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Interaction between the plant *Ap*Def$_1$ defensin and *Saccharomyces cerevisiae* results in yeast death through a cell cycle- and caspase-dependent process occurring *via* uncontrolled oxidative stress

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ABSTRACT

Background: Plant defensins were discovered at beginning of the 90s'; however, their precise mechanism of action is still unknown. Herein, we studied *Ap*Def$_1$-*Saccharomyces cerevisiae* interaction.
Methods: *Ap*Def$_1$-*S. cerevisiae* interaction was studied by determining the MIC, viability and death kinetic assays. Viability assay was repeated with hydroxyurea synchronized-yeast and pretreated with CCCP. Plasma membrane permeabilization, ROS induction, chromatin condensation, and caspase activation analyses were assessed through Sytox green, DAB, DAPI and FITC-VAD-FMK, respectively. Viability assay was done in presence of ascorbic acid and *Z*-VAD-FMK. Ultrastructural analysis was done by electron microscopy.
Results: *Ap*Def$_1$ caused *S. cerevisiae* cell death and MIC was 7.8 μM. Whole cell population died after 18 h of *Ap*Def$_1$ interaction. After 3 h, 98.76% of synchronized cell population died. Pretreatment with CCCP protected yeast from *Ap*Def$_1$ induced death. *Ap*Def$_1$-*S. cerevisiae* interaction resulted in membrane permeabilization, H_2O_2 increased production, chromatin condensation and caspase activation. Ascorbic acid prevented yeast cell death and membrane permeabilization. *Z*-VAD-FMK prevented yeast cell death.
Conclusions: *Ap*Def$_1$-*S. cerevisiae* interaction caused cell death through cell cycle dependentprocess which requires preserved membrane potential. After interaction, yeast went through uncontrolled ROS production and accumulation, which led to plasma membrane permeabilization, chromatin condensation and, ultimately, cell death by activation of caspase-dependent apoptosis *via*.
General significance: We show novel requirements for the interaction between plant defensin and fungi cells, *i.e.* cell cycle phase and membrane potential, and we indicate that membrane permeabilization is probably caused by ROS and therefore, it would be an indirect event of the *Ap*Def$_1$-*S. cerevisiae* interaction.

1. Introduction

Plant defensins are peptides which present antimicrobial activity and are found in several plant species. They comprise a superfamily of antimicrobial peptides (AMPs), which are structurally and functionally similar [1]. Peptides from this superfamily are found in several other organisms, including bacteria [2], fungi [3], insects [4], birds [5] and mammals [6]. Plant defensins were discovered more than two decades ago [7], and since then they were intensively studied. Therefore, many characteristics of this plant peptide family, *e.g.* primary, secondary and tertiary structures, gene expression patterns, tissue and cell localization and biological activities, are well known [1,8]. Taken together, the antimicrobial biological activity observed *in vitro*, the information of gene expression in response to pathogens and hormones [9] and the results of plants transformed with defensin genes [10,11], are now associated with a biological function in plant defense against pathogens [12].

Also for some plant defensins, many aspects of their mechanisms of action over fungi have been characterized [13]. In general, and

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especially in the case of plant defensins whose therapeutic potential was clearly demonstrated [14], the understanding of the mechanism of action of AMPs is important because: (1) it will facilitate their use as antimicrobial agents; (2) it will facilitate the identification of possible targets for antifungal therapy; (3) it will allow rational design of modifications and substitutions for minimization of instability and toxicity; and (4) it will improve the choice of the best delivery method to their sites of action. Therefore, the relevance of plant defensins to the medical application justifies the intense studies to unravel the mechanism of action of this plant peptide family. However, despite these extensive studies, the precise mechanism of action is still unknown.

Our group has previously purified and characterized a plant defensin from *Adenanthera pavonina* L. seeds called *Ap*Def$_1$ (*A. pavonina* Defensin one) [15]. The identification and characterization of *Ap*Def$_1$ provided us a biological tool to study the cell death mechanism occurring upon the *Ap*Def$_1$-*Saccharomyces cerevisiae* interaction.

2. Materials and methods

2.1. Biological material

Seeds from *Adenanthera pavonina* (L.) were harvested in natural meadows of Campos dos Goytacazes province, Rio de Janeiro, Brazil. The seeds were kept in closed flasks at room temperature until use.

Saccharomyces cerevisiae (1038) were conserved on Sabouraud agar (10 g/l peptone, 20 g/l D(+)glucose and 17 g/l agar) (Merck Millipore Brazil) at 30 °C and transferred to new medium, every three months.

2.2. Extraction and purification of ApDef$_1$

The extraction and purification of the *Ap*Def$_1$ were accomplished as described by Soares et al. [15].

2.3. Evaluation of the minimal inhibitory concentration of ApDef$_1$ over S. cerevisiae

The assay to determine the minimal inhibitory concentration (MIC) was done as described by Broekaert et al. [16] and Wiegand et al. [17] with modifications. Initially, cells of *S. cerevisiae* from a culture plate stock were replicated to a new Petri dish containing Sabouraud agar and grown for 24 h at 30 °C. After the growth period, colonies were suspended in Sabouraud broth (5 g/l peptone from meat, 5 g/l peptone from casein, and 20 g/l D(+)glucose) (Merck Millipore Brazil) and cells were counted in a Neubauer chamber (Laboroptik, United Kingdon) under an optical microscope (Axioplan.A2, Carl Zeiss). The obtained count was used for preparation of cell dilutions to be later used. *S. cerevisiae* cells, at density of 4000 cells/ml, were incubated in 100 μl of Sabouraud broth containing a two-fold dilution of *Ap*Def$_1$, as follows: 15.6, 7.8, 3.9, 1.9, 0.98 and 0.49 μM (this dilution concentration series was based in a previous assay described by Soares et al. [15]). These dilutions were used for both the growth inhibition assay and the MIC determination assay. The assays were done in cell culture plates of 96 wells (Nunc) and the growth was assessed by determination of the optical density at 620 nm, under a microplate reader (Gio. DG Vita EC DV 990DV6), at every 6 h for 36 h and at a temperature of 30 °C. Cells in Sabouraud broth without the addition of *Ap*Def$_1$ were employed as a growth control. Optical densities obtained from the different test samples, containing the *Ap*Def$_1$ diluted solutions, were plotted as a function of time (h). The experiment was repeated twice and the test for the particular MIC was repeated five times. The MIC was defined as the lowest *Ap*Def$_1$ concentration, in μM, which resulted in no visible cell growth, at the bottom of the well, to unaided eye, at the end of the experiment (at 36 h).

2.4. Analysis of S. cerevisiae cell viability after interaction with ApDef$_1$

To verify whether the cell growth inhibition was caused through a fungicidal or fungistatic effect, control cells were resuspended in Sabouraud broth, after 12 h of *Ap*Def$_1$ incubation (as described in Section 2.3), washed once in Sabouraud broth, diluted approximately 50 fold in Sabouraud broth and homogeneously spread on Petri dishes containing Sabouraud agar with a Drigalski spatula. The Petri dishes were incubated at 30 °C for a period of 24 or 36 h in order to establish colonies [18]. After this period, the colonies were counted and images of the Petri dishes were taken. The same procedure described above was applied to verify viability of cells incubated with the different concentrations of *Ap*Def$_1$. The number of colonies obtained in the control Petri dish was considered as the 100% viability. The assay was repeated twice.

2.5. Kinetic analysis of S. cerevisiae cell death induced by ApDef$_1$

After the elucidation of the fungicidal nature of *Ap*Def$_1$ mechanism of action over *S. cerevisiae* cells, we determined the minimal period necessary for *Ap*Def$_1$, at MIC level, to cause the viability loss. Cell preparation and incubation with *Ap*Def$_1$ were done exactly as described at Section 2.3 and the assay of cell viability loss was performed as described in Section 2.4, with the following modifications: from the initial incubation time (0 h), and at every 3 h until 21 h, the entire volumes of the control and test wells were used. The experiment was repeated twice.

2.6. Analysis of the cell cycle interference in S. cerevisiae cell death induced by ApDef$_1$

Cells of *S. cerevisiae* were transferred to a new Petri dish containing Sabouraud agar and grown for 24 h at 30 °C. Then, an inoculum was transferred to a 50 ml centrifuge tube containing 10 ml of Sabouraud broth and incubated for 16 h at 30 °C under shaking at 250 rpm. After this period, the cells were counted in a Neubauer chamber and 10^6 cells/ml were transferred to 5 ml of a new Sabouraud broth containing 500 mM hydroxyurea (HU) (Sigma) [19]. As a control, a second tube without HU was also made. The tubes were kept under constant agitation at 250 rpm, 30 °C for 4 h and, then, both control and HU treated cells were washed 3 times with Sabouraud broth. After a new counting of the control and HU treated cells, 4000 cells/ml were incubated in 100 μl Sabouraud broth containing *Ap*Def$_1$ at the MIC, as described at the Section 2.5 except for the fact that the cells were incubated only for 3 h. The analysis of cell viability loss was performed as described in Section 2.4, being the entire volume of the control and test wells used in this present purpose. The number of colonies obtained in the HU synchronized culture was considered as 100% of viability. The experiment was repeated three times.

2.7. Analysis of DNA binding capability of ApDef$_1$

S. cerevisiae DNA was extracted by DNeasy Plant Mini Kit (Qiagen) from a fresh *S. cerevisiae* culture in Sabouraud broth according to the manual and quantified by NanoDrop 2000 (Applied Biosystems). The assay is based on the binding of the protein to the DNA, what results in a complex that has a different electrophoretic mobility from the free DNA. The differential mobility of the complex is observed, in gel, as retardation mobility in regard to relative mobility of the unbound DNA. Gel shift assay was performed as described by Park et al. [20] by mixing 100 ng of yeast DNA and 7.8 μM *Ap*Def$_1$ in 20 μl of binding buffer (5% glycerol, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl, and 50 μg/ml BSA). The reaction was incubated for 30 min at 30 °C and then 4 μl of 6× DNA Loading Dye (10 mM Tris–HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA, Thermo Scientific) were added to the sample and the

entire volume was loaded on 0.8% agarose gel. Water was used as negative control and 100 μg/ml poly-L-lysine (Sigma-Aldrich), as positive control. The run was performed at a voltage of 4 V/cm^2 and the gel image was acquired using the imager ImageQuant LAS 500 (GE Healthcare). The gel was stained with GelRed (Biotium). The experiment was repeated twice.

