *Aspergillus giganteus* [19] is a small basic protein which has been shown to inhibit the growth of several filamentous fungi, mainly from the genera *Fusarium* and *Aspergillus* [11,22,27]. The protein consists of 51 amino acids and is folded into a small compact structure with four stabilizing disulfide bridges [15]. AFP causes membrane permeabilization in AFP-sensitive fungi by an as yet

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poorly understood mechanism, and its minimal inhibitory concentrations (MICs) range from 0.1 µg/ml for *Fusarium moniliforme* IfGB 39/1402 up to 200 µg/ml for *Aspergillus nidulans* G191. However, the antifungal activity of AFP is strongly diminished in the presence of cations [22].

The localization of AFP within sensitive and resistant fungi by means of immunofluorescent staining revealed that the protein binds to the cell wall or the membrane of AFP-sensitive fungi, and it is localized intracellularly in AFP-resistant fungi [22]. However, immunofluorescent staining with subsequent fluorescent microscopic analysis did not allow further characterization of the target site of AFP.

In the present study, we used transmission electron microscopy (TEM) for structural analysis of the AFP-sensitive fungus *A. niger* and the AFP-resistant fungus *P. chrysogenum* after treatment with AFP. We also localized the protein within these fungi by immunogold staining with subsequent TEM analysis in order to obtain more information on the cellular target site of AFP.

As AFP inhibits the growth of several plant pathogenic fungi in vitro, it might be suitable as a substitute for classical fungicides. We therefore tested the heat stability of AFP and sought to determine whether AFP acts in a fungistatic or fungicidal manner. Vila et al. reported that rice plants are protected against *Magnaporthe grisea* infection by direct application of AFP to rice leaves [26]. Another example for a possible future application of AFP would be the protection of tomato plants against the pathogen *F. oxysporum*, which is highly sensitive to AFP [22]. *F. oxysporum* is a soil inhabitant that colonizes and infects tomato roots and causes wilt diseases of worldwide economic importance [9]. We show in this study, that AFP also holds promise in protection of tomato plants, as the protein was found to prevent infection of tomato roots by *F. oxysporum* f. sp. *lycopersici*.

2. Materials and methods

2.1. Strains and materials

The fungal strains *A. giganteus* MDH 18894, *A. niger* IFGB15/1802, *F. oxysporum* f. sp. *lycopersici* and *P. chrysogenum* ATCC 10002 were selected from the strain collection of the Institut für Gärungsgewerbe, Berlin. The tomato seeds *Lycopersicon esculentum* (Harzglut F1) were purchased from Saatzucht Quedlinburg (Quedlinburg, Germany). The AFP-specific antibody (0.3 mg specific Ig Y/ml) was purchased from Biogenes (Berlin, Germany) and the gold-conjugated antibody (10 nm colloidal gold EM grade) was purchased from British Biocell International (Cardiff, UK). All chemicals used for immunogold experiments and transmission electron microscopic analysis were EM grade.

2.2. Protein purification and MIC assay

A. giganteus was grown for 96 h at 28 °C in 2-l Erlenmeyer flasks each containing 500 ml culture medium composed of 2% soluble starch, 1% beef extract, 2% peptone and 0.5% NaCl. The culture was subsequently incubated at 37 °C for 20 h. Both, the purification of AFP from the culture supernatant of *A. giganteus* and the quantification of its antifungal activity by the determination of minimal inhibitory concentrations (MIC) using *A. niger* as a test strain were performed as described previously [22]. The putative temperature sensitivity of AFP was analyzed by treating AFP, diluted in solution E (0.05 M sodium acetate, 0.1 M NaCl), at different temperatures, ranging from 60 to 100 °C. Preincubated AFP was subsequently assayed for its MIC on the test strain *A. niger*.

2.3. Analysis of the fungicidal and fungistatic effect of AFP

Conidia of the AFP-sensitive fungus *A. niger* were cultivated in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose, (pH 4.5)) containing different AFP concentrations, ranging from 1 ng/ml up to 100 µg/ml. After 48 h of incubation, the AFP solution was removed and conidia and mycelia were washed three times with YPG medium. Subsequently, fungi were incubated for an additional 72 h in YPG medium without AFP. For negative control, conidia of *A. niger* were cultivated in YPG medium lacking AFP for 48 h, washed and further cultivated for 72 h as described above. A fungistatic effect of AFP was assumed if growth of *A. niger* was reduced during AFP treatment, but comparable to the negative control after replacement of the medium. The growth inhibitory effect was considered fungicidal if no growth could be detected after 72 h incubation in YPG medium lacking AFP.

2.4. Transmission electron microscopy (TEM)

2.4.1. Cultivation of fungi

24-well microplates (TPP, Switzerland) containing 1 ml YPG medium per well were inoculated with 3×10^6 conidia. All subsequent steps, including AFP treatment and sample fixation were carried out in 24-well microplates. The fungi were cultivated for 18 h at 28 °C with continuous agitation at 120 rpm. Subsequently, 100 µl AFP solution was added to final protein concentrations of 1 µg/ml up to 300 µg/ml. For negative controls, 100 µl solution E without AFP was added. The supernatants were removed after 1 h and the hyphae were washed three times with 1 ml PBS buffer (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4; (pH 7.4)) for 10 min at room temperature without agitation. The mycelia were subsequently subjected to the fixation procedure.

2.4.2. Paraformaldehyde fixation for immunoelectron microscopy

After removing excess PBS buffer, the mycelia were treated with 4% paraformaldehyde in PBS buffer (w/v) (pH. 7.4) for 1 h at 4 °C without agitation. Subsequently, the

mycelia were washed three times with PBS buffer and were then subjected to the embedding procedure in methacrylate.

