Biochemical and Biophysical Research Communications 454 (2014) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Screening of a synthetic peptide combinatorial library to identify inhibitors of the appressorium formation in *Magnaporthe oryzae*

Aarón Rebollar [a], Jose F. Marcos [b], Belén López-García [a,*]

[a] Centro de Investigación en Agrigenómica (CRAG) CSIC-IRTA-UAB-UB, Campus UAB, Bellaterra (Cerdanyola del Vallés) 08193, Barcelona, Spain
[b] Instituto de Agroquímica y Tecnología de Alimentos (IATA) – CSIC, Apartado de Correos 73, Burjassot 46100, Valencia, Spain

ARTICLE INFO

Article history:
Received 27 September 2014
Available online 18 October 2014

Keywords:
Rice-blast
Appressoria
Oryza sativa
Antimicrobials

ABSTRACT

The rice blast disease caused by *Magnaporthe oryzae* is one of the most devastating diseases of cultivated rice. One of the most important stages in the infective cycle of *M. oryzae* is the formation of the dome-shaped structure called appressorium. The purpose of the present study was to identify novel peptides to control the rice blast disease by blocking the appressorium formation through screening of a synthetic peptide combinatorial library. As result of the screening, a set of 29 putative bioactive peptides were identified, synthesized and assayed in comparison with the previously identified peptide PAF104. The peptides MgAPI24, MgAPI40 and MgAPI47 showed improved inhibitory activity on the *M. oryzae* appressorium formation. Our data show that these peptides have a differential effect on two developmental structures: appressoria and appressorium-like structures. Antimicrobial assays against *M. oryzae* and other non-target microorganisms showed a weak or no toxicity of these peptides, demonstrating their specific activity blocking the appressorium formation. Therefore, the outcome of this research would be useful in the development of novel target-oriented peptides to use in plant protection.

1. Introduction

Plant infectious diseases caused by phytopathogenic fungi produce an important reduction of plant yield across many crop species, and require the continued use of fungicides for control. However, there are several problems associated to their use, such as the limited spectrum of action, the emergence of resistant fungal isolates and the negative impact on human health and the environment [1]. To substitute for or complement current fungicides, more pathogen-specific compounds with lower unspecific toxicity could be identified by screening for molecules which specifically block infection-related processes rather than inhibit vegetative hyphal growth. The identification and application of these target-oriented molecules has been proposed as an environmentally friendly alternative to avoid some drawbacks of current fungicides [1,2].

Natural and synthetic antimicrobial peptides (AMPs) have emerged as promising compounds to use in plant protection as alternative to fungicides [3–5]. The diversity available through combinatorial chemistry provides a powerful tool to identify novel peptides targeted to specific crop protection problems. "Non-defined" synthetic combinatorial libraries (SCL) enable the high-throughput (HTP) assay of millions of compounds without sequence restrictions in relatively short time [6]. In most cases, the primary peptide screens are cell-based assays of *in vitro* growth inhibition, which enables the HTP formats required for the analysis of compound libraries [7,8]. Indeed, the library used in this work was previously assayed against the phytopathogen *Penicillium digitatum*, identifying several peptides called PAFs [7]. However, few examples of peptide screens targeted to specific pathogen functions have been applied to plant pathogens [9–11].

This work focuses on the screening of a peptide library to identify inhibitors of an essential infection process of the rice blast fungus *Magnaporthe oryzae*, a devastating rice pathogen [12]. *M. oryzae* passes through a set of well-defined developmental stages to successfully invade and infect host plants: adhesion to plant surface and germination, appressorium differentiation and maturation, penetration and invasive growth. Multiple external signals and several transduction pathways, including Pmk1 and Mps1 mitogen-activated protein kinases (MAPK) and cAMP-dependent signaling pathways, regulate this developmental process [12–16].

