A Novel Cecropin A-derived Peptide as Specific Inhibitor of Appressoria in Magnaporthe oryzae

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Abstract
Magnaporthe oryzae causes crop losses around the world and is considered one of the most harmful pathogens in rice. The search for new types of antifungal compounds focuses on specificity in order to avoid toxicity to non-target species. In this work, we characterized the activity of the natural antimicrobial peptide Cecropin A and its derived peptide MgAPI16 as inhibitors of appressorium formation in M. oryzae. These peptides were able to control the development of blast disease in rice plants. Several lines of evidence indicated the different mode of action of both peptides. The addition of inducers of appressorium formation interfered with the inhibitory effect of MgAPI16 but not with Cecropin A. Moreover, antimicrobial activity assays showed a weak or no toxicity of MgAPI16 against bacteria and fungi suggesting high specificity in inhibiting appressorium formation. By fluorescence confocal microscopy, we observed a preferential binding of MgAPI16 to germininal tubes and appressoria causing the formation of aberrant non-functional appressorium structures. Based on our results, MgAPI16 is proposed as a potential target-oriented peptide that specifically blocks appressorium formation and control rice blast disease, being a promising compound with potential application in plant protection.

Keywords. Rice blast; Appressorium; Oryza sativa

Introduction
Rice (Oryza sativa) is an important crop in the world and staple food for many countries. One of the major limitations to rice production is the blast disease caused by the fungus Magnaporthe oryzae [1]. Different strategies are required for rice blast management, such as cultural strategies for prevention, resistant varieties, biological control, and chemical fungicides [2]. But even with the integration of these techniques rice blast disease is still difficult to control, largely because M. oryzae presents a high pathogenic variability and a large number of fungal races [3-5]. Nowadays, the use of some chemical fungicides is being restricted due the long-terms repercussions in environment and human health. Legislation on the use of fungicides is becoming more restricted; many chemical fungicides are being reviewed for safety and efficacy and some of them may be deregistered as dangerous to human health. Legislation on the use of fungicides is becoming more restricted; therefore has to potential for control of rice blast diseases. Moreover, the effect of this peptide has been compared with the lead peptide CecA.

An obligatory feature of novel low risk fungicides is pathogen specificity, which will avoid unspecific toxicity and prevent undesirable effects on the environment. More pathogen-specific fungicides could be identified by the screening for molecules which specifically block infection-related processes such as appressorium formation, an essential developmental stage in the pre-penetration phase of some phytopathogenic fungi. Appressorium development in M. oryzae is a complex morphogenetic process regulated by multiple external signals (surface hydrophobicity, hardness, cutin monomers, and leaf waxes), and different transduction pathways (Pmk1 and Mps1 MAPK and cAMP-dependent signaling pathways) [1,6,7]. In previous studies, we have identified small synthetic peptides able to control rice blast disease by blocking appressorium formation in M. oryzae [8,9]. The proposed mode of action for one of these peptides, PAF104, is affecting the Pmk1 pathway by repression of the gene expression of MoMSB2, which encodes a surface sensing protein, and the mitogen-activated protein/extracellular signal-regulated kinase kinase kinase MST11 [8]. Our previous results further support the hypothesis that peptides blocking a specific target have lower unspecific toxicity and suggest that the application of target-oriented antifungal compounds might be an environmentally sustainable strategy for plant protection.

One promising alternative to the classic chemical fungicides are antimicrobial peptides (AMPs), peptides broadly distributed in nature as innate defense molecules in all organisms [10-12]. The first family of antibacterial peptides clearly related to a bacteria-induced immunity in animals was cecropins in insects [13]. Cecropin A (CecA) is a linear and cationic peptide isolated from the hemolymph of Hyalophora cecropia which has antimicrobial activity against numerous pathogenic bacteria and fungi [13-16]. Moreover, several reports have shown that the transgenic expression of genes encoding CecA enhance resistance to plant pathogens [17,18].

The fact that CecA display cytototoxic activity against a wide range of microorganisms demonstrates its lack of specificity. An approach to identify more pathogen-specific peptides is the modification of known AMP sequences by residue substitution and/or reduction of size [19-22]. This rational design allows us to obtain new peptides with the favorable characteristics of the natural peptide but avoiding undesirable secondary effects.

In this work, we have identified a synthetic CecA-derived peptide called MgAPI16 that specifically blocks appressorium formation in M. oryzae and therefore has to potential for control of rice blast diseases. Moreover, the effect of this peptide has been compared with the lead peptide CecA.