2.4.3. Combined glutaraldehyde and reduced osmium tetroxide fixation for standard electron microscopy

Fungi were fixed with 2.5% glutaraldehyde in cacodylate buffer (v/v) (pH 7.3) for 1 h at 4 °C without agitation. After washing with cacodylate buffer (21.4 g/l sodium-cacodylate × $3H_2O$; (pH 7.3)) for 10 min at room temperature, samples were secondarily fixed with reduced osmium tetroxide (0.5% OsO_4 and 0.75% potassium ferrocyanide in cacodylate buffer (pH 7.3)) for 1 h at room temperature. After three washing steps with cacodylate buffer for 10 min at room temperature, fungi were subjected to the embedding procedure in epoxy resin.

2.4.4. Embedding for immunoelectron microscopy

Mycelia were dehydrated in standard ethanol series (15 min treatment with 50, 70, 90% ethanol each, and 3 × 15 min with 100% ethanol). Resin infiltration was carried out by preincubation of 50% Lowicryl in 100% ethanol (v/v) for 1 h at 4 °C and pure Lowicryl incubation for 20 h at 4 °C without agitation. Mycelia then were transferred into gelatine capsules (Merck, Darmstadt) and were polymerized for 48 h under UV light.

2.4.5. Embedding for standard electron microscopy

Samples that were fixed with glutaraldehyde and reduced OsO_4 were dehydrated in standard ethanol series, preincubated in 100% ethanol containing 50% of Spurr's low-viscosity resin (v/v) for 18 h at 4 °C, finally infiltrated twice with 100% Spurr resin [21] for 2 h at room temperature and polymerized at 60 °C for 48 h.

2.4.6. Preparation of ultrathin sections

Both methacrylate and epoxy blocks were trimmed with a Leica Ultratrim (Leica). Semithin (300 nm), and ultrathin sections (50–90 nm) were cut with an Ultracut UCT (Leica) using diamond knives. Methacrylate sections were mounted on gold slot grids using pioloform support films and immunostained (see below). Epoxy sections were mounted on regular copper hexagonal grids, double-stained with uranyl acetate and lead citrate and examined on a Zeiss 902 TEM.

2.4.7. Immunogold staining

All steps of the immunogold staining were carried out at room temperature. Lowicryl sections were treated twice with blocking solution (BS: 0.1 M PBS containing 1% bovine serum albumin (pH 7.3)) for 15 min. Further incubation for 1 h in the primary antibody (chicken anti AFP, dilution 1:100 and 1:200 in BS) was followed by three washing steps with BS for 5 min. Secondary, gold-conjugated antibody (rabbit anti-chicken, dilution 1:50 in BS) was applied for 1 h. To reduce background staining, sections were rinsed with blocking solution for 5 min, followed by 0.1 M PBS for 2 min each and three washing steps with distilled H_2O for 5 min each. Sections were then dried under red light, double-stained with uranyl acetate and lead citrate and examined with a Zeiss 902 TEM.

2.5. Cultivation and infection of tomato plants

Artificial cultivation and infection of tomato seedlings with spores of *F. oxysporum* f. sp. *lycopersici* was performed according to the protocol of Dolej [4]. Tomato seeds were grown in a glass Petri dish filled with sterile, wet silica sand. The seeds were washed with sterile water and subsequently sowed with tweezers. After 2 days at 28 °C in the dark, the plants were incubated for an additional 8 days with a day/night rhythm of 16/8 h at 25 °C. Tomato seedlings were subsequently transferred to test tubes containing 10 ml liquid fertilizer (0.88 g/l $Ca(NO_3)_2{\cdot}4H_2O$, 0.126 g/l $NaH_2PO_4{\cdot}2H_2O$, 0.39 g/l K_2SO_4, 0.31 g/l $MgSO_4{\cdot}7H_2O$, 0.0031 g/l EDTA, pH adjusted to 5.8 with 1 M KOH). The liquid fertilizer was supplemented with 10 µl trace element solution (1.01 mg/l $MnSO_4{\cdot}4H_2O$, 0.56 mg/l H_3BO_3, 0.098 mg/l $CuSO_4{\cdot}5H_2O$, 0.124 mg/l $ZnSO_4{\cdot}7H_2O$, 0.322 mg/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) and different AFP concentrations ranging from 1 to 100 µg/ml. The test tubes were sealed with parafilm, and the tomato seedlings were transferred through a 4 mm hole in the parafilm. The roots of the seedlings were completely covered with liquid fertilizer. Each AFP concentration was tested on four tomato seedlings. After 10 days of further cultivation, the liquid fertilizer containing AFP was sucked off. The roots of the seedlings were washed three times with 10 ml distilled H_2O. Subsequently, the test tubes were filled with 10 ml fresh liquid fertilizer containing 2×10^7 *Fusarium oxysporum* conidia. The tomato seedlings were cultivated for an additional 10 days and were visually monitored for signs of vascular wilt disease.

3. Results

3.1. Ultrastructural analysis of AFP-treated fungi

AFP-treated cells were analyzed by TEM to investigate whether treatment of fungi with AFP leads to alterations in cellular structures. The experiments were carried out using both the AFP-sensitive fungus *A. niger* whose growth is completely inhibited at an AFP concentration of 1 µg/ml (MIC), and the AFP-resistant fungus *P. chrysogenum* [22]. After 1 h of treatment with AFP, mycelia of *A. niger* were fixed with 2.5% glutaraldehyde and subsequently with 0.5% OsO_4. This procedure yielded a very clear cellular ultrastructure as illustrated in Fig. 1. AFP-untreated cells contained one or two vacuoles and several nuclei (Fig. 1a). Furthermore, the cell wall of *A. niger* was surrounded by an outer layer. In contrast, *A. niger* hyphae treated with 100 µg/ml AFP showed a very different ultrastructure (Fig. 1b).