We have previously characterized the heptapeptidePAF104 as an inhibitor of appressorium formation in *M. oryzae* that affects the expression of genes coding for components of the Pmk1 pathway [17]. PAF104 was identified as a derivative of PAF26, and both peptides resulted from combinatorial screens to find

* Corresponding author at: Futureco Bioscience, S.A., 08799 Olèrdola, Barcelona, Spain.
E-mail address: crag.blg@gmail.com (B. López-García).

http://dx.doi.org/10.1016/j.bbrc.2014.09.145
0006-291X/

inhibitors of fungal growth [7,18]. The purpose of this study was the improvement of the properties of PAF104 through combinatorial chemistry by screening of a "non-defined" hexapeptide library specifically against appressorium formation. As a result of the screening, the improved peptides MgAPI24, MgAPI40 and MgAPI47 have been identified.

2. Materials and methods

2.1. Microbial strains and growth conditions

M. oryzae isolate PR9 (CIRAD collection, Montpellier, France) was used for the *in vitro* appressorium inhibition assays. The fungus was grown on complete medium at 25 °C under 16 h/8 h (light/dark) photoperiod. The composition of complete medium (CM) is minimal medium (MM, 6 g/l $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4 \cdot 7H_2O$, 1.52 g of KH_2PO_4, 0.001% thiamine, 0.1% trace elements supplemented with 10 g/l D-glucose) supplemented with 2 g/l peptone, 1 g/l yeast extract, and 1 g/l casamino acids. Conidia were collected from 11 to 13 days-old culture with sterile water, filtered through miracloth and titrated with a Neubauer chamber.

Other microorganisms used were a fungal strain of *Fusarium proliferatum* isolated from rice and the laboratory strain of *Escherichia coli* (DH5α). *F. proliferatum* was cultured in potato dextrose agar (PDA) plates at 25 °C and conidia were collected as described above. *E. coli* was grown in Luria-Bertani (LB) medium at 37 °C to exponential phase, diluted to the appropriate concentration, and used in the antimicrobial assays.

2.2. Peptide library and individual synthetic peptides

The synthesis of the library used in this work was described previously [7]. Peptide mixtures were dissolved in 5% dimethylsulfoxide (DMSO). Individual peptides (Table 1) were purchased at >90% purity from GenScript Corporation (New Jersey, USA) and dissolved in sterile miliQ-water.

2.3. Appressorium formation assay

Development of *M. oryzae* appressoria on hydrophobic surface was monitored by microscopic examination as previously described [17]. Briefly, 8 µl-drops of a conidial suspension adjust to 5×10^5 conidia/mL were placed on the hydrophobic surface and then 2 µl of each mixture of peptide library or sequence-defined peptides were added to reach different final concentrations from 5× stock solutions. In the assay of the peptide library, two concentrations of peptide mixtures were assayed (0.5 mg/ml and 1 mg/ml), and the same volume of 5% DMSO was added to some drops as mock to confirm that it did not significantly affect the appressorium formation.

Pictures of 5 random fields of every drop were taken with an Olympus Stereoscope microscope SZX16 and the percentage of conidia induced to form appressorium was determined by microscopic examination for at least 100 conidia per replicate. The percentage of appressorium formation after each peptide treatment was calculated as the number of appressoria formed relative to the number in control samples without peptide. At least two independent experiments were carried out.

2.4. In vitro germination and antimicrobial activity assays

The percentage of *M. oryzae* germinated-conidia after peptide treatment was determined as previously described [17]. Briefly, conidia suspensions (10^6 conidia/mL) were incubated with 20 µM of each peptide and, after 4 h incubation at 28 °C, germinated and non-germinated conidia were quantified by direct microscopic counts.

Table 1
Amino acid sequences of the peptides obtained of the analysis of the library.