2.8. Analysis of S. cerevisiae membrane potential interference in cell death induced by ApDef$_1$

This experiment was performed as described in the Section 2.3, until *S. cerevisiae* quantitation, with the following differences: cells were incubated with 30 μM of carbonylcyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich) for 1 h 30 min at 30 °C [21]. After this period, *Ap*Def$_1$ was added and the assay continued for 18 h. Non-treated cells and cells treated only with CCCP were taken as controls. At the end of the assay, treated cells were plated as described for the Section 2.5. This experiment was repeated three times.

2.9. Analysis of the influence of temperature on the activity of ApDef$_1$ over S. cerevisiae

Cells for this assay were obtained as described in Section 2.3, but *Ap*Def$_1$ was incubated with *S. cerevisiae* cells for 18 h at 4 °C. Control was done with incubation at 30 °C for 18 h. After this period, cells were treated as described in Section 2.5. The experiment was repeated twice.

2.10. Analysis of H_2O_2 production in S. cerevisiae induced by ApDef$_1$

This experiment was done as described at the Section 2.3, until the quantitation of the *S. cerevisiae*. A concentration of 40,000 cells/ml was transferred to 96 wells plate and incubated with *Ap*Def$_1$ at 7.8 μM and 10 μl of a freshly prepared 1 mg/ml 3,3′-diaminobenzidine (DAB) (Sigma) solution. A higher cell density was used in this assay to allow microscopic visualization of cells. The preparation of the DAB staining solution and the staining process followed procedures described by Thordal-Christensen et al. [22] and Liu et al. [23]. After 3 h of incubation at 30 °C with *Ap*Def$_1$, the cells were analyzed by DIC microscopy (Axio Imager.A2, Zeiss). Cells treated with 200 mM of acetic acid were used as positive controls. The experiment was repeated twice.

2.11. Analysis of the importance of oxidative stress for the S. cerevisiae death induced by ApDef$_1$

This experiment was done, until the quantitation of the *S. cerevisiae* cells, as described at the Section 2.3 and after incubation as described for the Section 2.5, with the following differences: a concentration of 4000 cells/ml was transferred to 96 wells cell plate and incubated with *Ap*Def$_1$ at 7.8 μM and 100 mM antioxidant ascorbic acid solution (Sigma-Aldrich), diluted from a 1 M stock aqueous solution and filter sterilized (Millex-GV 0.22 μm, Merck Millipore), for periods of 3, 9 and 18 h. Ascorbic acid was added at the same time as *Ap*Def$_1$. Controls with only either 100 mM ascorbic acid or cells in medium were taken as negative controls and the experiment was repeated three times.

2.12. Analysis of S. cerevisiae cell membrane permeabilization by ApDef$_1$

Cell membrane permeabilization induced after *Ap*Def$_1$ treatment was evaluated through the use of the fluorescent dye Sytox green (Molecular Probes, Life Technologies) [24]. This experiment was done, until the quantitation of the *S. cerevisiae*, exactly as described in Section 2.3. A concentration of 40,000 cells/ml was transferred to 96 wells cell plate and incubated with *Ap*Def$_1$ at 7.8 μM for 5, 10, 15, 30 min and 1 h. A higher cell density was used in this assay to allow their microscopic visualization. The Sytox green dye [0.5 μM final concentration diluted from a 50 μM stock solution in dimethyl sulfoxide (DMSO) (Sigma-Aldrich)] was added 5 min before completion of the incubation time and the plate was protected from light. Cells were then mounted on microscope slides and visualized under a differential interference contrast (DIC) epifluorescence microscope (Axio Imager.A2, Zeiss) equipped with a fluorescence filter set (excitation 450–490 nm; emission 500 nm). The baseline membrane permeability of *Ap*Def$_1$ untreated cells was taken as control. All the fluorescence images were obtained with the same exposure time at the AxionVision LE version 4.8.2 (Zeiss) with Axio Can MR5 (Zeiss). The percentage of permeabilized cells was calculated based on the total number of cells in the DIC and fluorescent views of random microscopic fields for each sample, assuming the total cell number in bright field of each sample as 100%. The experiment was repeated three times. This assay was repeated in the presence of 100 mM of ascorbic acid only for the time of 1 h. The experiment was repeated twice.

2.13. Analysis of S. cerevisiae cells ultrastructural alterations after exposure to ApDef$_1$

S. cerevisiae cells grown for 18 h in Sabouraud broth in the presence or absence of 7.8 μM *Ap*Def$_1$, as described in Section 2.3, were fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde (V/V) and 4% paraformaldehyde (V/V), in 100 mM phosphate buffer, pH 7.2. After fixation, the cells were washed, post-fixed in 1% (W/V) osmium tetroxide in 100 mM phosphate buffer, pH 7.2, for 30 min at room temperature. The samples were dehydrated in a graded acetone series (30%, 50%, 70%, 90% and 100% (V/V)) and embedded in Epoxi resin (Polybeded). Ultrathin sections (0.1 mm) were laid on copper grids, stained with uranyl acetate for 10 min, followed by lead citrate for five min, and were then observed with a ZEISS 900 transmission electron microscope (TEM) (Zeiss company, Germany) operating at 80 kV. The experiment was repeated three times.

2.14. Detection of chromatin condensation in S. cerevisiae treated with ApDef$_1$

This experiment was done, until the quantitation of the *S. cerevisiae*, exactly as described at the Section 2.3. The nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) was described by Madeo et al. [25]. A concentration of 40,000 cells/ml was transferred to a 96 wells cell culture plate and incubated with 7.8 μM of *Ap*Def$_1$ for 18 h. A higher cell density was used in this assay to allow microscopic visualization of cells. For nuclear staining, after incubation time, cells were washed with PBS, incubated with 1 μg/ml DAPI in PBS for 10 min, rinsed three times with PBS and then visualized under a DIC epifluorescence microscope (Axio Imager.A2, Zeiss), equipped with a fluorescence filter set (excitation 365 nm; emission 397 nm). A control with cells heated at 100 °C for 5 min was done. The experiment was repeated three times.

2.15. Caspase activity detection

Detection of active caspase was performed using the CaspACE, FITC-VAD-FMK *In Situ* Marker (Promega), as described in the manual. This fluorescent marker is a derivative of the fluorescein isothiocyanate (FITC) group substituted at the carbobenzoxy (Z) N-terminal blocking group of the pan caspase inhibitor *Z*-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone). Forty-thousand cells/ml were treated with 7.8 μM *Ap*Def$_1$ for 30 min, 3, 9 and 18 h and then they were harvested by centrifugation, washed once in 1 ml PBS, and resuspended in 50 μl of the staining solution containing 50 μM FITC-VAD-FMK. A higher cell density was used in this assay to allow microscopic visualization of cells. After incubation for 20 min at 30 °C, cells were washed in 1 ml PBS and resuspended in

10 μl PBS. The cells were observed by fluorescence microscopy as described at the Section 2.12. The experiment was repeated three times.

2.16. Analysis of the importance of caspase activity for S. cerevisiae death induced by $ApDef_1$

This experiment was done, until the quantitation of the *S. cerevisiae*, as described at the Section 2.3 with the following differences: $ApDef_1$ was incubated for 2 h with the cells and after this period, Z-VAD-FMK (50 μM) was added and the incubation continued for 18 h. After this period, cells were treated as described in Section 2.4. Three samples were employed: (1) only cells and Sabouraud broth (control), (2) cells treated with $ApDef_1$ and (3) cells and Z-VAD-FMK. The experiment was repeated twice.

2.17. Statistical analysis

Assays were statistically determined using a one-way ANOVA with means differences at $P < 0.05$ or $P < 0.01$ were considered to be significant. Analyses were done with the Gene software [26].

3. Results and discussion

In this report, features of the $ApDef_1$-*S. cerevisiae* interaction which are involved and required for the defensin deleterious effects over yeast cells were evaluated. We had previously purified $ApDef_1$ from H_{11} fraction, which contained $ApDef_1$ and a yet uncharacterized protein [15]. Based on the parameters of 83% of growth inhibition and fungicide effect of H_{11} fraction over *S. cerevisiae* at 80 μg/ml, we started the two-fold dilution of $ApDef_1$ from 80 μg/ml (15.6 μM) (Fig. 1), in order to determine the MIC of $ApDef_1$ over *S. cerevisiae*. The growth inhibition assay indicated that the concentrations of 15.6, 7.8 and 3.9 μM of $ApDef_1$ completely inhibited the growth of *S. cerevisiae*. The other concentrations were less efficient in causing this inhibition effect (Fig. 1A). Images from the wells of the cell plate assay at 36 h showed no visible mass of cells by unaided eye, at the concentrations of 15.6 and 7.8 μM. At the concentration of 3.9 μM, a tiny mass of cells could be seen to unaided eye; however, it was not captured by the camera resolution (Fig. 1B). The tiny mass of cells was also not detected by the device used for measuring absorbance readings, what led to an artefact result of complete inhibition, in the growth inhibition assay at the presence of 3.9 μM of $ApDef_1$. This fact occurred due to the resolution limitation of the used device which did not allow to differentiate this tested concentration from the blank well (only medium). Based on these two results, we assumed the 7.8 μM of $ApDef_1$ as the MIC for *S. cerevisiae*, in the conditions tested. No growth of *S.cerevisiae* was observed since 12 h, in the presence of 15.6, 7.8 and 3.9 μM of $ApDef_1$ (Fig. 1A). To confirm whether the growth inhibition caused by $ApDef_1$ was due to fungistatic or fungicidal effect, an aliquot of the samples from Section 2.4 was taken at 12 h of incubation and tested for cell viability. No colony was obtained for the samples treated with $ApDef_1$ at concentrations of 15.6 and 7.8 μM. To all other concentrations, the number of grown colonies was inversely proportional to the $ApDef_1$ concentration (Table 1). This result corroborates with the determined MIC of 7.8 μM and confirmed that the previous fungicidal effect of the H_{11} fraction [15] is in fact imparted by $ApDef_1$. Accordingly, the results presented in Table 1 corroborate with the presence of viable cells treated with 3.9 μM of $ApDef_1$, despite no growth being detected by absorbance readings and even by the camera resolution. The MIC determination has advantages in clinical practice for the evaluation of the antimicrobial properties of new substances as well as for the assessment of microbial susceptibility and resistance [17]. In our case, we associated the MIC to a biological effect, *i.e. S. cerevisiae* cell death after interaction with 7.8 μM of $ApDef_1$, and, therefore, we assure that the observed events which we have studied, such as membrane permeabilization, ROS and apoptosis inductions, are in fact relevant and associated with the *S. cerevisiae* cell death induced by $ApDef_1$. This precaution was taken to avoid misinterpretation of the signal involved in the cell death, since concentrations of $ApDef_1$ below MIC did not cause *S. cerevisiae* cell death, probably because yeast cells, in the presence of low $ApDef_1$ concentrations, are able to