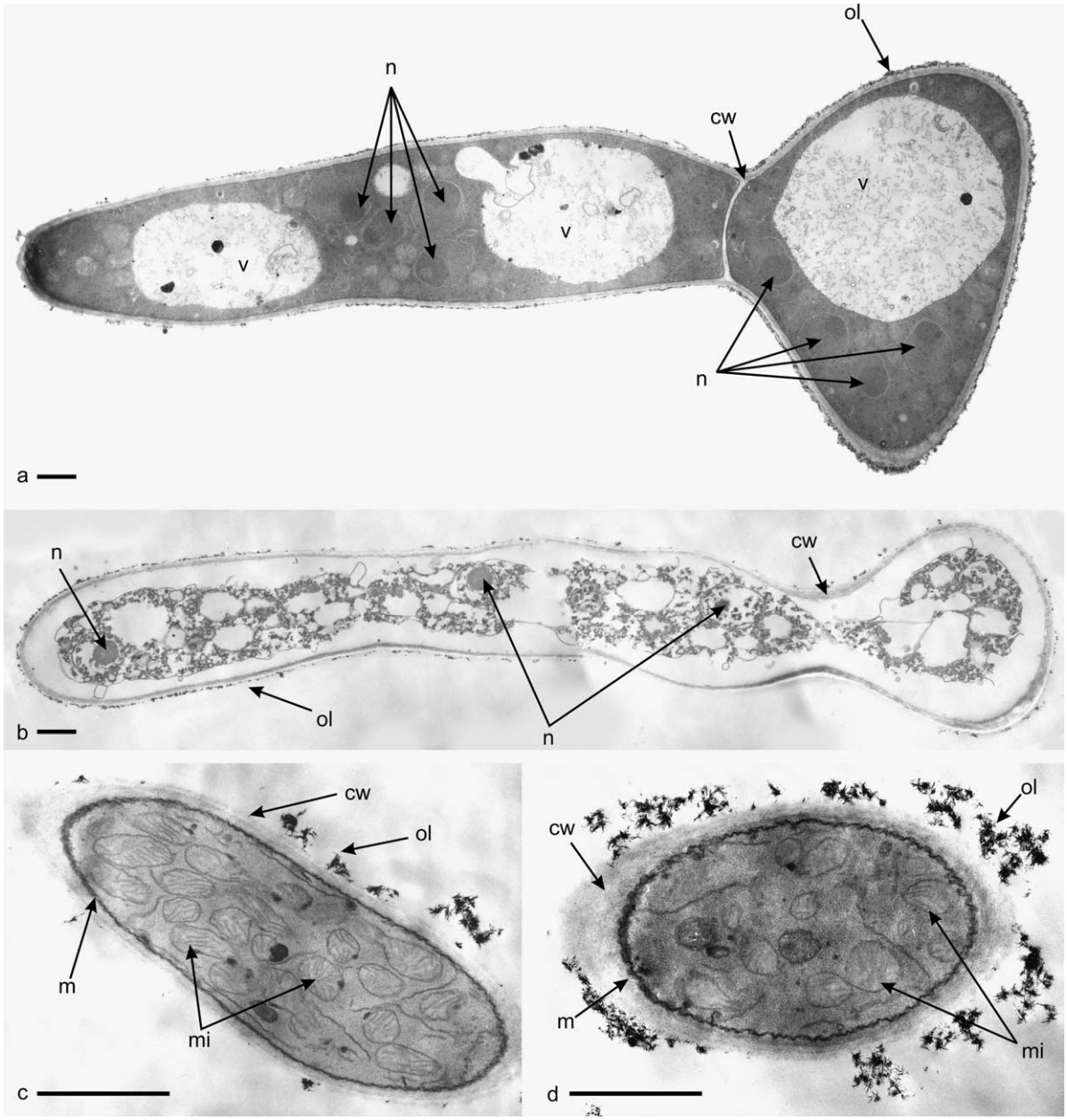

Fig. 1. Ultrastructure of *A. niger* and *P. chrysogenum*. (a) Two compartments of an AFP-untreated *A. niger* hyphae. The cells display clearly defined vacuoles, several nuclei inside one compartment, an intact cell wall and a cell-wall-associated outer layer. (b) Treatment of *A. niger* with 100 μg/ml AFP for 1 h led to cell death, indicated by a collapsed cytoplasm and a large number of aberrant vacuoles. The outer layer disappeared partially. (c) Ultrastructure of *P. chrysogenum* without AFP treatment. (d) Treatment of *P. chrysogenum* with 100 μg/ml AFP for 1 h did not lead to any detectable ultrastructural alterations. cw: cell wall; cp: cytoplasm; ol: outer layer; v: vacuole; n: nucleus; m: membrane; mi: mitochondrium. Bars: 1 μm.

Although the cell wall seemed to be intact, the cytoplasm of the cells had collapsed and all intracellular structures had started to disintegrate. Many vacuoles were detected, most of which showed an aberrant shape. The nuclei were still present but partially lacked nuclear membranes. Furthermore, the outer layer had partially disappeared. In summary, these hyphae showed signs of cellular death.

Analysis of semi-thin sections demonstrated that approximately 25% of all hyphae showed a similarly altered ultrastructure after treatment with 100 μg/ml AFP for 1 h compared to only 2% of the untreated *A. niger* cells (data not shown), demonstrating that the observed alterations were due to treatment of the cells with AFP.

The ultrastructure of the untreated AFP-resistant *P. chrysogenum* (Fig. 1c) varied significantly from that of *A. niger*. The main differences were the higher amount of mitochondria and the different structure of the plasma membrane. Remarkably, while the outer layer of *A. niger* completely surrounded the hyphae, only scattered fragments were found at *P. chrysogenum* hyphae (Figs. 1c and 1d). Treatment of *P. chrysogenum* with 100 μg/ml AFP for 1 h led neither to a detectable alteration in membrane structure, nor to any