Peptide	Sequence
PAF26	NH_2-RKKWFW-COOH
PAF104	**NH_2-WRKKWFW-COOH**
#21	NH_2-WRKKWEW-COOH
#22	NH_2-WRKKWGW-COOH
#23	NH_2-WRKKWHW-COOH
#24	**NH_2-WRKKWIW-COOH**
#25	NH_2-WRKKWMW-COOH
#26	NH_2-WRKKWPW-COOH
#27	NH_2-WRKKWTW-COOH
#28	NH_2-WRKKWVW-COOH
#29	NH_2-WRKKWWW-COOH
#30	NH_2-WRKKEFW-COOH
#31	NH_2-WRKKFEW-COOH
#32	NH_2-WRKKFGW-COOH
#33	NH_2-WRKKFHW-COOH
#34	NH_2-WRKKFIW-COOH
#35	NH_2-WRKKFMW-COOH
#36	NH_2-WRKKFPW-COOH
#37	NH_2-WRKKFTW-COOH
#38	NH_2-WRKKFVW-COOH
#39	NH_2-WRKKFWW-COOH
#40	**NH_2-WRKKRFW-COOH**
#41	NH_2-WRKKREW-COOH
#42	NH_2-WRKKRGW-COOH
#43	NH_2-WRKKRHW-COOH
#44	NH_2-WRKKRIW-COOH
#45	NH_2-WRKKRMW-COOH
#46	NH_2-WRKKRPW-COOH
#47	**NH_2-WRKKRTW-COOH**
#48	NH_2-WRKKRVW-COOH
#49	NH_2-WRKKRWW-COOH

Residues differential to those of heptapeptide PAF104 are underlined. Peptides selected for further studies are in bold letter.

In vitro antifungal activities of the peptides were determined using a microtiter plate assay as previously described [19,20]. The assay mixture contained 2.5×10^4 conidia/ml in a final volume of 100 µL of PDB (Potato Dextrose Broth, Difco, Detroit, USA) diluted one half (50% PDB) or one fifth (20% PDB) for *M. oryzae* or *F. proliferatum*, respectively. Peptides were added in a 10 µL volume from a 10× peptide solution to reach different final concentrations. The assay mixture contained 30 µg/ml chloramphenicol per well to avoid bacterial contamination. 96-well plates were incubated at 25 °C and growth was determined by measuring optical density (OD) at 492 nm in a SpectraMax M13 (Molecular Devices, CA, USA) microplate reader at different times. In all the experiments, three replicates were prepared and the mean and standard deviation (SD) were calculated for each treatment.

A solution killing assay was performed to determine survival of the bacterium *E. coli* after peptide treatment. Bacterium in exponential phase growth was suspended to 10^6 cells/ml in 20% LB, different concentrations of peptides were added and samples were incubated at 37 °C. At different incubation times, serial 10-fold dilutions of cells were prepared and aliquots of 10 µL of each sample were dotted on peptide-free LB agar plates to determine viability.

3. Results and discussion

3.1. Screening of a PS-SCL for inhibition of appressorium formation in M. oryzae

The PS-SCL represent 47 millions of peptides and consisted of six sub-libraries as previously described [7,20]. Each sub-library

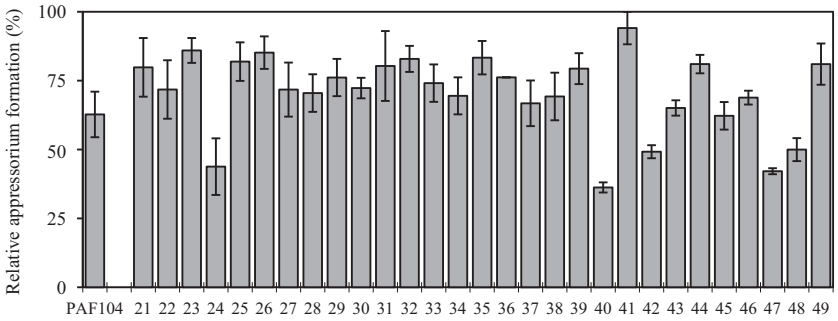

Fig. 1. Effect of the peptides labeled in the *x*-axis on the *in vitro* appressorium formation in *M. oryzae* on a hydrophobic surface. The panel shows the mean relative appressorium formation ± SD after 6 h of treatment with 10 μM of peptide.

is composed of 19 complex mixtures, each one specifying one of the natural amino acids (except cysteine) at each one of the peptide position. The assay of the peptide mixtures provides information about the most important amino acid residue at each position of the peptide. In this work, the PS-SCL was tested for the *in vitro* inhibition of appressorium formation in *M. oryzae* (Fig. S1). Clear differences were observed between the different mixtures that composed each sub-library, suggesting that inhibitory activity is due to individual peptide(s) present in the mixture.