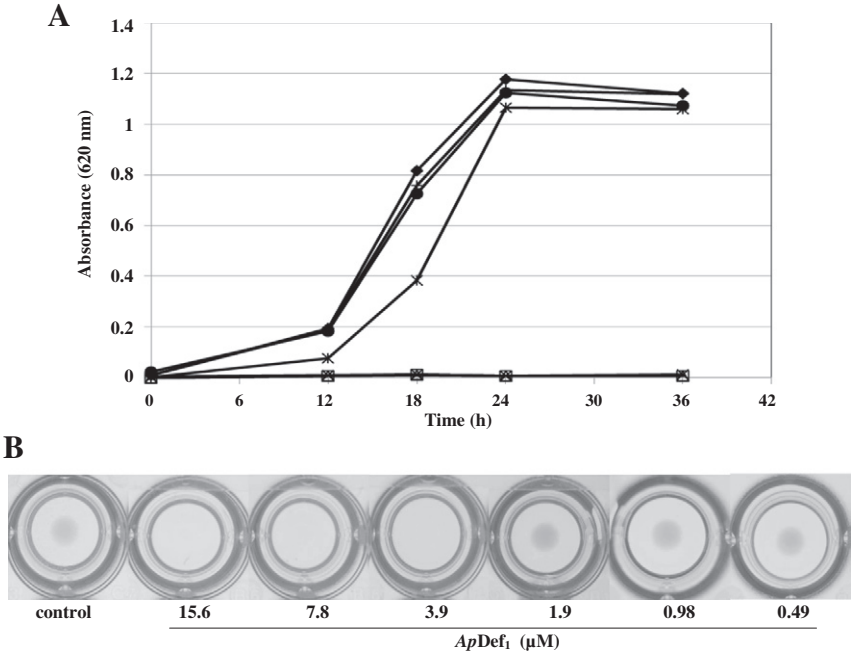


Fig. 1. Antifungal effect of $ApDef_1$ over *Saccharomyces cerevisiae* and minimal inhibitory concentration determination. (A) Graphic visualization of the growth inhibition assay of *S. cerevisiae* yeast incubated for 36 h in the presence of $ApDef_1$ at different concentrations as follows: (□) 15.6 μM[b], (△) 7.8 μM[b], (○) 3.9 μM[b], (×) 1.9 μM[a], (●) 0.98 μM[a] and (+) 0.49 μM[a] of $ApDef_1$. (♦) Control[a] (without addition of $ApDef_1$). Graphic was generated using data of one independent experiment out of two (n = 2). Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$. (B) Images of the plate wells at the end of the growth inhibition assay (at 36 h), showing the growth pattern of *S. cerevisae* cells at the bottom of the wells, in the absence (control) and presence of different $ApDef_1$ concentrations. Images shown were generated using data of one independent experiment out of two (n = 2).

Table 1
Viability of the *Saccharomyces cerevisiae* cell culture* after 12 h of treatment with *Ap*Def$_1$. The number of colonies obtained in the control was assumed to be 100% of viability.

*Ap*Def$_1$ (μM)	Number of colonies	Viability (%)
0.0 (control)	999	100
0.49	606	60.6
0.98	652	65.3
1.9	515	51.5
3.9	83	8.3
7.8	0	0
15.6	0	0

* This assay was done with an aliquot of the MIC assay take after 12 h incubation with *Ap*Def$_1$.

mount a defense response which reverses the pathway leading to *Ap*Def$_1$-induced cell death. Corroborating with this supposition of a response event reversing the pathway leading to cell death at concentrations of *Ap*Def$_1$ below MIC, membrane permeabilization (see S.I. 1) and ROS induction (see S.I. 2) have occurred. Countermeasure responses were characterized in some interactions with AMPs and fungi, such as the *Na*D1 (plant defensin from *Nicotiana alata* flower) and the HOG pathway (a stress-responsive pathway which protects fungi from osmotic stress) in *C. albicans* [27] and histatin-5 (AMP from human saliva) and the HOG pathway [28]. Additionally, we characterized the antifungal effect of *Ap*Def$_1$ against *S. cerevisiae* as fungicidal. This characteristic is favorable over fungistatic compounds for biotechnological applications, because fungistatic effect may contribute to the development of resistance [29,30].

After this initial parameters determination, we proceed to study the characterization of the possible mechanisms underlining the interaction between *Ap*Def$_1$ and *S. cerevisiae*. Initially, based on the fungicidal effect of *Ap*Def$_1$ on *S. cerevisiae*, we determined the time, after the initial contact of *Ap*Def$_1$ and *S. cerevisiae,* which led to cell death. In this experiment, the entire volume of each sample (100 μl) was used, instead of only an aliquot as in the prior analysis, in order to include the whole initial cell population (Fig. 2). From 3 h after *Ap*Def$_1$ interaction, a *S. cerevisiae* cell subpopulation died, as indicated by the decreased number of colony forming unities (CFU) in the experimental samples. For the next interval periods of 3 h, the result was the same; a cell subpopulation died. This result was repeated to the other incubation times until no colony growth could be observed at 18 h after the initial incubation with *Ap*Def$_1$. We assumed that, in the condition tested, the *Ap*Def$_1$ induces cell death of all cell population after 18 h treatment. This observation led us to question why the cells after contact with *Ap*Def$_1$ died at different times and not all at once, as expected. We assumed this as expected, because of the AMPs universal physicochemical characteristics ruling their interaction with microorganisms, *i.e.* their positive charge at neutral pH and amphiphilic tridimensional structure [31]. Despite the fact that the overall three dimensional structure of plant defensins does not present the amphiphilic scaffold of the linear or helical AMPs, several works have pointed out that a minimal sequence of amino acid residues is required for the activity of

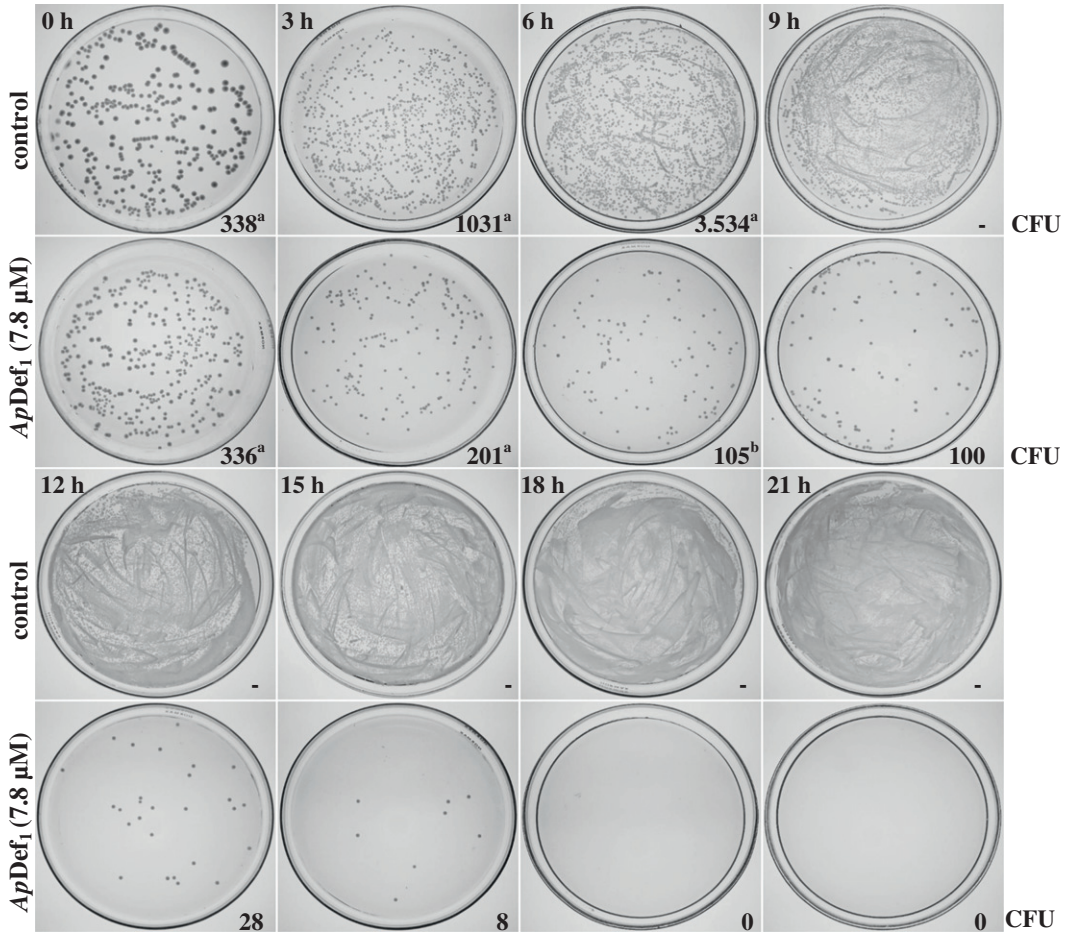


Fig. 2. Kinetic of the *Saccharomyces cerevisiae* cell death incubated with *Ap*Def$_1$. Images of the Petri dish where *S. cerevisiae* cells were incubated with *Ap*Def$_1$ at the MIC (7.8 μM) for 21 h. Note that in the Petri dishes containing the test samples, the number of viable colonies decreased with the increase of time until the complete initial cell population lost viability, at 18 h. Also note that the initial cell population is not homogeneously affected at the same time. Below the Petri dish images are shown the colony forming unities (CFU) in each time interval and (−) indicates an excessive number of grown colonies which impeded colony counting. Images shown are representative of one independent experiment out of two (n = 2). Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$.