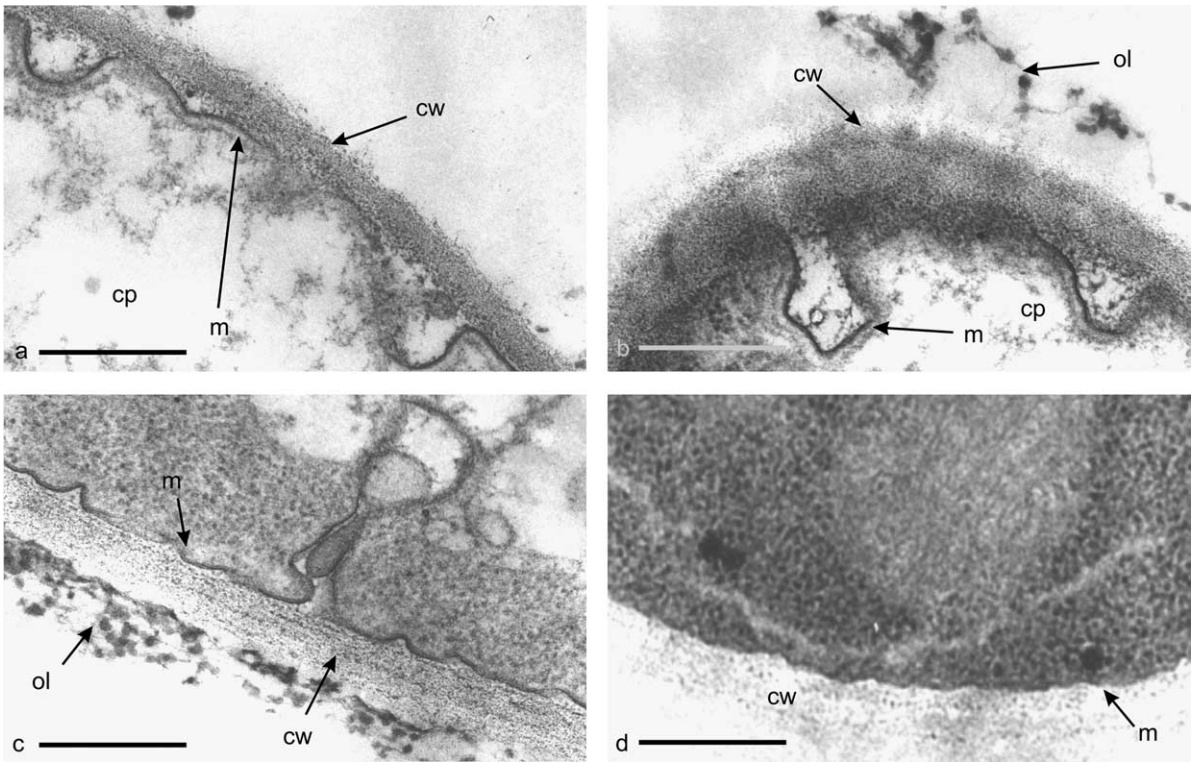

Fig. 2. Plasma membrane alterations after AFP treatment. TEM analysis of cross-sections of *A. niger* treated with 1 µg/ml AFP for 1 h. (a, c) Altered membrane structure on lateral wall. (b) Enhanced alteration of the plasma membrane at hyphal tips. (d) Ultrastructure of *A. niger* without AFP treatment. cp: cytoplasm; cw: cell wall; ol: outer layer; m: plasma membrane. Bars: 0.5 µm.

change in the structure of the mitochondria or other intracellular structures (Fig. 1d), clearly demonstrating that *P. chrysogenum* cells are not affected by AFP.

Analysis of *A. niger* cells treated with a concentration corresponding to the MIC (1 µg/ml) revealed that this was large enough to cause membrane alterations to *A. niger* (Fig. 2). Striking changes in the membrane, comprising "bulb-like" structures, were often observed at the lateral plasma membranes (Figs. 2a and 2c) and also at the hyphal tip (Fig. 2b). These alterations in the membrane were not detected within untreated *A. niger* hyphae (Fig. 2d), indicating that their occurrence is provoked by AFP.

3.2. Localization of AFP by immunogold staining

Localization of AFP by immunogold staining was carried out with *A. niger* and *P. chrysogenum* which were treated with 1 µg/ml AFP for 1 h, fixed with 4% paraformaldehyde and embedded in Lowicryl resin as described in Section 2.

TEM analysis of sections fixed with paraformaldehyde revealed loss of ultrastructural information (Figs. 3–5) in comparison to fixation with glutaraldehyde and subsequent treatment with OsO_4 (Figs. 1, 2). However, the latter method of fixation as well as fixation with glutaraldehyde alone prevented binding of the AFP-specific antibody. Therefore, paraformaldehyde fixation was preferred.

As can be seen in Figs. 3b and 3c, a considerable amount of AFP was detected within the outer layer and the cell wall of *A. niger*. Minor amounts of the protein were associated with the membrane (Fig. 3b). Some AFP was detected inside a vacuole (Fig. 3d), but this was only rarely observed. In general, no AFP was detected in mitochondria or nuclei. Interestingly, AFP seemed to accumulate at distinct areas within the cell wall of *A. niger* (Fig. 3b). Fig. 3e shows an *A. niger* hyphae which was not treated with AFP. The absence of any labeling clearly demonstrates specific detection of AFP within *A. niger* and rules out cross-reactions of the antibodies used.

Immunogold staining with the AFP-resistant fungus *P. chrysogenum* showed that some AFP was internalized by *P. chrysogenum*, as reported in Fig. 4e. It had not bound to a specific intracellular organelle, and was thus localized in the cytoplasm. A very small amount of AFP was localized at the vacuolar membrane or inside the vacuoles (Figs. 4c and 4e), the cell wall (Fig. 4d), and to some extent at the outer layer (Fig. 4b). No labeling of any structures was observed when immunogold staining with *P. chrysogenum* hyphae not treated with AFP was carried out (Fig. 4f).