The so-called deconvolution process of the library consisted in selecting the putative relevant amino acid residues for each position. In our case, very similar (and significant) activities were observed between mixtures within position 3, indicating that no clear-cut assignment could be made at this position of the peptide. Based on the data from the screening of the PS-SCL at both concentrations 0.5 mg/ml (Fig. S1) and 1 mg/ml (data not shown), the initially selected residues for the other positions were: glutamic acid, asparagine and arginine at position 1; arginine at position 2;alanine, phenylalanine, and arginine at position 4; glutamic acid, glycine, histidine, methionine, proline, threonine, valine and tryptophan at position 5; and alanine and glutamic acid at position 6 (colored in black in Fig. S1).

3.2. Selection of sequence-defined peptides and assay of their appressorium inhibitory activity

We have previously characterized a synthetic heptapeptide (WRKKWFW, PAF104) that reduced the appressorium formation in *M. oryzae* [17]. PAF104 was identified as a derivative sequence of the lead hexapeptide PAF26 (RKKWFW) [18] which was previously identified by the screening against the growth of the filamentous fungus *P. digitatum* of the same PS-SCL used in this study [7]. Considering the amino acid sequence of these peptides, we used the data from the screening of the PS-SCL (Fig. S1) to rationally design a set of 29 heptapeptides that are derived from the PAF104 sequence (Table 1).

Unexpectedly, the mixtures corresponding to tryptophan at position 4 and phenylalanine at position 5 (Fig. S1) (numbering as in PAF26, which correspond to positions 5 and 6 of PAF104, see Table 1) showed only a limited inhibition of appressorium formation at the lowest concentration used (i.e. 0.5 mg/ml). This discrepancy is likely related to the different activities that were assayed in the screening of the PS-SCL: inhibition of fungal growth to identify PAF26/PAF104 [7,18] versus inhibition of appressorium formation (this work). Thus, these positions were combinatorialized, whereas the other five positions 1, 2, 3, 4, and 7 were conserved in the primary structure of PAF104, due to the promising inhibitory activity on appressorium formation of this heptapeptide [17]. Residues selected for substitutions have chemical properties representative of the active mixtures. The residues used in position 5 were phenylalanine (as hydrophobic amino acid) and arginine (as positively charged), that correspond to two of the three most active peptide mixtures at position 4 of the combinatorial library (Fig. S1). For position 6, the residues selected were isoleucine, methionine, proline and valine (as hydrophobic amino acids), histidine, threonine and tryptophan (as polar amino acids), glutamic acid (as negatively charged) and glycine (due to its high activity in position 5 of the combinatorial library). As a result of our focused deconvolution process, we selected 29 heptapeptide sequences with potential inhibitory activity of appressorium formation (Table 1, numbered #21 to #49). The peptides are divided in three series depending of the residue used in position 5 of PAF104: tryptophan (from #21 to #29), phenylalanine (from 130 to #39) and arginine (from #40 to #49).

The set of 29 individual peptides (Table 1) were assayed against the appressorium formation of *M. oryzae* on a hydrophobic surface and PAF104 was used as a reference control (Fig. 1). From this screening, we selected the peptides #24, #40 and #47 (called MgAPI24, MgAPI40 and MgAPI47 from now on) with improved inhibitory activity on the appressorium formation of *M. oryzae* than PAF104.