plant defensins. This sequence was shown to be located in a stretch region at the loop between the β2 and β3-strands, and encompasses approximately 19 amino acids [32–34]. These stretches present polar (RHGS) and apolar (VFPA) features which have been demonstrated to be important to the antifungal activity of *Rs*-AFP2 (plant defensin from *Raphanus sativus*) [33]. Sagaran et al. [34] have demonstrated that cationic (hydrophilic) and hydrophobic amino acid residues in this stretch are important to the antifungal activity of MsDef1 (plant defensin from *Medicago sativa*) and MtDef4 (plant defensin from *Medicago truncatula*). AMPs interaction with microorganism has been demonstrated to occur firstly by opposite charge attraction between the positively charged AMP and negatively charged targets in the microorganism surface, *e.g.* the head of negatively charged phospholipids [35]. Secondly, the AMP hydrophobic face interacts with the hydrophobic membrane core and, in this moment, the AMPs are capable of inserting themselves into the membrane, causing its disorganization and consequent permeabilization [35]. Several studies corroborate with this view. For example: (1) salts, such as NaCl and $MgCl_2$, are known to block the antimicrobial effect of AMPs due to disruption of the initial opposite charge attraction [28,36]; (2) mutant microorganisms lacking negatively charged components in their membranes are more resistant to the action of AMPs, such as the *Leishmania donovani,* deficient in the production of the anionic lipophosphoglycan [37]; (3) some bacteria evolved defense mechanisms which alter the negative charge of surface molecules, such as teichoic acid or lipopolysaccharides and also alter the membrane fluidity to avoid the AMP interaction [38]. These are particularly interesting mechanisms because the deliberate modifications induced by these bacteria in response to AMPs perception are done in the sites where AMP-microorganism interaction was theoretically and practically proved to occur. For the plant defensin NaD1 (from *Nicotiana alata*), it has been suggested that the resistance mechanism of a *S. cerevisiae agp2*Δ mutant would be related to the raise of positively charged molecules at cell surface, and these, by their turn, would repulse NaD1 and other AMPs [8]; (4) the amphipathicity of AMPs can be loaned to other molecules endowing them with antimicrobial activity [39]. For these reasons, we would expect a higher uniformity in the outcome of the *Ap*Def_1-*S. cerevisiae* interaction in relation to the death time. Additionally, we know that some heterogeneity could be observed because of the naturally desynchronization of the *S. cerevisiae* cell cycle [40–42].

Based in all exposed reasons and in our results, we hypothesize that the action of the *Ap*Def_1 that leads to the process of *S. cerevisae* cell death was dependent of the *S. cerevisiae* cell cycle. To test this hypothesis, the same assay was repeated with *S. cerevisiae* cells, synchronized with 500 mM HU. The synchronized *S. cerevisiae* cells, when incubated with *Ap*Def_1 for 3 h, showed 98.76% of cell death (Table 2). Comparing the colony number of the synchronized culture (4 colonies in average, Table 2) with the colony number of the unsynchronized one (201 colonies in average, see Fig. 2 after 3 h o f incubation with *Ap*Def_1), both treated with *Ap*Def_1, we can conclude that the synchronized cells were more susceptible to the action of *Ap*Def_1. The time required for *S. cerevisiae* cell division (generation time) in the conditions tested was about 3.4 h (see S.I. 3), and accordingly, within only one generation time 98.76% of synchronized cells died. Therefore, it is possible to suggest that a cell cycle phase, in which cells are in synchronization, favors the action of *Ap*Def_1. This result may indicate that the *Ap*Def_1 induced *S. cerevisiae* cell death is cell cycle dependent. Note that 500 mM of HU caused substantial toxicity to *S. cerevisiae* cells, reflected by the fewer developed colonies (321.67 colonies against 1299 in the control, *i.e.* 75.23% of cell death due to HU treatment) (Table 2). Because of the toxicity observed in the synchronized culture of *S. cerevisiae* due to the HU treatment, we did not use synchronized cultures in the next assays. This precaution was taken to avoid possible misinterpretation between the signals involved in the *S. cerevisiae* cell death induced by *Ap*Def_1 and HU. HU is an inhibitor of cell division specifically at the G1/S phase, which inhibits DNA synthesis by impairing the activity of ribonucleotide reductase in the conversion of ribonucleotides into deoxyribonucleotides. After the treatment period with HU, all cells are synchronized in the same phase of the cell cycle [19,43]. This synchronization is reversible and when cells are transferred to new growth medium, lacking the drug, they continue the cell cycle from the arrested phase. The synchronized cells were treated with the *Ap*Def_1 for 3 h and, after this period, 98.76% of the cells lost viability (Table 2). Based on our results, we hypothesize that the *Ap*Def_1 acts in a certain moment of the *S. cerevisiae* cell cycle what clearly is reinforced by the total time of 18 h required for 100% loss of viability of unsynchronized cells (Fig. 2, *Ap*Def_1 (7.8 μM)). Hultmark et al. [44] reported that the antibacterial proteins, attacins, (from hemolymph of immunized pupae of the moth *Hyalophora cecropia*) might target the cell cycle of *Escherichia coli* envA chain forming mutant.

One of the possible explanations for the influence of cell cycle stage to the cell death induced by *Ap*Def_1 could be the interference with the metabolism of anionic macromolecules, such as DNA, by its direct binding or by its synthesis inhibition. The binding hypothesis was investigated by a DNA shift assay. Our result indicates that *Ap*Def_1 was not able to prevent the mobility of the *S. cerevisiae* DNA, showing that *Ap*Def_1 does not bind to DNA (result not shown). There are reports of AMPs which interfere with or halt the DNA synthesis as the mechanism underlining their antimicrobial activity. It has been demonstrated that [^{3}H]-thimidine incorporation into the DNA was stopped in *E. coli* by PR-39 (AMP from small intestine of pig) [45] and in *Entamoeba histolytica* by cryptdin-2 (defensin from mouse Paneth cell) [46]. Also the AMPs indolicitin (from cytoplasmic granules of bovine neutrophils) and buforin-II (derived from the Asian toad *Bufo bufo garagriozans* buforin I) were demonstrated to bind directly to DNA [20,47]. As our result shows that *Ap*Def_1 does not bind to *S. cerevisiae* DNA (result not shown), we infer that other mechanism must be involved in the toxic cell-cycle dependent effect of *Ap*Def_1. Another possibility for the cell cycle dependence of *Ap*Def_1 action was demonstrated by Lobo et al. [48], which showed, *via* yeast two-hybrid screening system, that the *Ps*D1 (plant defensin from *Pisum sativum* seeds) interacts with cyclin F of the fungus *Neurospora crassa*, a protein related to the cell cycle control. This would destabilize the nuclear translocation of cyclin B, thus interfering with the normal cell cycle progression [49,50]. *In vivo* studies using a developing retinal tissue of neonatal rats, as a model for cell cycle progression, have shown that *Ps*D1 blocked the cyclin F role in the transition from S to G2 phases, impairing the cell cycle progression by promoting disturbing nuclear migration and endoreduplication [48]. Therefore, a possible explanation for the dependence of the *Ap*Def_1 activity on the cell cycle stage might be the interaction of this molecule with one of the several components of the cell cycle control, such as cyclins. Unfortunately, the cyclin binding site of *Ps*D1 is not mapped, what impaired us to make a more precise analysis. However, the similarity of *Ap*Def_1 and *Ps*D1 is indicated by the sharing of 17 identical amino acid residues (35% of similarity) and 22 positive amino acid residues (44% of similarity) between the two sequences (see S.I. 4). This hypothesis should be experimentally tested.

Table 2
Cell cycle dependence of the inhibitory effect of *Ap*Def_1. Counting of the number of grown colonies after 3 h of incubation of unsynchronized, synchronized with hydroxyurea and *Ap*Def_1-treated synchronized *Saccharomyces cerevisiae* cells. Note that the used concentration of hydroxyurea caused some toxicity to the cells but it is still possible to see that a cell cycle stage influences positively the action of the defensin. The colony number for unsynchronized cells treated with *Ap*Def_1 is shown in Fig. 2.

Samples	Number of colonies	Percentage of viability
Unsynchronized cells	1299 ± 41^a	–
Synchronized cells	321.67 ± 48^a	100
Synchronized cells + *Ap*Def_1	4 ± 2.6^b	1.24

Table was generated using data of three independent experiments (n = 3). Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.01$.

Other reported relevant feature to the AMP-microorganism interaction is the electrical potential in the plasma membrane. Therefore, to verify if the membrane potential is required for the *Ap*Def$_1$-*S. cerevisiae* interaction, we have used the uncoupler and protonophore CCCP as a membrane depolarizing agent [21]. Our results indicate that the pretreatment of *S. cerevisiae* cells with CCCP protected 16.37% of cells from death (Fig. 3A). This result indicates that an intact membrane potential is necessary for the activity of *Ap*Def$_1$. To analyze the effect of the temperature, the incubation of *S. cerevisiae* cells with *Ap*Def$_1$ was done at 4 °C for 18 h. We have observed that the cells incubated at low temperature did not have their viability affected by the treatment with the *Ap*Def$_1$ (Fig. 3B). The opposite could be seen with control treated cells incubated at 30 °C, where, after 18 h of treatment, a complete cell death occurred. This indicates that the low temperature influenced negatively the fungicidal activity of *Ap*Def$_1$ over *S. cerevisiae*. The AMPs PR-39 [45] and attacins [44] did not present antimicrobial activity against non-growing bacteria. The membrane potential was seen to be necessary for some AMPs in order to exert their toxic effects, as demonstrated for *E. coli* cells pretreated with 100 mM of the uncoupler CCCP, which were resistant to the killing ability of indolicitin [21] and *C. albicans* petit mutant (deficient in respiration due to mitochondrial DNA mutation), which was resistant to the action of histatin-5 [51]. The same low temperature protection has been reported by Gyurko et al. [51] in respect to *C. albicans* when exposed to histatin-5 at 4 °C, being the observed effect suggested as a result of a low metabolic state. Therefore, our result demonstrates that the membrane potential plays a role in the interaction of the *Ap*Def$_1$ and *S. cerevisiae* cell.