Immunogold staining was also performed with mycelia from *A. niger* treated with 300 µg/ml AFP, which is far above the MIC (Fig. 5). The protein was localized at the outer layer, the cell wall and the membrane. Only minor amounts of the protein were detected inside living cells (Fig. 5a). Notably, many cells showed the first signs of cell death, as the intracellular structures of these cells had started to disintegrate (Fig. 5b). Here, a high amount of AFP was

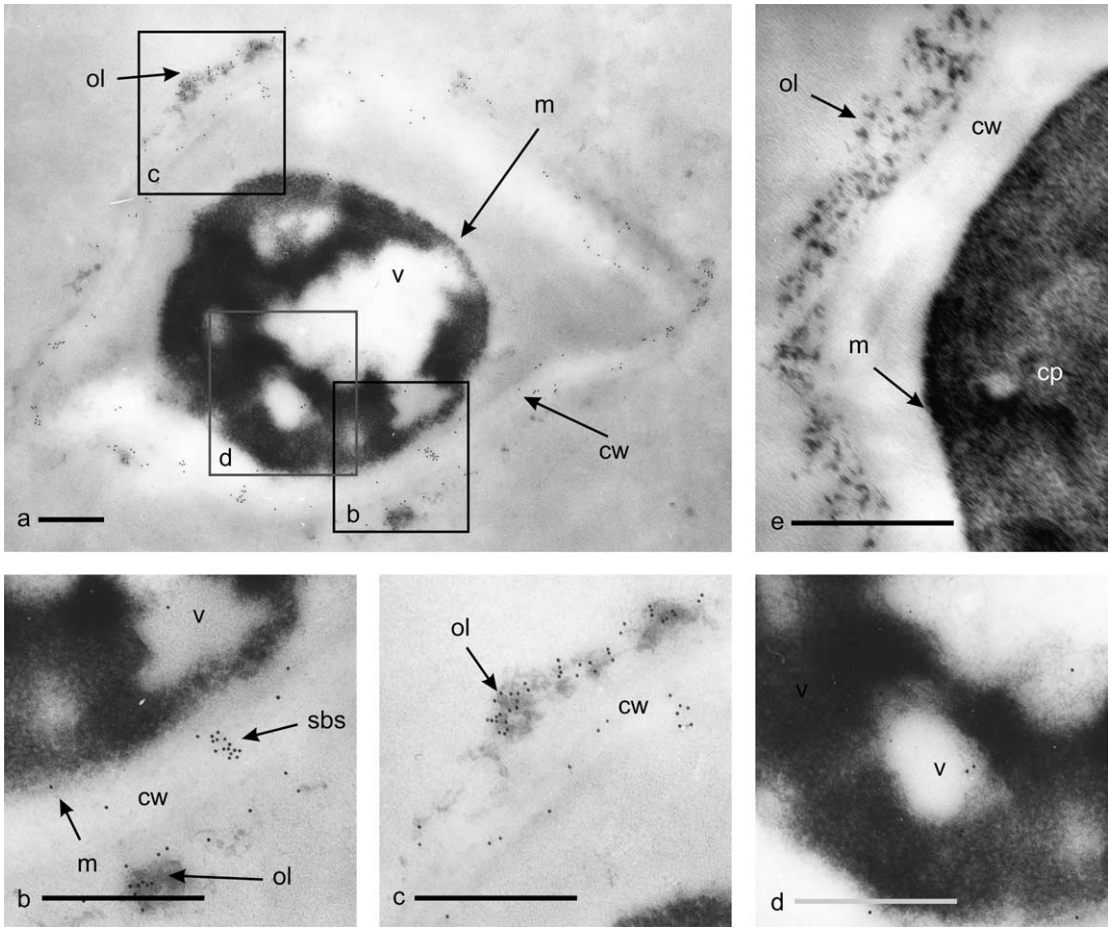

Fig. 3. Localization of AFP within *A. niger* by immunogold staining. TEM analysis of immunogold-stained cross-sections of *A. niger* treated with 1 µg/ml AFP for 1 h. Due to the fixation and embedding protocol the cell wall is not stained and appears as a light area. Black dots represent gold-stained AFP. (a) Cross-section of *A. niger* hyphae (overview). (b) Detailed view of (a), showing localization of AFP at putative specific binding structures (sbs) within the cell wall of *A. niger* and binding of AFP to the membrane and the outer layer. (c) Detailed view of (a), showing binding of AFP to the cell wall and the outer layer. (d) Detailed view of (a), showing minor amounts of AFP at the plasma membrane and inside a vacuole of *A. niger.* (e) Negative control; *A. niger* without AFP treatment. cp: cytoplasm; cw: cell wall; m: plasma membrane; ol: outer layer; sbs: specific binding structure; v: vacuole. Bars: 0.5 µm.

detected within these cells, although most still bound to the cell wall and the outer layer. Finally, Fig. 5c shows a dead cell. The cytoplasm had completely collapsed and no intact organelles could be detected. Moreover, these cells had lost most of the outer layer. Only small amounts of AFP were still bound to the cell wall, whereas the majority were found inside the cell.

3.3. Fungistatic and fungicidal activity of AFP

According to previous studies, AFP has a fungistatic effect [27], since AFP-sensitive fungi were not killed by the protein but their growth was inhibited. However, the present study revealed that AFP is able to act both in a fungistatic manner and fungicidally. No growth was detected for conidia which had been treated with AFP concentrations equal to or higher than the MIC (1 µg/ml), thus indicating a fungicidal activity of AFP. In contrast, a fungistatic effect was observed for conidia treated with AFP concentrations below 1 µg/ml. Here, the replacement of the AFP-containing media with fresh media that lack AFP led to growth comparable to that of the negative control, indicating that the toxicity of AFP is reversible (data not shown). A fungicidal effect of AFP was also observed with mycelia of *A. niger*. Treatment of hyphae with an AFP concentration of 80 µg/ml for 1 h completely inhibited fungal growth. After removal of AFP, no further mycelial growth was detected (data not shown). This data is in agreement with the TEM analysis in which treatment of *A. niger* with an AFP concentration of 100 µg/ml for only 1 h already caused cellular death to approximately 25% of the hyphae.