3.3. Comparative appressorium inhibition activities of selected peptides with PAF104

Firstly, we evaluated the effect of different concentrations of the selected peptides MgAPI24, MgAPI40 and MgAPI47, in comparison with PAF104, at an initial stage of the appressorium formation, i.e. 6 h after conidia germination when the appressorium is differentiated from the tip of the germ tube. We observed a reduction of appressorium formation that correlated with an increase of peptide concentration for all the peptides tested (Fig. 2), as previously

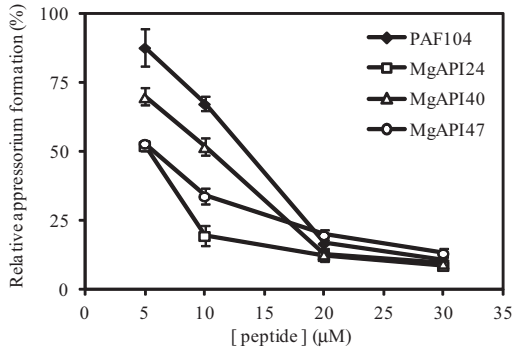

Fig. 2. Relative appressorium formation in *M. oryzae* after 6 h of incubation with different concentrations of PAF104 (black diamond), MgAPI24 (white square), MgAPI40 (white triangle) or MgAPI47 (white circle). Results are shown as mean values ± SD of one representative experiment from at least three experiments with similar results.

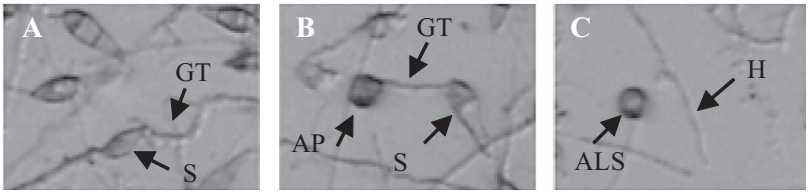

Fig. 3. Microscopic visualization of the morphologically distinct structures of *M. oryzae* observed after incubation 24 h on hydrophobic surfaces: germinated spore (A), melanized appressoria formed at the tip of germ tubes (B), and appressorium-like structures at hyphal tips (C). Arrows point to (S) spores, (GT) germ tubes, (AP) appressoria and (ALS) appressorium-like structures.

published for PAF104 [17]. At low concentration of peptides (5 μM and 10 μM), the novel peptides showed an improved activity over PAF104, thus fulfilling one of the objectives of the present study.

To characterize the appressorium blocking activity in more detail, we studied the inhibitory activity of the selected peptides at a later stage when the mature appressorium is already formed. At this stage, careful visualization of microscopic images showed morphologically distinct structures after 24 h inoculation of conidia on hydrophobic surface: spores that are able to germinate but not to form appressoria, appressoria from germ tubes, and appressorium-like structures at hyphal tips (Fig. 3). As previously reported, in addition of melanized appressoria formed at the tip of germ tubes (AP), *M. oryzae* is able to develop appressorium-like structures at hyphal tips (ALS) [21,22]. In this work, all these structures were observed after 24 h incubation of *M. oryzae* in the absence or presence of the four peptides (PAF104, MgAPI24, MgAPI40 and MgAPI47). At first sight, all peptides reduce the amount of AP formed although increase the number of ALS structures observed. Therefore, both structures (AP and ALS) were quantified separately in samples treated with different concentrations of peptides PAF104, MgAPI24, MgAPI40 and MgAPI47 (Fig. 4 and Table S1). Our data show that all peptides have a similar inhibitory profile at this maturation stage. At increasing concentration of peptides, we observed a clear reduction on the amount of mature AP developed from germ tube. However, peptide treatment induced the formation of ALS from hyphal tips. For example, after 24 h