In this part of the work, we investigate the possible mechanism of *Ap*Def$_1$ action which leads to the *S. cerevisiae* cell death. There are increasing evidences indicating that AMPs have intracellular targets, including plant defensins [48] and additionally, to some of them, it has been demonstrated their entrance in fungal cytoplasm [52,53]. Once in the cytoplasm, translocated AMPs can induce cell damages, such as the inhibition of DNA, RNA and protein synthesis, inhibition of cell wall synthesis, inhibition of enzymatic activity and inhibition of cytoplasmic membrane septum formation [54–56]. Furthermore, cell death may be an independent event, which occurs solely, or complementary to other mechanisms of action [57].

Accumulating evidences suggest that several AMPs exert their antimicrobial action by inducing excessive intracellular ROS accumulation, what causes molecular damages including cell death. The plant defensins *Na*D1 [52], *Rs*-AFP2 (from *Raphanus sativus* seeds) [58], *Pv*D1 (from *Phaseolus vulgaris* seeds) [59] and *Hs*-AFP1 (from *Heuchera sanguinea* seeds) [60] display ability to induce ROS in target organisms and some of those works have shown a direct link between ROS generation and the antifungal effects of those plant defensins. ROS, such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl

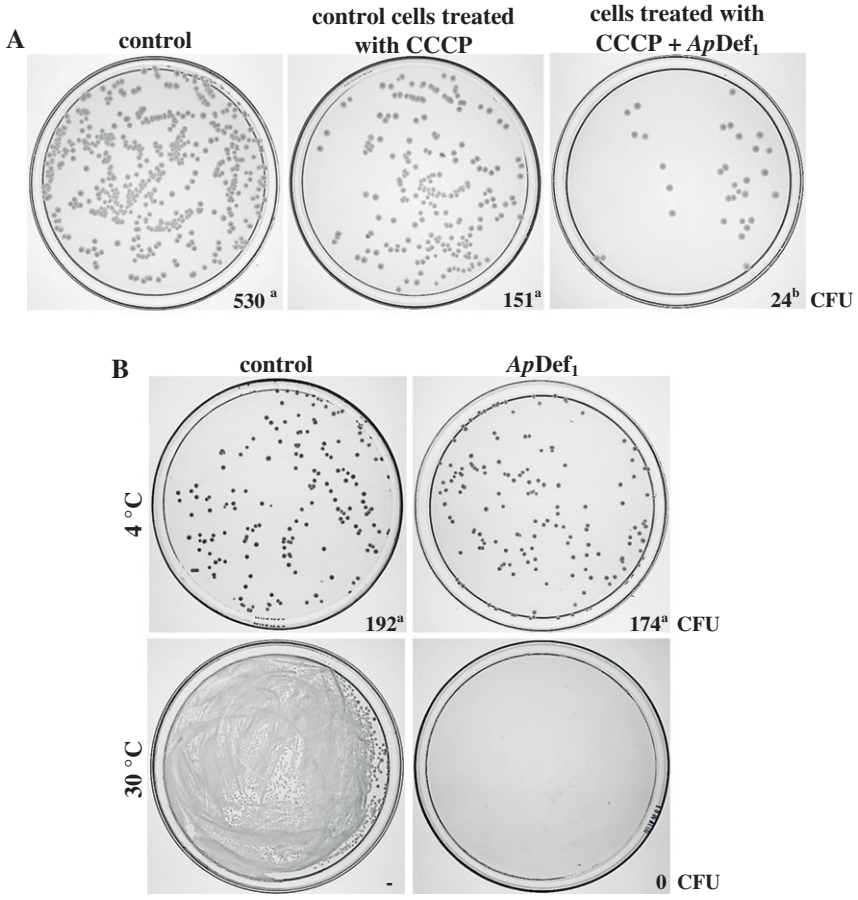


Fig. 3. (A) Membrane potential is necessary to the activity of *Ap*Def$_1$. Image of the Petri dish of *S. cerevisiae* after incubation with 7.8 μM *Ap*Def$_1$, in the presence of CCCP for 18 h. Note that CCCP rescued 16.63% of the cell viability loss induced by *Ap*Def$_1$ (Fig. 3C, 30 °C). The colony forming unities (CFU) are shown for each sample. Images shown are representative of one independent experiment out of three (n = 3). Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$. (B) Low temperature blocks the toxic effect of *Ap*Def$_1$. *S. cerevisiae* cells treated with 7.8 μM of *Ap*Def$_1$ and incubated at 4 °C for 18 h did not loss their viability. For comparison, a control incubated at 30 °C for 18 h, where all cells died (please, also refer this control for Fig. 4A). The CFU are shown for each sample. (−) indicates an excessive number of grown colonies which impeded colony counting. Images shown are representative of one independent experiment out of two (n = 2). Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$.

radical ($OH^{\bullet}$), are reactive molecules which are generated during normal cellular metabolism [61]; failure in the detoxification of ROS leads directly or indirectly to DNA damage, oxidation of proteins, carbohydrates and lipids [62]. Based on these observations, we speculate that the mechanism involved in *S. cerevisiae* death would be the induction of uncontrolled ROS production. Accordingly, we tested whether *S. cerevisiae* cells treated with *Ap*Def_1 had increased ability of H_2O_2 production by analyzing DAB polymerization. DAB is taken up by the cells and reacts with H_2O_2 when peroxidase activity is present and upon oxidation, DAB polymerizes instantly into a light brown insoluble polymer. After 3 h of treatment, we observed a light brown color labeling, similar to that seen in cells treated with 200 mM acetic acid, the positive control for reactivity of DAB (Fig. 4A), indicating the increase of H_2O_2 production by the cells. In the control, there was no generation of H_2O_2, thus suggesting that the production was specifically caused by *Ap*Def_1.

Since *Ap*Def_1 induces H_2O_2 production in *S. cerevisiae* cells, we verified whether the resulting oxidative stress is in fact relevant for the *Ap*Def_1 induced *S. cerevisiae* cell death. Then a test using ascorbic acid, a widely known ROS scavenger, was done to verify whether the treatment of cells with this compound would protect *S. cerevisiae* cells from the toxic effect of *Ap*Def_1. In this assay, ascorbic acid was added at the same time of *Ap*Def_1 to the cell culture medium and evaluated for 18 h, which was the estimated time for reaching 100% of loss viability of *S. cerevisiae* cells. We observed, in the presence of the antioxidant, that the cells did not have their growth affected by *Ap*Def_1, during the

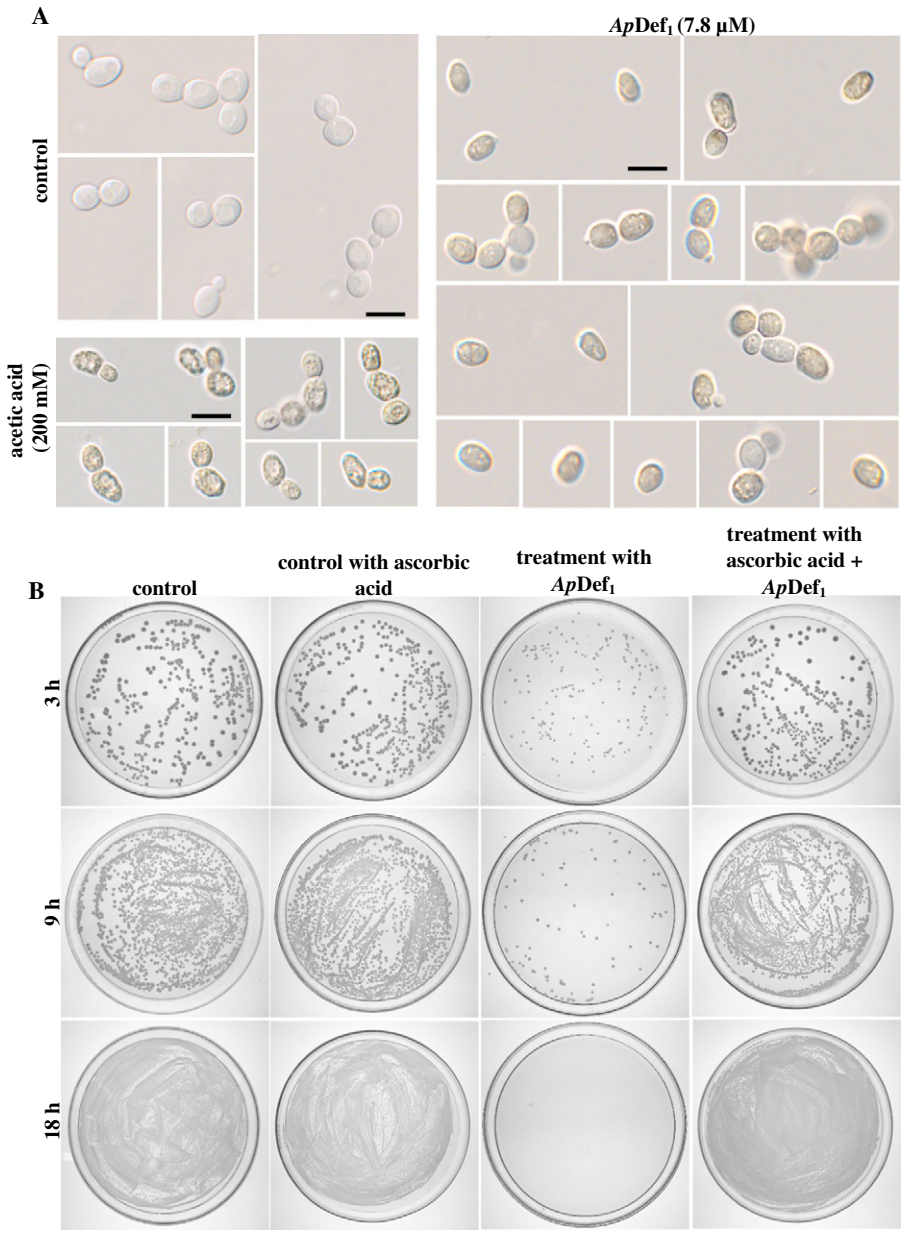
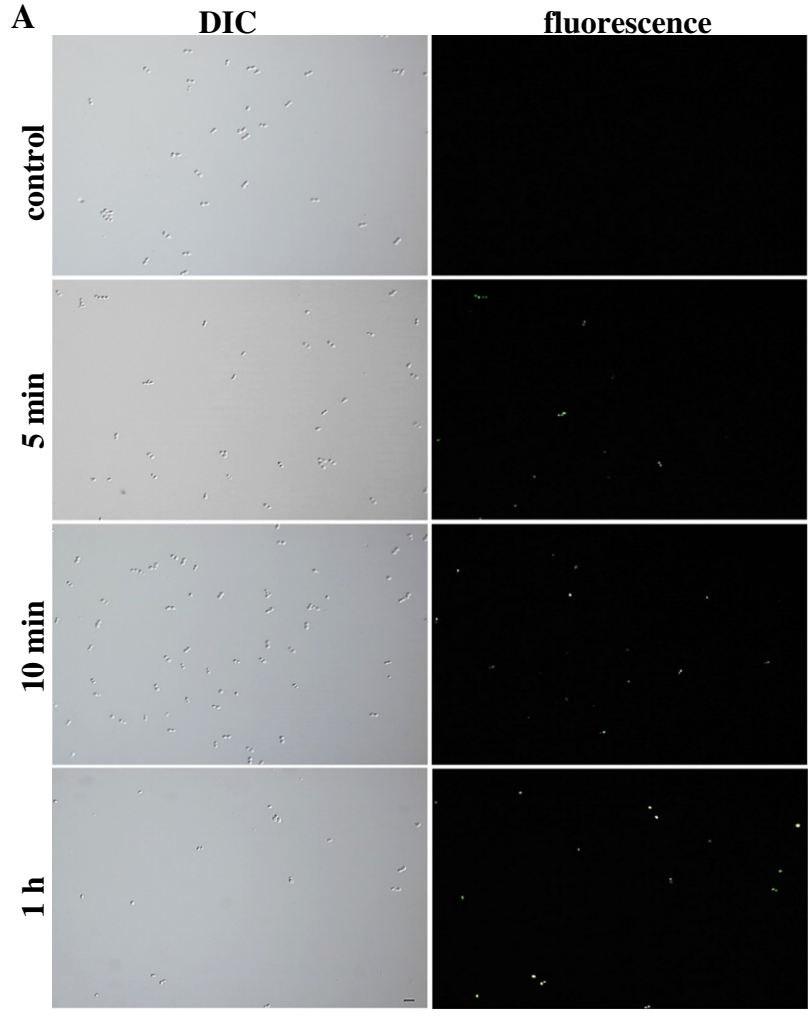