In addition, we tested whether AFP retains its antifungal activity after treatment at high temperatures. AFP was preincubated at different temperatures, ranging from 60 °C to 100 °C for 15–60 min and was then applied to conidia of *A. niger* and *P. chrysogenum*. No change in protein activity was detected, even after 1 h of treatment at temperatures up to 80 °C (data not shown). However, the MIC of AFP preincubated for 15 min at 100 °C on the test strain *A. niger* increased 10-fold, i.e., from 1 up to 10 µg/ml, indicating that

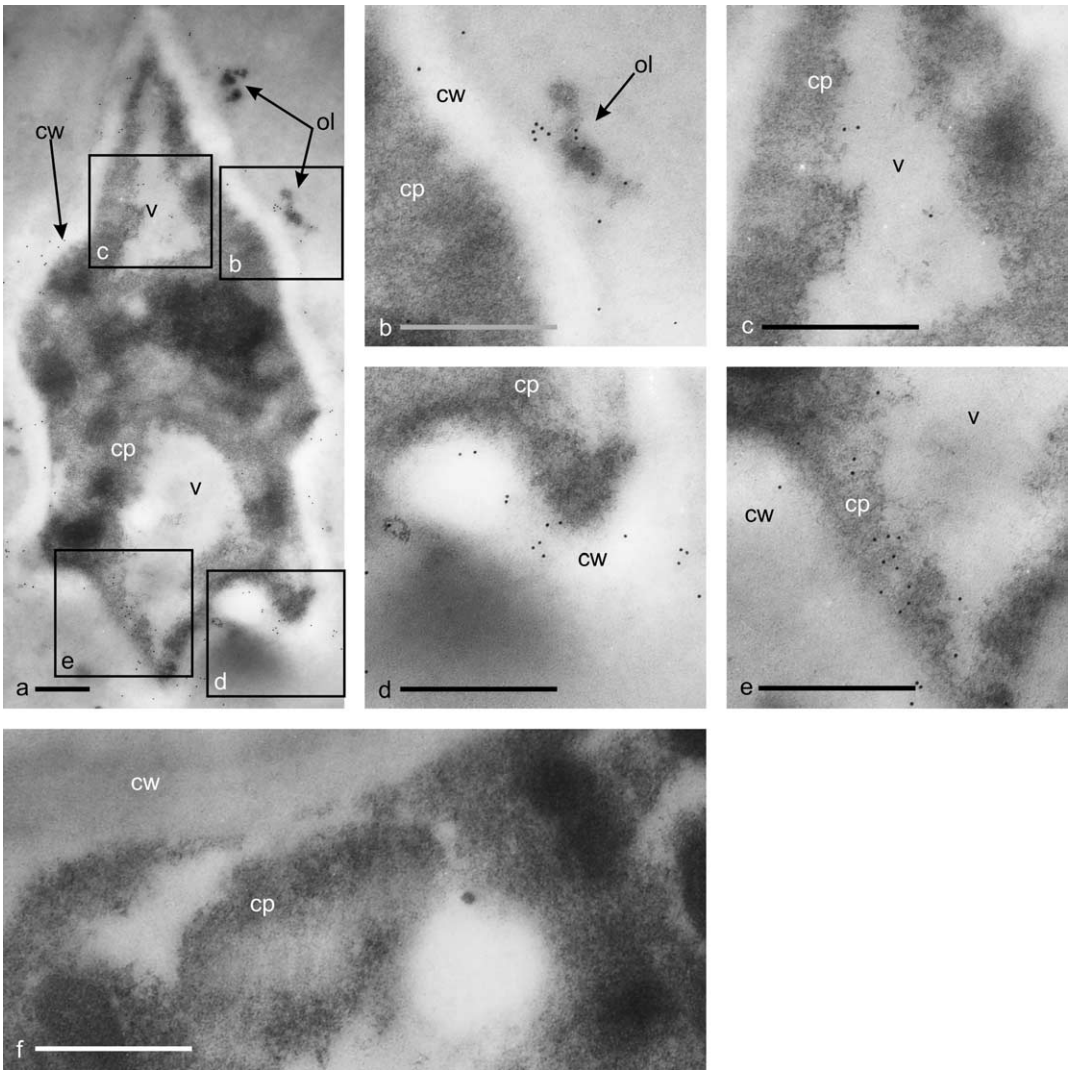

Fig. 4. Localization of AFP within *P. chrysogenum* by immunogold staining. TEM analysis of immunogold-stained cross-sections of *P. chrysogenum* treated with 1 µg/ml AFP for 1 h. Due to the fixation and embedding protocol the cell wall is not stained and appears as a lightened area. Black dots represent gold stained AFP. (a) Cross-section of *P. chrysogenum* hyphae (overview). (b) Detailed view of (a), showing localization of AFP at the cell wall of *P. chrysogenum* and binding of AFP to the outer layer. (c) Detailed view of (a), showing localization of AFP at the edge of a vacuole. (d) Detailed view of (a), showing a localization of AFP inside the cell wall of *P. chrysogenum*. (e) Detailed view of (a), showing cytoplasmatic localization of AFP. (f) Negative control; *P. chrysogenum* without AFP treatment. cp: cytoplasm; cw: cell wall; ol: outer layer; v: vacuole. Bars: 0.5 µm.

the protein had lost approximately 90% of its antifungal activity. Prolonged treatment of AFP at 100 °C for up to 60 min did not result in further loss of activity.

3.4. In vivo application of AFP

Tomato seedlings preincubated for 10 days with different AFP concentrations were inoculated with the plant pathogenic fungus *F. oxysporum* f. sp. *lycopersici*. Incubation of the plants with an AFP concentration up to 100 µg/ml had no negative impact on the growth of the plants (data not shown). Treatment of plants with 1 µg/ml AFP and 10 µg/ml AFP did not prevent infection with *F. oxysporum* f. sp. *lycopersici*. After 10 days of incubation with the fungal pathogen, these plants showed the same signs of vascular wilt disease as the AFP-untreated control (Figs. 6c, 6e and 6g). In contrast, treatment of tomato plants with 100 µg/ml AFP for 10 days was sufficient to protect these plants against *F. oxysporum* f. sp. *lycopersici* infection (Figs. 6a and 6b). Their growth and appearance resembled those of the controls which were not inoculated with *F. oxysporum* f. sp. *lycopersici* (Figs. 6b, 6d, 6f and 6h).