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Materials and Methods

Microbial strains

*Magnaporthe oryzae* isolates PR9 and FR13 (kindly provided by Dr. Tharreau, CNRAS, Montpellier, France) were grown on complete medium at 28°C under 16h/8h (light/dark) photoperiod for 11-13 days as previously described [9]. A fungal strain of *Fusarium proliferatum* isolated from rice was cultured in potato dextrose agar (PDA) plates at 25°C for 7 days. The laboratory strain of *Escherichia coli* (DH5a) was grown in Luria-Bertani (LB) medium at 37°C to exponential phase.

Synthetic peptides

Peptides (Table 1) were purchased at >90% purity (GenScript, New Jersey USA). Peptide MgAPI16 was also synthesized labeled with tetramethyl rhodamine at the N terminus (TMR-MgAPI16) by the same company. Peptide solutions were prepared in sterile millQ-water at the appropriate concentration.

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

| Peptide | Sequence |
|---------|----------|
| CecA    | KWKLFKIEKVGQNRDGIKGAPAVAVGGATQIAK |
| #1 (MgAPI16) | KWKLFKIEKVGQNRDG |
| #2     | QNIRDGIKGAPA |
| #3     | GAPAVAVGGATQIAK |

Appressorium formation assay

Development of *M. oryzae* appressorium was monitored by microscopic examination as previously described [8]. Briefly, 8 µl drops of a conidial suspension adjusted to 5x10^5 conidia/mL and 2 µl of 5x peptide solutions (or sterile water as control) were placed on a hydrophobic surface. Pictures of 5 random fields of every drop were taken with an Olympus Stereo microscope SZX16 after 6h and 24h of incubation and the percentage of appressorium formation was determined by microscopic examination for at least 100 conidia per replicate. The data were shown as the relative percentage of appressorium formed after peptide treatment compared to the appressorium formed in control samples without peptide. At least three independent experiments were carried out.

To evaluate the effect of appressorium development inducers on the inhibitory activity of the peptides, 2.4 mM CAMP (Sigma-Aldrich, Missouri USA) or 10 µM 1,16-hexadecanediol (Sigma-Aldrich, Missouri USA) were added in determined assays.

Growth inhibition assay

**In vitro** antifungal activity of the peptides was determined using a microtitre plate assay as previously described [9]. Fungus at 2.5x10^5 conidia/ml was grown in a 96-well plate in a final volume of 100 µl of 50% PDB with 10 µl of a 10x peptide solutions (or sterile millQ-water as a control for fungal growth). The plate was incubated at 28°C, and fungal growth was determined by measuring optical density (OD) at 492 nm in a SpectraMax M13 (Molecular Devices, California USA) microplate reader. For bacteria, 10^5 cells/ml were grown at 28°C, and bacterial growth was determined by measuring optical density at 600 nm. In each antifungal experiment, the mean and standard deviation (SD) of three replicates were calculated for each treatment and were repeated at least three times.

For further characterization, we evaluated the inhibitory activity of CecA after 24h incubation, when mature appressorium is already formed. At this stage, in addition to melanized appressorium formed at the tip of germ tubes (AP), we observed that *M. oryzae* PR9 is able to develop appressorium-like structures at hyphal tips (ALS), as previously reported [9,23]. Both structures (AP and ALS) were visualized at different times using an Olympus FV1000 confocal laser scanning microscope (Tokyo, Japan), using excitation at 488 nm and emission at 505 to 530 nm for visualization of gfp-M. oryzae and excitation at 543 nm and emission at 575 nm for TMR-MgAPI16.

Results

**In vitro** inhibition of appressorium formation in *Magnaporthe oryzae* by CecA and its derived peptide MgAPI16

Firstly, the effect of the antimicrobial peptide CecA on *M. oryzae* appressorium formation in vitro was evaluated. Incubation of the strain PR9 with 10^5 conidia/ml was treated with 10 µM of labeled peptide (TMR-MgAPI16) at a polyethylene hydrophobic plate and incubated at 28°C in dark conditions. The appressorium formation stages were visualized at different times using an Olympus FV1000 confocal laser scanning microscope (Tokyo, Japan), using excitation at 488 nm and emission at 505 to 530 nm for visualization of gfp-M. oryzae and excitation at 543 nm and emission at 575 nm for TMR-MgAPI16.

**Interaction peptide-fungi assay**

To characterize the interaction of the peptide MgAPI16 with fungal cells, a *M. oryzae* Guy11 isolate that constitutively produces GFP protein was used (gfp-M. oryzae; kindly provided by Dr. Sesma). Fungus at 10^5 conidia/ml was treated with 10 µM of labeled peptide (TMR-MgAPI16) in a polystyrene hydrophobic plate and incubated at 28°C in dark conditions. The appressorium formation stages were visualized at different times using an Olympus FV1000 confocal laser scanning microscope (Tokyo, Japan), using excitation at 488 nm and emission at 505 to 530 nm for visualization of gfp-M. oryzae and excitation at 543 nm and emission at 575 nm for TMR-MgAPI16.