Fig. 4. (A) *Ap*Def_1 triggers H_2O_2 production. Images of the *S. cerevisiae* cells showing H_2O_2 production, after incubation with 7.8 μM of *Ap*Def_1 or with 200 mM acetic acid, as positive control, for 3 h by optical microscopy. Note the light brown DAB reaction in the treated samples. Bars: 10 μm. Images shown are representative of one independent experiment out of two (n = 2). (B) Dependence on the cells oxidative stress status for *Ap*Def_1 induced *S. cerevisiae* death. Images of the Petri dish of *S. cerevisiae* after incubation with 7.8 μM *Ap*Def_1 and 100 mM ascorbic acid for 3, 9 and 18 h. Note that the treatment with ascorbic acid recovers the cell viability loss induced by *Ap*Def_1, indicating the dependence on the oxidative stress for the occurrence of this process. Images shown are representative of one independent experiments out of three (n = 3).

18 h treatment (Fig. 4B). Therefore, ascorbic acid was able to completely reverse the pathway that leads to cell death, indicating that the toxic effect caused by *Ap*Def$_1$ on the cells occurs *via* oxidative stress.

Taking relevant literature information as basis for our understanding of defensin mechanism of action, we would like to highlight two major apparently dissonant piece of information. First, it is known that defensins exert their antifungal activity over many fungal species by inducing membrane permeabilization [53,58,59,63]; these data corroborate with the available information about AMP-membrane interaction, as exposed in the second paragraph of the Section 3, and additionally with the report that *Ps*D1 can interact with membranes [64]. In contrast, *Rs*-AFP2, at concentrations ranging from 20 to 50 μg/ml, does not interact with and neither causes leakage of carboxyfluorescein from small unilamellar vesicles with lipid composition containing different

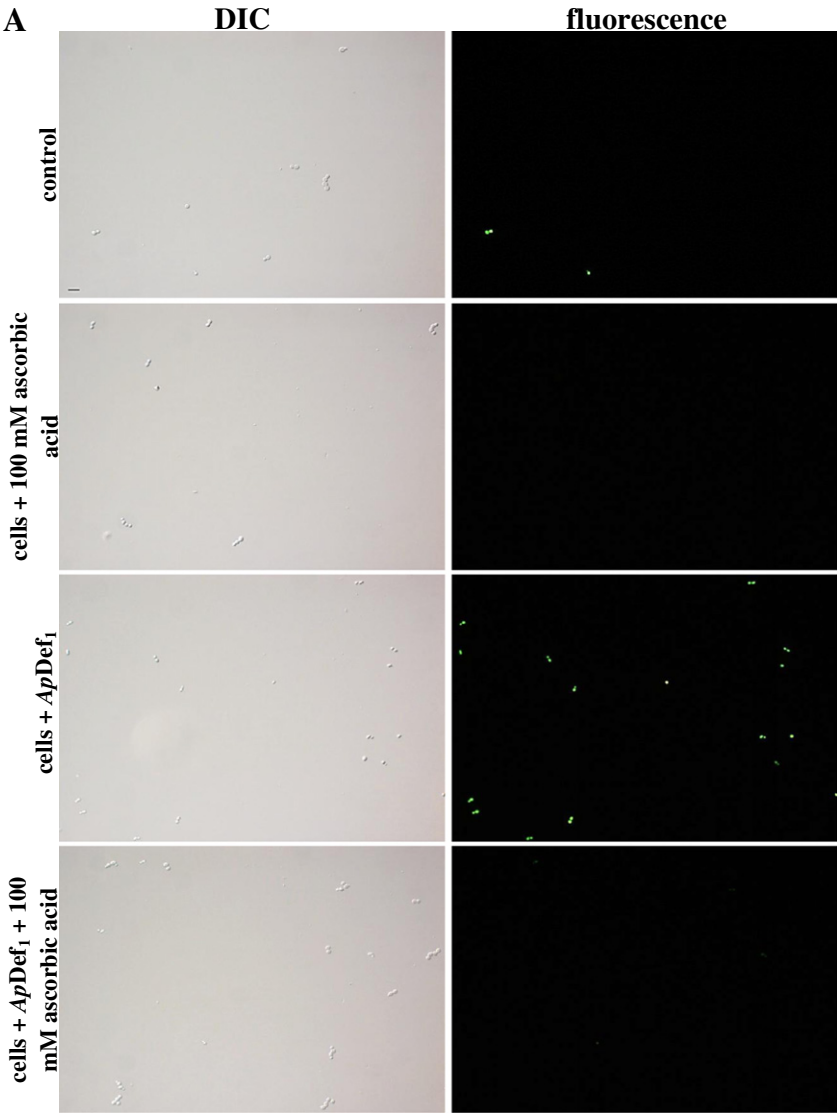
A



B

samples	cell number in DIC	fluorescent cell number	percentage of permeabilized cells
control	48.8 ± 10.72	1.2 ± 0.96^{bc}	2.46
5 min	125.8 ± 19.76	24.4 ± 6.48^{c}	19.4
10 min	62.6 ± 9.52	17.2 ± 3.76^{ab}	27.48
15 min	49.8 ± 4.64	28.6 ± 3.28^{ab}	57.43
30 min	32.4 ± 3.92	21.4 ± 3.92^{a}	66.05
1 h	16.4 ± 6.08	12.6 ± 3.92^{ab}	76.83

Fig. 5. (A) Membrane permeabilization of *Saccharomyces cerevisiae* cells treated with *Ap*Def$_1$. Images of the *S. cerevisiae* cells treated with *Ap*Def$_1$ for 5 min, 10 min and 1 h and incubated with the fluorescent dye Sytox green to determine plasma membrane permeabilization by optical microscopy. The green fluorescence indicates the presence of permeabilized cells. All images had the same exposure time. Bar: 20 μm. Images shown are representative of one independent experiment out of three (n = 3). (B) Percentage of permeabilized *S. cerevisiae* cells was determined by the total cell number in random fields of the DIC and fluorescence views of the samples obtained from the membrane permeabilization assay, being the cell number in the DIC of each sample considered as 100%. Table data are representative of one independent experiment out of three (n = 3). Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.01$.



B

samples	cell number in DIC	fluorescent cell number	percentage of permeabilized cells
control (cells + medium)	11.2 ± 1.8	1.6 ± 0.88[a]	14.28
cells + 100 mM ascorbic acid	17 ± 2.8	0.2 ± 0.32[a]	1.17
cells + *Ap*Def$_1$	21.6 ± 2.72	20.6 ± 3.12[b]	95.37
cells + *Ap*Def$_1$ + 100 mM ascorbic acid	17.6 ± 3.92	2.8 ± 2.16[a]	15.9

Fig. 6. (A) Membrane permeabilization of *Saccharomyces cerevisiae* cells treated with *Ap*Def$_1$ in the presence of ascorbic acid. Images of the *S. cerevisiae* cells treated with *Ap*Def$_1$ for 1 h in the presence of ascorbic acid and incubated with the fluorescent dye Sytox green to determine plasma membrane permeabilization by optical microscopy. The green fluorescence indicates the presence of permeabilized cells. All images had the same exposure time. Bar: 20 μm. Images shown are representative of one independent experiment out of two (n = 2). (B) Percentage of permeabilized *S. cerevisiae* cells determined by the total cell number in random fields of the DIC and fluorescence views of the samples obtained from the membrane permeabilization assay, being the cell number in the DIC of each sample considered as 100%. Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.01$.

molecules such as cholesterol, phosphatidylserine and phosphatidic acid and even glucosylceramide (GlcCer) (the known binding target of this plant defensin in the *C. albicans* membrane) [58]. This result suggests that the membrane permeabilization may be an indirect event of *Rs*-AFP2-fungus interaction. Taking together these literature data and our results on the preventive effect of ascorbic acid towards the *Ap*Def$_1$ induced *S. cerevisiae* cell death and considering the classic known toxic effects of ROS to cell components [62], we questioned whether permeabilization could be indirectly caused by *Ap*Def$_1$. Firstly, we analyzed membrane permeabilization by Sytox green staining. This dye fluoresces after interacting with nucleic acids and penetrates only in cells with compromised membranes. Our results indicated a gradual increase in the number of fluorescent cells as the incubation period with *Ap*Def$_1$ progresses, showing 19.4% of permeabilized cells within the first 5 min of interaction and reaching 76.8%, after 1 h (Fig. 5A and B). This assay revealed that the *Ap*Def$_1$-*S. cerevisiae* interaction caused *S. cerevisiae* plasma membrane permeabilization. These results are in accordance with the reports of Thevissen et al. [30], which showed that plant defensins are able to permeabilize the membranes of yeast and filamentous fungi [24]. Mello et al. [59] have also shown that the *Pv*D1 is able to permeabilize the membrane of the filamentous fungi *Fusarium oxysporum, Fusarium solani*, *Fusarium laterithium* and the yeasts *Candida parapsilosis*, *Pichia membranifaciens*, *Candida tropicalis*, *Candida albicans*, *Kluyveromyces marxiannus* and *S. cerevisiae*.