4. Discussion

The analysis of immunogold-labeled AFP by TEM showed that AFP binds to the cell-wall-associated outer layer which surrounds hyphal cells of *A. niger*. Most species of the *Ascomycotina*, *Basidiomycotina* and *Zygomycotina* are surrounded by an outer layer of glycoproteins [1,3]; however, its composition is not yet known. Interestingly, the

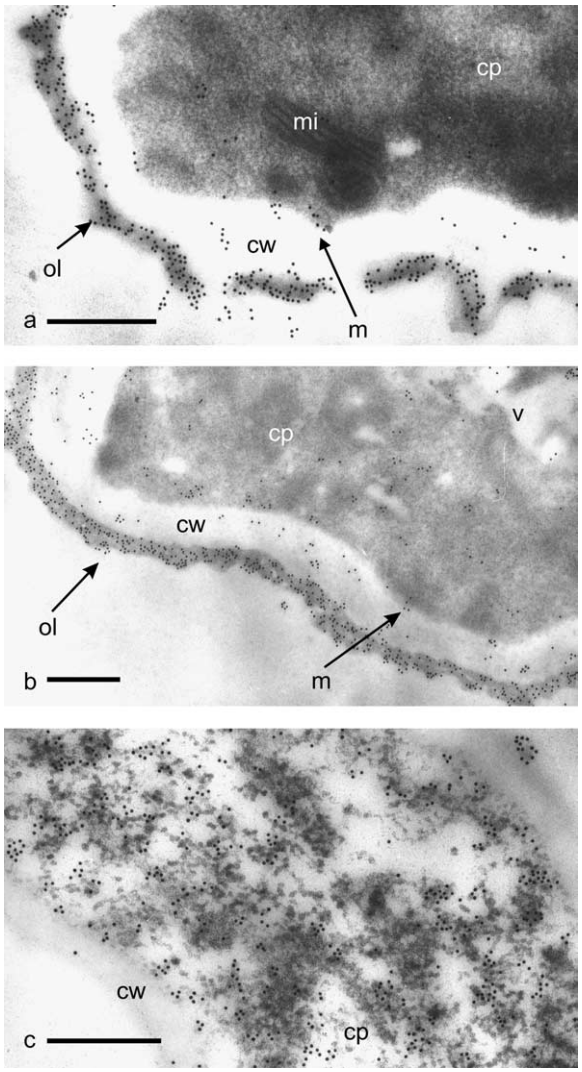

Fig. 5. Effects of high AFP concentrations on *A. niger* analyzed by immunogold staining. TEM analysis of immunogold-stained cross-sections of *A. niger* hyphae treated with 300 µg/ml AFP for 1 h. Panels (a)–(c) show selected cells demonstrating different stages of the antifungal activity of AFP. (a) Binding of AFP to the outer layer, the cell wall and the membrane of an intact cell. A small amount of AFP was localized cytoplasmatically, but not associated with organelles, e.g., mitochondria. (b) A cell affected by AFP. The cytoplasm has started to collapse and a large amount of AFP has entered the cell. (c) In a dead cell with a completely unstructured cytoplasm, the majority of AFP was found inside the cell. No outer layer was present at this stage. cw: cell wall; m: plasma membrane; mi: mitochondria; ol: outer layer; v: vacuole. Bars: 0.5 µm.

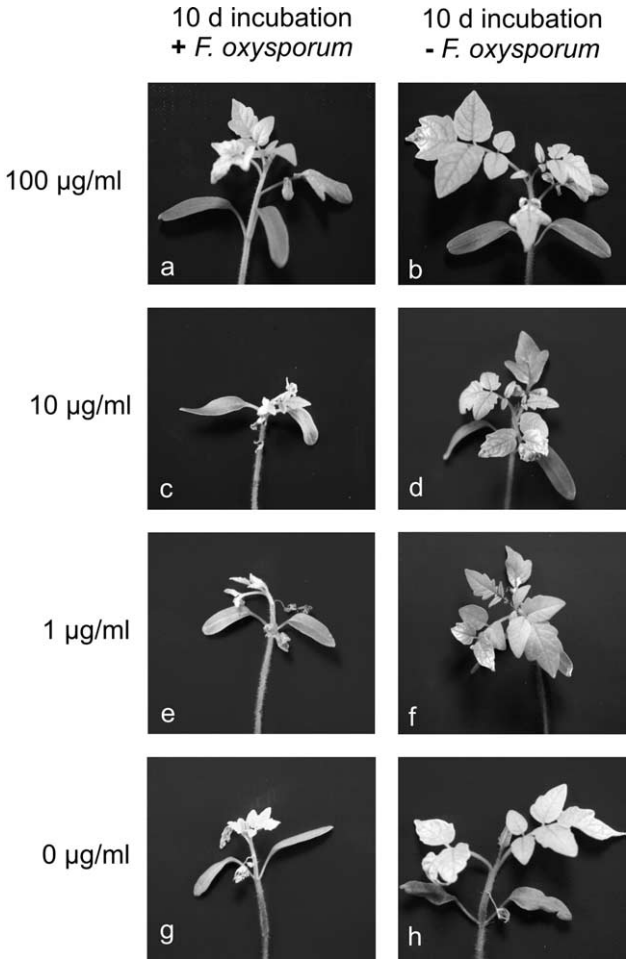

Fig. 6. Tomato seedlings after inoculation with *F. oxysporum* f. sp. *lycopersici*. 30-day-old tomato plants whose roots were treated with different AFP concentration prior to inoculation with *F. oxysporum* f. sp. *lycopersici*. Plants in panels (a) and (b) were incubated with 100 µg/ml AFP, in panels (c) and (d) with 10 µg/ml, and in panels (e) and (f) with 1 µg/ml. Plants shown in panels (g) and (h) were not treated with AFP. Panels (b), (d), (f), and (h) show plants that were treated with AFP but not inoculated with *F. oxysporum* f. sp. *lycopersici*.

outer layer is sparsely distributed on the cell wall surface of *P. chrysogenum*, and less AFP has been detected there. These data indicate that the outer layer might contain an initial binding site for AFP.