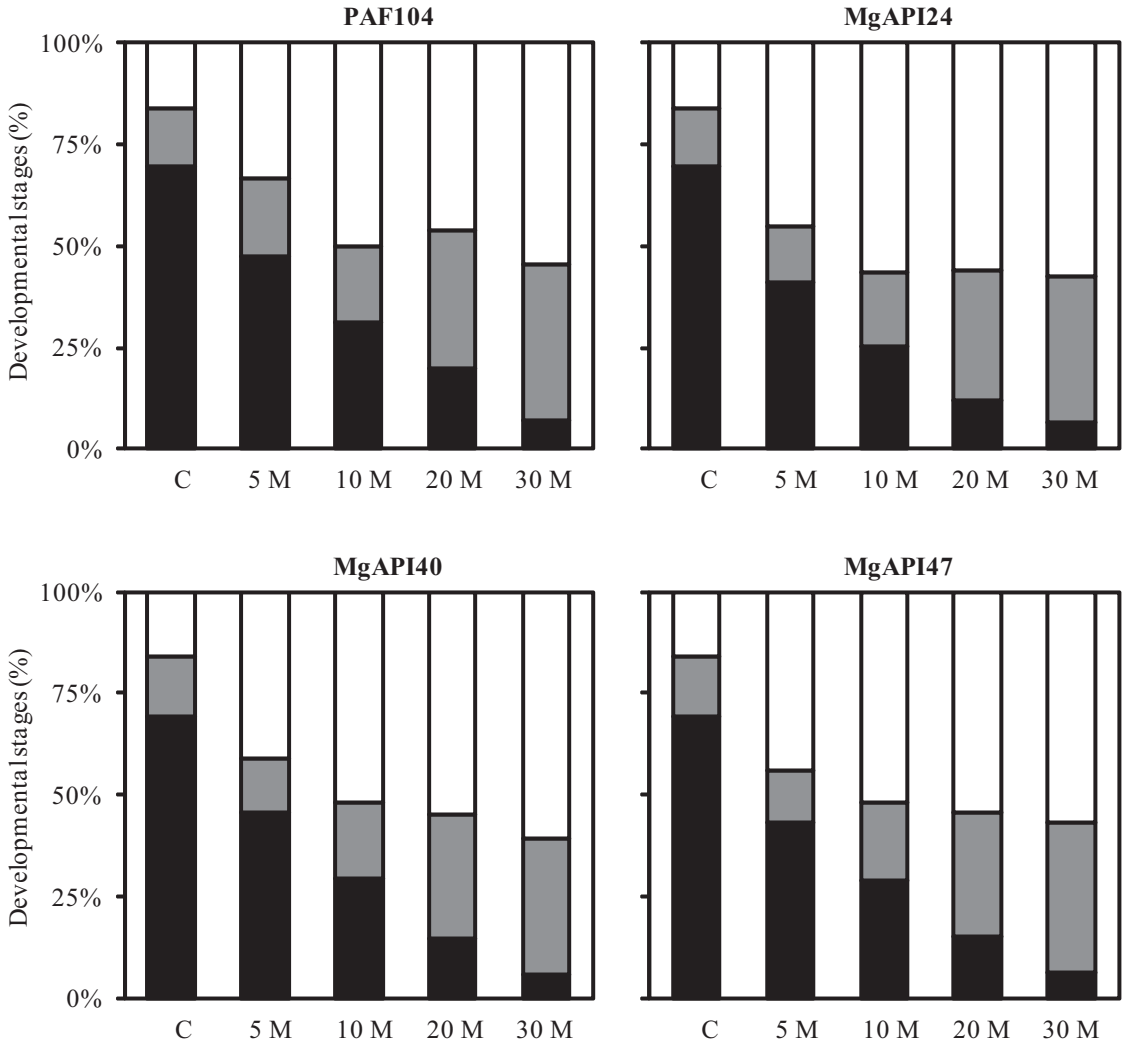

Fig. 4. Quantification of different developmental stages after 24 h incubation of *M. oryzae* on a hydrophobic surface in the absence (C) or presence of increasing concentrations of PAF104, MgAPI24, MgAPI40 and MgAPI47. The developmental stages are appressoria (black), appressorium-like structures (gray) and germinated spore (white).

Table 2
Percentage of growth of *F. proliferatum* treated with peptides.