We have further repeated the membrane permeabilization assay in the presence of ascorbic acid. Surprisingly, cells were not permeabilized (Fig. 6A and B). Therefore, our result strongly suggests that the membrane permeabilization is an indirect event of the *Ap*Def$_1$-*S. cerevisiase* interaction and possibly caused by ROS accumulation.

It is known that almost 90% of the produced ROS come from mitochondria [65], and in our ROS detection assay *S. cerevisiae* cells were only treated with *Ap*Def$_1$ and incubated with DAB. Accordingly, we can assert that H_2O_2, whose concentration was strong enough to allow DAB polymerization, was endogenously produced. We can also deduce the presence of active peroxidases due to the requirement of their activity for DAB polymerization [22]. Therefore, the ROS scavenger systems of *S. cerevisiae*, at least those based on enzymatic reactions, are functioning. Enzymatic mechanisms remove ROS produced during the normal growth conditions and maintain a reducing environment. The ROS scavenging system in *S. cerevisiae* is based on enzymatic reactions

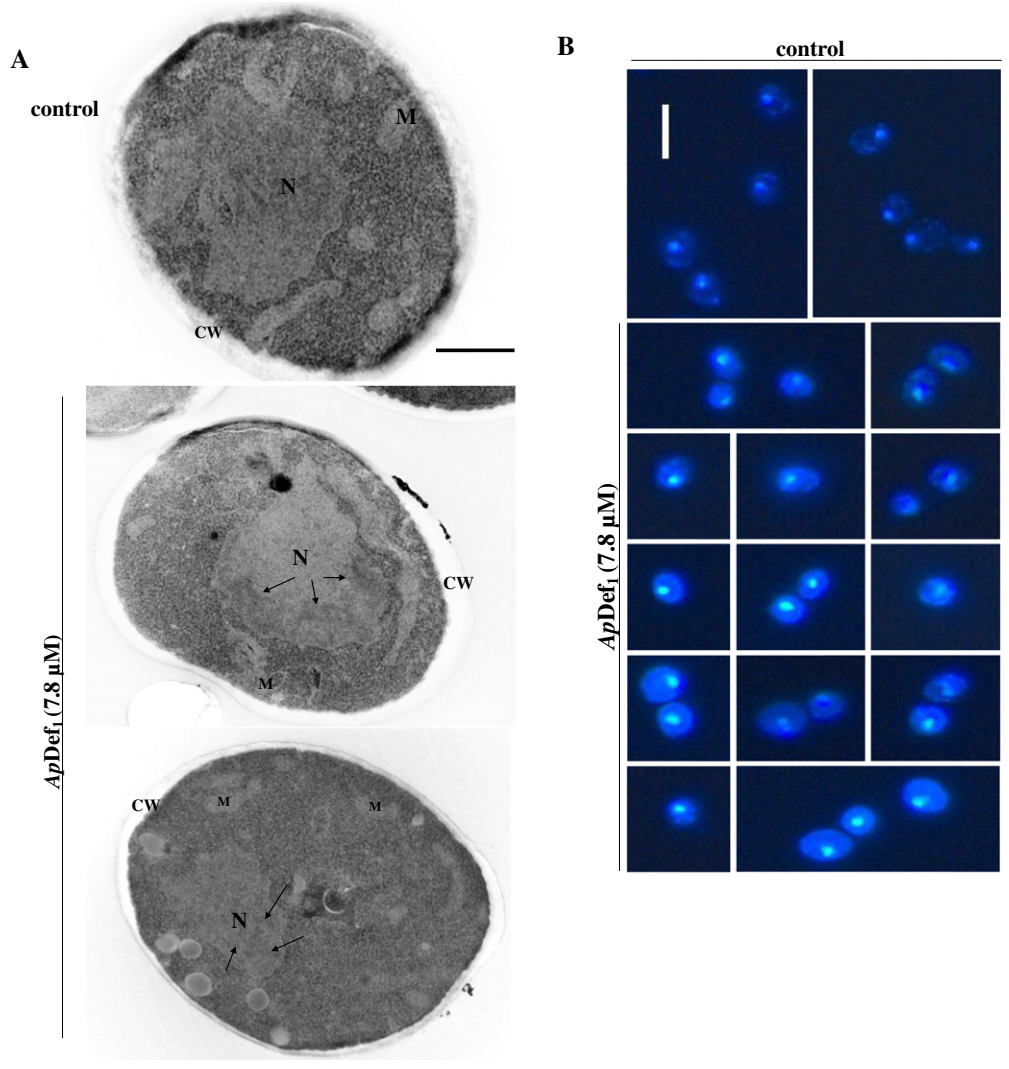
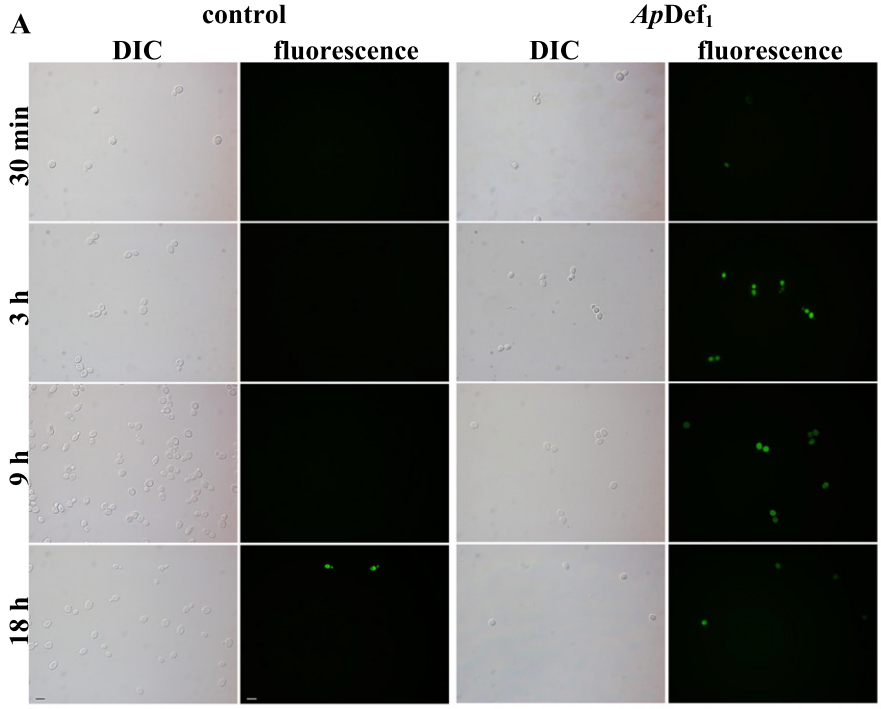


Fig. 7. (A) Ultrastructural alterations in *Saccharomyces cerevisiae* cells induced by *Ap*Def$_1$ at 18 h of incubation. Electron microscopy images of control cells and cells treated with *Ap*Def$_1$. In the interaction of *Ap*Def$_1$, nuclei present areas with dark staining what indicates chromatin condensation (arrows). The mitochondria are swollen and pale stained, with barely seen cristae within them. Control = without *Ap*Def$_1$. M = mitochondria, N = nuclei, CW = cell wall. Images shown are representative of one independent experiment out of three (n = 3). (B) Chromatin condensation. DAPI staining of control cells and cells treated during 18 h with 7.8 μM of *Ap*Def$_1$. Bar: 10 μm. Images shown are representative of one independent experiment out of three (n = 3).

and antioxidant substances which act together. Among these enzymes are superoxide dismutases, catalases, GPXs and TRX peroxidases/peroxiredoxins, glutaredoxins and peroxidases [61,66]. During the respiration, electrons are transported by electron transport chain and eventually some of them may escape from this transport system and react inappropriately with oxygen, what renders the formation of $O_2^{\bullet-}$. From these $O_2^{\bullet-}$, an *anti*-oxidant cascade generates H_2O_2 and $OH^{\bullet}$[61]. Therefore, ROS production can be linked to a mitochondria dysfunction [67]. Our TEM results demonstrated structural damage in the mitochondria of *Ap*Def$_1$ treated *S. cerevisiae* cells. The mitochondria were swollen and cristae were barely seen within them (Fig. 7A). This result indicates that the mitochondria could be a probable ROS source and target of *Ap*Def$_1$.