In a further approach, three CecA-derived peptides were synthesized based on its primary structure (Table 1) and their effect on appressorium formation was compared with CecA (Figure 1C). Peptides #2 and #3 barely reduced *M. oryzae* appressorium formation, but 10 µM of peptide #1 performed equal or slightly better than the same concentration of CecA. This result supported the idea that appressorium inhibitory effect of CecA lies in the N-terminal site of the peptide. For further studies the peptide #1 was selected and renamed as MgAPI16.
Appressorium formation inducers bypass the inhibitory effect of MgAPI16

Some substances have been reported as *M. oryzae* appressorium formation inducers due their implication in one or more pathways involved on appressorium development, such as 1,16-hexadecanediol (a cutin monomer of the plants leaf cuticle) and cAMP (a regulator of appressorium morphogenesis) [1]. We evaluated the effect of these inducers on the inhibitory activity of CecA and MgAPI16 on a hydrophilic surface (Figure 1D). Hydrophobicity is one of most important signals sensed by the conidia to trigger appressorium formation. Our results showed that the fungus cannot form appressoria in hydrophilic conditions without the inducers. *M. oryzae* PR9 treated with CecA was unable to form appressoria even if it is supplemented with exogenous inducers. This result was consistent with the mode of action of CecA, i.e. forming channels in the membrane [24], killing the fungus and making it impossible to generate appressoria. However, *M. oryzae* PR9 treated with the peptide MgAPI16 was capable to develop appressorium when is supplemented with cAMP (93.5%) or 10 µM 1,16-hexadecanediol (47.6%). Similar behavior was observed after treatment of another strain of *M. oryzae*, the FR13 strain, with MgAPI16 (data not shown). These results suggested a specific inhibitory effect of MgAPI16 on *M. oryzae* appressorium formation.

Application of CecA or MgAPI16 reduces the blast disease in rice plants.

An infection assay on *japonica* rice plants cv. Senia was developed to evaluate if *M. oryzae* treated with CecA or MgAPI16 is still able to infect rice plants. Figure 2A shows representative leaves from the inoculated plants. Under the same experimental conditions, a clear reduction of the infection symptoms was observed when the fungus was treated with each peptide. Plant disease was also quantified by determining the percentage of the leaf area affected by blast lesions (Figure 2B). In control conditions, the fungus infected around 25% of the leaf surface (24.7% ± 9.6) (mean ± SD) at 7 days post-inoculation. However, CecA and MgAPI16 treatments significantly reduced the percentage of infected surface (1.4% ± 2.8 and 1.9% ± 2.2 respectively) in agreement with eyesight perception. This result was consistent with our previous data of reduction of appressorium formation by the peptides in vitro, which prevent to start the infection. Our data also showed that both peptides have similar efficacy inhibiting appressorium formation and controlling blast disease development.

Growth inhibitory activity profile of MgAPI16 and CecA

Indirectly, microscopic visualization of *M. oryzae* treated with peptides pointed to a lack of toxicity of MgAPI16 on fungal cells. In order to characterize a target-oriented peptide which specifically interferes with appressorium formation, the toxic effect of MgAPI16 was tested against two representative microorganisms and compared with the antimicrobial activity of CecA (Figure 3). The growth of *Escherichia coli* DH5α isolate was measured after incubation with different concentrations of each peptide (from 0.5 µM to 5 µM) at 37°C (Figure 3A). Results showed that bacterium is unable to grow at 2 µM and 5 µM of CecA; even the lesser tested concentration, i.e.
0.5 µM, slightly reduced the viability of the bacterium. By contrast, MgAPI16 seemed harmless to the bacterium even at the highest tested concentration.

A similar experiment was performed in the eukaryotic organism Fusarium proliferatum (Figure 3B). In these assays, CecA inhibited the fungal growth with a minimum inhibitory concentration of 5 µM of peptide. Treatment with 2 µM CecA delayed fungal growth around 40h but at the end of the assay, i.e. 72h, fungal growth was no longer inhibited. On the other hand, no inhibitory activity was observed for the peptide MgAPI16 at the tested concentrations.