		% Growth[a]		
		24 h	48 h	72 h
PAF104	50 μM	63.1 ± 25.9	73.9 ± 21.0	88.6 ± 19.8
	75 μM	5.1 ± 1.9	49.4 ± 1.7	86.1 ± 11.7
MgAPI24	50 μM	105.5 ± 13.2	103.0 ± 15.5	108.5 ± 16.8
	75 μM	6.7 ± 2.4	12.5 ± 0.1	56.0 ± 2.1
MgAPI40	50 μM	35.7 ± 8.9	60.3 ± 4.8	82.2 ± 4.6
	75 μM	3.6 ± 2.0	52.6 ± 4.0	95.8 ± 4.7
MgAPI47	50 μM	137.3 ± 9.1	103.0 ± 15.5	108.5 ± 16.8
	75 μM	49.7 ± 11.1	67.3 ± 5.7	88.7 ± 2.0

[a] Data are shown as mean values ± SD from one representative experiment with three replicates.

treatment with 30 μM of peptides, around 6% and 36% of conidia develop AP and ALS, respectively, compared with 69.5% and 14.4% of AP and ALS formed without peptide treatment. Our data show a differential effect on AP and ALS, supporting previous evidences that the formation of both developmental structures involve different molecular mechanisms [21].

We have previously published that PAF104 reduced the pathogenicity of *M. oryzae* on rice although it was not able to completely block appressoria *in vitro* [17]. One possible explanation is the formation of non-functional appressorium structures. As reported, the ALS structures are less efficient than mature AP in penetration of barley leaves [21]. Our present results regarding the reduction of AP and induction of ALS after peptide treatment support this possible explanation.

3.4. Growth inhibition activity profiles of heptapeptides

The previous assays based on the 24 h incubation of fungus with peptides indicated no inhibition on conidial germination and mycelial growth, as previously published for PAF104 [17]. To further confirm this, the number of germinated conidia was quantified after peptide treatment and compared with that of control treatment; for example, the percentage of germinated conidia was 91.6 ± 0.8 for sample treated with 20 μM MgAPI24 versus 92.7 ± 0.6 for control. Our data showed that conidial germination remained unaffected at concentrations at which appressorium inhibition was observed.

In addition, we tested the effect of different concentrations of peptides on the mycelia growth of *M. oryzae* and another filamentous fungus *F. proliferatum*. In these assays, no or very limited inhibition activity against *M. oryzae* and *F. proliferatum* was observed for the different peptides tested, even at the maximum peptide concentration used (Fig. S2 and Table 2). Looking in more detail, we only observed limited growth inhibition of *F. proliferatum* after 24 h of incubation at 75 μM of peptides (Table 2). However, the effect of peptides on the fungal growth was negligible at longer incubation times (until 72 h), with the unique exception of MgAPI24. MgAPI24 was the peptide showing higher antifungal activity against *F. proliferatum* along the time; after 72 h incubation with 75 μM of MgAPI24, the fungus was able to grow around 50% relative to growth in absence of peptide.

The antimicrobial activity of peptides was also determined against the bacterium *E. coli*. Similarly, a limited effect was observed on bacterial growth after short incubation time (2 h) for peptides PAF104 and MgAPI24 at 75 μM although no inhibition activity was observed at longer incubation time (4 h) (Fig. S3).

Finally, it would be very important to reinforce the specific effect of the selected peptides on the appressorium formation. All the four peptides (PAF104, MgAPI24, MgAPI40 and MgAPI47) have limited inhibitory potency of the growth of the microorganisms tested. Due to their lower antimicrobial activity, our studies advice selection of MgAPI47, over PAF104, MgAPI24 and MgAPI40, as promising compound to control rice blast disease by specifically blocking appressorium formation and without toxicity to non-target organisms.

Acknowledgments

We thank Dr. M. Coca and Dr. B. San Segundo for their helpful comments during the course of this investigation. *M. oryzae* PR9 isolate was kindly provided by Dr. Tharreau from CIRAD, Montpellier, France. This work was supported by grant AGL2010-16847 and BIO2012-34381 from the Ministerio de Ciencia e Innovación (MICINN, Spain).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.145.

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