Similarly, AFP was localized within the cell wall of *A. niger* and, to a lesser extent, in the cell wall of *P. chrysogenum*. Liu et al. reported that AFP can be purified by chitin affinity chromatography [12]. As chitin is the main structural component of fungal cell walls, it is most likely that binding of AFP to the cell wall is due to its affinity to chitin. Although AFP shows no sequence similarity to chitin binding proteins, it does share several features with these proteins. For example, chitin binding proteins are often small and basic and inhibit fungal growth at concentrations of 1 µg/ml [6, 10]. As is the case for AFP, their antifungal activity is often antagonized by monovalent and divalent cations [2,16,22]. Notably, Vila et al. reported AFP-mediated growth inhibition of the oomycete *Phytophtora infestans* [26]. Thus, if binding of AFP to chitin is involved in its antifungal activity, it cannot be the only cause for fungal growth inhibition, since *Oomycetes* contain little or no chitin within their cell walls.

Remarkably, an accumulation of AFP at distinct areas within the cell wall was only detected for the AFP-sensitive fungus *A. niger*. This accumulation might hint at specific binding to structures within the cell wall and could therefore be involved in the antifungal effect of AFP. It remains

unclear whether these structures are proteins which reside at specific sites of the cell wall or whether they are built up by AFP itself.

A former study revealed that AFP is able to permeabilize the plasma membrane of the AFP-sensitive fungus *A. niger* [22]. In accordance with this finding, small amounts of AFP were localized at the plasma membrane of *A. niger*. The activity at the membrane seemed to be dependent on the concentration of the protein. The observed alterations in the membrane structure at a concentration of 1 μg/ml, which corresponds to the MIC for *A. niger*, might be due to AFP affecting the membrane curvature. Membrane acting proteins have been reported to cause curvature stress by expanding the phospholipid head groups of membrane lipids [5], thereby resulting in membrane thinning [7]. However, it is also possible that curvature stress is reduced by the formation of the observed "bulb-like" structures which were ascertained in membranes of *A. niger* treated with AFP. In this regard, it is interesting to note that Thevissen and colleagues reported that the glycosphingolipid composition of the fungal membrane is involved in resistance and susceptibility of *Saccharomyces cerevisiae* and *Pichia pastoris* to the plant defensins DmAMP1 and RsAFP2, respectively [24,25]. As AFP shares similarities with defensins in terms of size, basic character, three-dimensional structure and disulfide bridge formation, it would be interesting in future experiments to test whether the glycosphingolipid composition differs in *A. niger* and *P. chrysogenum* and whether it contributes to sensitivity or resistance to AFP.

The effect of AFP on membranes was very prominent when *A. niger* was treated with high concentrations (300 μg/ml) of AFP. Numerous dead hyphae were detected where large amounts of AFP had entered the cell. We previously reported that a concentration higher than the MIC leads to rapid membrane permeabilization in sensitive fungi [22]. The absence of membrane permeabilization at AFP concentrations below the MIC would be a reasonable explanation for the fungistatic activity of AFP. Internalization as a consequence of membrane interaction has been described for many antimicrobial proteins [8,14,17,20,28]. Martinez del Pozo et al. reported that AFP displays characteristic features of the oligonucleotide–oligosaccharide binding motif (OB fold) found in proteins that bind nucleic acids as well as oligosaccharides [13]. Indeed, AFP is able to bind DNA in vitro and promotes charge neutralization and condensation of DNA. It was concluded that similar binding of AFP to RNA and DNA might be involved in its antifungal activity. However, in our study we did not observe a nuclear or mitochondrial localization of AFP when *A. niger* was incubated at the MIC. Only when a 300-fold higher amount of AFP was applied to *A. niger* was AFP localized intracellularly. Thus it remains questionable, whether the binding affinity of AFP to DNA contributes to its antifungal activity.

In the AFP-resistant fungus *P. chrysogenum*, no membrane alterations after AFP treatment were observed, which also points toward a possible role of the membrane composition in AFP-mediated growth inhibition of AFP-sensitive fungi. Interestingly, AFP was not only localized in minor amounts at the cell wall and scattered outer layer of *P. chrysogenum*, but also within the cytoplasm. Thus far, we have not been able to provide an explanation for this phenomenon. It is conceivable that AFP is internalized and used as a nutritional source by *P. chrysogenum*. Degradation products of AFP which are still antigenic might subsequently be recognized by the AFP-specific antibody used in immunogold staining.

This study provides evidence of in vivo activity of AFP, as vascular wilt disease of *L. esculentum* caused by *F. oxysporum* was prevented by preincubation of tomato roots with AFP. The amount of conidia deployed in the artificial model system does not reflect environmental conditions, since the concentration of *F. oxysporum* conidia within the soil is normally much lower. However, it was necessary to use a high concentration of conidia for this model system, as only a few plants would otherwise have been infected. This might have led to an erroneous statistical analysis. Moreover, signs of vascular wilt disease occur much earlier in plant development when the plants are treated with high amounts of the pathogen. The high concentration of conidia most probably contributed to the observation that only an AFP concentration of at least 100 μg/ml was sufficient to protect *L. esculentum* against *F. oxysporum*. This concentration is about 1×10^2 times higher than the previously estimated MIC of AFP on *F. oxysporum* conidia in vitro. Moreover, MICs were determined in a different cultivation medium which also has an impact on the antifungal activity of AFP [22]. The mechanism by which AFP is able to prevent infection of treated tomato seedlings with *F. oxysporum* was not further analyzed. It is conceivable that the protective effect might result from minor amounts of the protein which remained attached outside on the plant roots even though the plants were washed with H_2O after treatment with AFP.

In conclusion, this study indicates that determinants for the species-specificity of AFP reside in the outer layer and cell wall of sensitive fungi and that the plasma membrane might represent the primary target for antifungal activity. Due to its fungicidal mode of action and its high temperature stability, AFP might be a promising candidate for use in food preservation and for the generation of pathogen-resistant plants [18,26], as AFP is highly specific to diverse plant-pathogenic fungi, e.g., *Fusarium* spp. Since AFP also inhibits the growth of opportunistic human pathogens, e.g., *A. fumigatus*, (MIC = 10 μg/ml; unpublished), the protein might also be attractive for use in clinical applications.

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