**Preferential binding of MgAPI16 to fungal hyphae and appressorium structure**

Considering previous data from this study regarding the lack of toxicity of MgAPI16 to other microorganisms, this peptide was selected for further studies. In an attempt to know MgAPI16 mode of action, gfp-expressing M. oryzae conidia were incubated on a hydrophobic surface with MgAPI16 labeled with tetramethylrhodamine (TMR-MgAPI16). The location of TMR-MgAPI16 in gfp-M. oryzae was followed by live-cell imaging using fluorescence confocal microscopy (Figure 4). At the first stages of the conidia development, the red signal from the peptide was located at the surface of the conidia with
a high preference for conidia tips (Figure 4A, panels a-d). At later developmental stages, when spores were completely germinated and the hyphae were growing up, the peptide was located surrounding the emerging germ tubes and hyphae (Figure 4A, panels e-h). These results indicated a preferential binding of the peptide where performed its inhibitory effect on appressorium formation. After 6h of incubation, although appressorium number was severely reduced, TMR-MgAPI16 preferentially bound at the surface of formed appressoria (Figures 4A, panels i-l). By confocal microscopy, we also observed that most of appressoria did not present the classic dome-shape structure after 6h treatment with MgAPI16; treated appressoria presented aberrant physical structure (tips or clearly shape deficiencies, Figure 4B).

**Discussion**

Nowadays, we are within a new legal and ecological framework in which the traditional fungicides do not meet all the imposed requirements. One problem refers to their toxicity to other species of the ecosystem. Current chemical fungicides are focused to kill fungal microorganisms, and frequently they present broad-spectrum activity showing toxicity to other organisms. The application of target-oriented antifungal compounds may be an environmentally sustainable strategy to control phytopathogenic diseases. Even more interesting could be the idea of a very specific compound that only affects the infective stage of the pathogen. During *M. oryzae* infective phase, conidia sense the hydrophobicity and the cutin composition of the rice leaf, triggering the formation of appressorium, a specialized structure for the cleavage of the leaf cuticle allowing the mesophyll invasion. However, if the conditions for infection are not suitable, conidia will germinate and become fungal hyphae. Therefore, blocking exclusively appressorium formation could prevent rice infection without causing a toxic undesirable effect on surrounding organisms.

Initially, we characterized appressorium inhibitory activity of a well-known antimicrobial peptide Cecropin A (CecA). The peptide inhibited the formation of appressoria (AP) while apparently promoting the formation of appressorium-like structures (ALS). In a previous work, we have demonstrated a similar behavior for other short peptides [9], suggesting that a possible secondary effect of the inhibition of AP structures would be the formation of ALS, a less efficient structure to penetrate into the plant [23]. Our data support the idea that the formation of both developmental structures involves different molecular mechanisms [23].

In this work, we also identified a novel CecA-derived peptide (MgAPI16) with similar appressorium inhibitory activity than CecA but without antimicrobial activity against the microorganisms tested. CecA is a lytic peptide whose channel-forming properties resides in its double α-helix structure linked by a flexible hinge region [24-26]. The non-antimicrobial peptide MgAPI16 corresponds with the first 16 amino acids of the N-terminal region of CecA, losing the ability to form the helix-hinge-helix structure. This fact supports that the typical structure of CecA is important for its high antimicrobial activity. Many CecA derived peptides have been designed in order to increase antimicrobial properties [19,20,25,27]. But, in our knowledge, this is the first report of a CecA-derived peptide targeted to block a specific pathogenic step, i.e. appressorium formation, but without antimicrobial activity.

Our results obtained for CecA and MgAPI16 after the addition of appressorium inducers clearly suggest their different mode of action. Appressorium inhibitory activity of CecA was due mainly to its lytic activity, being the fungus unable to form appressorium even after the addition of an inducer. However, the inhibitory activity of MgAPI16 was bypassed by the addition of CAMP that restored appressorium formation at the same level of the control sample. By contrast, *M. oryzae* partially overcame MgAPI16 inhibition effect after the addition of 1,16-hexadecanediol, suggesting that this peptide may affect the fungal ability to recognize external signal such as hydrophobicity and cutin monomers.

By fluorescence confocal microscopy, we observed that TMR-MgAPI16 preferentially binds to the germinal tubes and appressoria, whose cell wall presents different composition of polysaccharides; specifically, they are rich in α1,3-glucan which is not observed in the conidia cell wall [28]. Whether the different composition of cell wall, or the presence of a specific receptor, would explain the preferential binding of MgAPI16 remains to be elucidated.

It has been reported the importance of the endosomal system and the autophagy in appressorium formation in *M. oryzae* [29-31]. During germination and appressorium development, small vacuoles in the apical cell of conidia move into the germ tube and nascent appressorium, as our confocal microscopic images shown. However, the appressoria formed after MgAPI16 incubation did not present the classic dome-shape structure (Figure 4B). The clear reduction of blast disease symptoms on rice plants after peptide treatment suggests that these aberrant appressoria are not able to perform the infection process.

Taking together, the results present in this report suggest that MgAPI16 specifically block appressorium formation and could be a promising compound with potential application for the control of rice blast disease. This is the first step to develop new kind of target-oriented antifungal compounds without toxicity to other organisms, such as beneficial microorganisms or host plants.

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