ROS or H_2O_2 can act as primary triggers of apoptosis in yeasts [68–71] and some studies have linked the accumulation of ROS to the induction of apoptosis. Madeo et al. [72] proved that the induction of



B

time	control		*Ap*Def$_1$ (7.8 μM)		percentage of labelling cells	
	cell number in DIC	fluorescent cell number	cell number in DIC	fluorescent cell number	control	test
30 min	29 ± 6.8	0.2 ± 0.32[a]	26 ± 5.5	16.25 ± 2.25[b]	0.69	62.5
3 h	84.75 ± 7.12	0.5 ± 0.64[a]	54 ± 6.4	50.4 ± 7.92[b]	0.59	93.33
9 h	353 ± 40.72	5.2 ± 1.76[a]	13.5 ± 0.5	12.5 ± 1.5[a]	0.14	92.59
18 h	1646 ± 138	73.33 ± 7.55[a]	22 ± 4	21.66 ± 4.22[b]	4.45	98.45

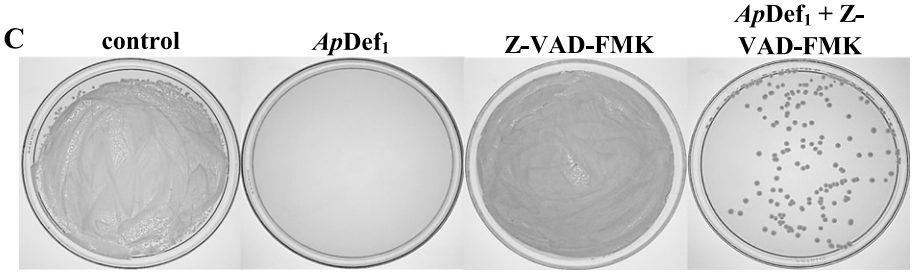


Fig. 8. (A) Involvement of caspase activity in *Saccharomyces cerevisiae* cells viability loss induced by *Ap*Def$_1$. Images of control cells and cells treated with 7.8 μM of *Ap*Def$_1$ incubated with FITC-VAD-FMK and analyzed for active caspases by fluorescence microscopy. The green fluorescence indicates positive staining for caspases activity. Images represent one independent experiment out of three (n = 3). Bar: 10 μm. (B) Percentage of caspase activity in *S. cerevisiae* cells determined by the total cell number in random fields of the DIC and fluorescence views, being the cell number in the DIC of each sample considered as 100%. Different letters denote significant differences and same letter denotes no difference, by one-way ANOVA and Tukey's pairwise comparison, $P < 0.01$. (C) Images of the Petri dish showing *S. cerevisiae* cells treated with 7.8 μM *Ap*Def$_1$ in the presence of 50 μM of the caspase inhibitor Z-VAD-FMK. As controls, cells without treatment (control), cells treated with *Ap*Def$_1$ (*Ap*Def$_1$) and cells only treated with inhibitor (Z-VAD-FMK) were used. Images represent one independent experiment out of two (n = 2).

apoptotic death by various stimuli generates ROS accumulation in yeast cells and showed them to be necessary and sufficient to induce an apoptotic phenotype in yeast.

Furthermore, in higher organisms, chromosomal DNA condensation and fragmentation are often an integral part of apoptosis [73]. In this study, besides the demonstration of ROS accumulation, *Ap*Def$_1$ induces other marker of apoptotic cells. An intense concentrated nuclear fluorescence was detected after treatment with *Ap*Def$_1$, what indicates chromatin condensation as confirmed by TEM (Fig. 7A) and also by DAPI staining method (Fig. 7B). DAPI is a cell permeable DNA binding substance which specifically interacts with the double-strand DNA minor groove and becomes fluorescent. DNA or nuclear fragmentation and chromatin condensation are considered two of the most representative phenomena in late-stage apoptotic cells [25].

Apoptosis is a type of programmed cell death, which is regulated by a complex network of proteins and metabolic pathways. The central core of this process is regulated by a family of proteins named caspases [74]. To get more insight about apoptotic features of cell viability loss induced by *Ap*Def$_1$, we assessed the involvement of caspases in this process, by using the FITC-VAD-FMK marker. FITC-VAD-FMK enters into the cell where it acts as a pseudo-substrate which irreversibly inhibits caspases through binding to the cysteine residue at their active site and becomes fluorescent. We showed that the exposure of *S. cerevisiae* cells to *Ap*Def$_1$ resulted in activation of caspases (Fig. 8A and B). Furthermore, several recent reports have suggested that AMPs, such as plant defensins *Rs*AFP2 [75] and *Hs*AFP1 [60], human lactoferrin [76], melittin (from *Apis mellifera* venon) [77] and papiliocin (from *Papilio xuthus* larvae) [78], exert their antimicrobial activity by promoting apoptosis in *C. albicans*. Additionally, we have analyzed whether apoptosis induction had a role in the *S. cerevisiae* cell viability loss or not. Our results indicate that the caspase inhibitor Z-VAD-FMK (a cell-permeant pan caspase inhibitor which irreversibly binds to the catalytic site of caspase proteases and can inhibit induction of apoptosis) alleviated cell viability loss, corroborating with the evidence of caspase activation and their requirement to the *Ap*Def$_1$ induced *S. cerevisiae* cell death (Fig. 8C). This shows that active caspases or caspase-like proteases play an important role for the antifungal mode of action of *Ap*Def$_1$. The collective data presented in this study indicate that *Ap*Def$_1$ caused apoptosis in *S. cerevisiae* cells displaying several key markers of yeast apoptosis, including an increase of ROS generation, chromatin condensation and the presence of active caspases.

4. Conclusion

In this work, we shed some light on the mechanism of action of the *Ap*Def$_1$ induced *S. cerevisiae* cell death and propose a model which would resume our data and ideas (Fig. 9). We suggest that the *Ap*Def$_1$-*S. cerevisiae* interaction occurs from outside to inside cell, initially by the interaction of *Ap*Def$_1$ with some *S. cerevisiae* cell wall component (Fig. 9). This initial cell wall interaction site has been shown to be necessary to the activity of AMPs [79–81]. From this initial cell wall binding site of AMPs (which is still unknown in the case of *Ap*Def$_1$), they must reach the plasma membrane where their initial toxic effects were described to take place. Peschel [38] argues that the transmembrane potential may participate in AMPs migration from the cell wall to the plasma membrane, concentrating them in the plasma membrane. This hypothesis is corroborated with the decreased membrane potential which protects some cells from the toxic effect of AMPs [21,51]. This effect may also govern *Ap*Def$_1$ migration since CCCP and low temperature block the *S. cerevisiae* cell death. Once in the membrane, positively charged AMPs interact with the external surface of the negatively charged phospholipids or other molecules. Some targets for plant defensins have been identified, in the fungal plasma membrane, such as membrane sphingolipids [82]. *Dm*-AMP1 (plant defensin from *Dhalia merckii*) [83], *Ah*-AMP1 (plant defensin from *Aesculus hippocastanum*) and *Ct*-AMP1 (plant defensin from *Clitoria ternatea*) [82,84,85] were

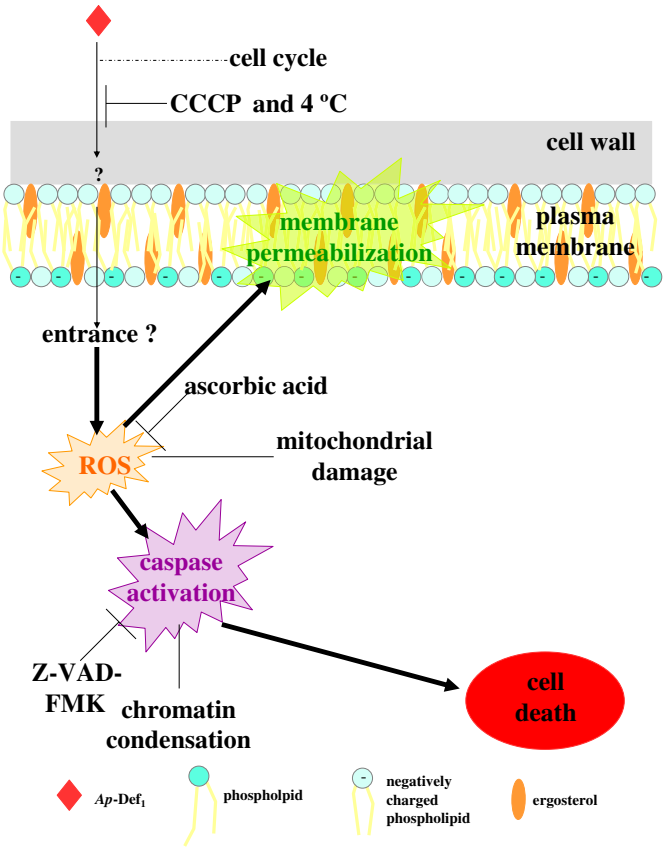


Fig. 9. Suggested model of *Ap*Def$_1$-*Saccharomyces cerevisiae* interaction and mechanism of action based on results presented in this work. (- ———, dependence), (├——, blockade), (————, associated with), (→, induction).

seen to interact with mannosyldiinositolphosphorylceramide (M(IP)2C) in *S. cerevisiae* plasma membrane. Additionally, *Rs*-AFP2 interacts with GlcCer in *C. albicans* membranes [86]. Therefore, we speculate that the fungicidal activity of *Ap*Def$_1$ may depend on a secondary interaction target in the yeast cell membrane, which is still unknown in the case of *Ap*Def$_1$ (Fig. 9). To some AMPs, cytoplasmic targets were demonstrated [48,87,88], implying that these AMPs enter into cells. Antimicrobial activity of histatin-5 in *C. albicans* cells was associated with the AMP cell entrance through the polyamine transporters, Dur3p and Dur31p [89]. A transregulator of polyamine uptake was also associated with the antifungal activity of plant defensin *Na*D1 [8]. There are evidences that the plasma-membrane potential might govern the rate of uptake of the polyamines transporters and that CCCP strongly depresses the uptake of polyamine compounds [90–92]. These results corroborate with the protective effect of CCCP on the *Ap*Def$_1$-*S. cerevisiae* interaction and indicate that a transporter may be involved in *Ap*Def$_1$ entrance (Fig. 3A). Whether *Ap*Def$_1$ enters into the *S. cerevisiae* cell remains to be determined.

In conclusion, our results indicate that *Ap*Def$_1$-*S. cerevisiae* interaction causes cell death through a cell cycle dependent-process which requires preserved membrane potential. After interaction, the yeast suffers an uncontrolled ROS production and accumulation which leads to plasma membrane permeabilization and chromatin condensation and, ultimately, cell death by the activation of a caspase-dependent apoptosis pathway. The general significance of our work is that we show new requirements for the interaction between plant defensin and fungi cells *i.e.* cell cycle stage and membrane potential, and we indicate that the membrane permeabilization is probably caused by the ROS and, therefore, it would be an indirect event of the *Ap*Def$_1$-*S. cerevisiae* interaction and not a direct event, as previously thought [24,52,53]. The

understanding of the dependence on the target cell cycle stage for the antimicrobial effect of *Ap*Def$_1$ may be crucial for the design of new antifungal molecules.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary information

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2016.09.005.

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