Biochemical and Biophysical Research Communications 417 (2012) 56–61

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

# Sensitivity of *Saccharomyces cerevisiae* to the cell-penetrating antifungal peptide PAF26 correlates with endogenous nitric oxide (NO) production

Lourdes Carmona, Mónica Gandía, Belén López-García [1], Jose F. Marcos *

*Departamento de Ciencia de los Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA)-CSIC, Avda. Agustín Escardino – 7, 46980 Paterna, Valencia, Spain*

ARTICLE INFO

*Article history:*
Received 9 November 2011
Available online 19 November 2011

*Keywords:*
Antimicrobial peptide
Reactive oxygen species
Nitric oxide
*Saccharomyces cerevisiae*
Antifungal mechanism

ABSTRACT

PAF26 is a synthetic fungicidal hexapeptide with cell-penetration properties and non-lytic mode of action. We demonstrate herein the endogenous accumulation of reactive oxygen species (ROS) and nitric oxide (NO) in the model fungus *Saccharomyces cerevisiae* treated with PAF26. However, the *S. cerevisiae* deletion mutant of *YAP1* – the major inductor of defense to oxidative stress – did not show high sensitivity to PAF26 but rather increased resistance, and its ROS accumulation did not differ from that of the parental strain. Cross-protection experiments suggest that the oxidant $H_2O_2$ and PAF26 kill yeast through different pathways. Overall, the data indicate that ROS are not the primary antifungal mechanism of the peptide. On the contrary, the PAF26-induced intracellular production of NO was blocked in two distinct resistant mutants: the above mentioned Δ*yap1*, which had the induction of NO disrupted, and the previously reported Δ*arg1* from the biosynthetic pathway of arginine, which has reduced basal NO levels. The NO synthase inhibitor L-NAME partially restored yeast growth in the presence of PAF26. These findings correlate antifungal activity of PAF26 with NO production and provide a plausible explanation for the resistance phenotype of Δ*arg1* through its involvement in NO biosynthesis.



## 1. Introduction

Antimicrobial peptides (AMPs) are widespread in nature and are considered promising molecules to combat pathogenic microorganisms of medical, agricultural, and food relevance [1–3]. Most AMP are cationic and amphipathic peptides with the capability to permeate biological membranes and lyse living cells. Over the last decade, however, it has been shown that some AMP have non-lytic effects on their target microorganism [4–6]. Significant examples include binding to membrane components, inhibition of the biosynthesis of macromolecules such as DNA, RNA and proteins, interaction with chaperone like proteins, or the induction of DNA damage and apoptosis. Also, some AMP can enter microbial cells in a non-disruptive manner and are therefore considered cell-penetrating peptides [6–8].

Several studies show the induction of apoptosis markers in fungal cells treated with AMP of very diverse origins [9–14]. In some of these reports, mutations of pro-apoptotic genes enhance resistance to AMP, indicating that induced microbial death is part of the peptide killing mechanism [9–11]. Intracellular rise of reactive oxygen species (ROS) is a marker of cell death and apoptosis in fungal cells. Further studies have increased the number of AMP for which antifungal action is associated with intracellular ROS production [15–18]. In the case of the synthetic VS2/VS3 peptides ROS accumulation was coincident with internalization of into *Candida albicans* [18]. However, the precise role of ROS in the antifungal mechanism of AMP remains controversial as exemplified in the case of histatin 5 [19,20]. Studies have not clearly determined whether intracellular ROS production is either the (primary) cause or just a marker of fungal death initiated by other pathways.

Nitric oxide (NO) has diverse roles in biological systems, including vasodilation, signaling, defense and microbe killing. NO release is part of the defense of higher organisms against invading microbial pathogens [21]. Interaction of NO with ROS generates reactive nitrogen species (RNS) that result in nitrosative stress and cytotoxicity. Therefore, RNS are often associated with ROS and contribute to harmful effects. The involvement of NO as part of AMP action is underexplored. Treatment of *C. albicans* with the pea defensin PvD1 resulted in the intracellular rise of ROS and NO detected by microscopy [15], although the relevance to antimicrobial activity was not further analyzed.

PAF26 is a synthetic *de novo*-designed hexapeptide that was identified in a combinatorial screen and shares sequence similarity with other AMP from natural or synthetic origin [22]. It has activity against plant pathogenic fungi and several microorganisms of clinical relevance [23]. PAF26 is a cell penetrating peptide that enters hyphal cells and conidia of *Penicillium digitatum* or *Saccharomyces cerevisiae* yeast cells in a non-disruptive way [24,25]. The precise

* Corresponding author. Fax: +34 963 636 301.
*E-mail address:* jmarcos@iata.csic.es (J.F. Marcos).
[1] Present address: Centre de Recerça en Agrigenòmica (CRAG) CSIC-IRTA-UAB-UB, Campus UAB, 08193 Bellaterra (Cerdanyola del Vallès) Barcelona, Spain.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.11.050

mechanism of action of PAF26 is being investigated. A transcriptome study on *S. cerevisiae* has identified cell components and intracellular pathways that modulate sensitivity [25]. Among them, several *ARG* genes of the arginine metabolism modulate sensitivity and are specifically induced by PAF26 but not by the cytolytic peptide melittin. Deletion mutants of *ARG* genes showed increased resistance to PAF26 including the mutant Δ*arg1*, which codes for the cytosolic argininosuccinate synthetase that catalyzes the formation of argininosuccinate from citrulline and aspartate in the arginine biosynthetic pathway.

The objective of this study was to investigate the role of ROS and NO in the PAF26 mechanism of antifungal action. We demonstrated production of these reactive agents upon *S. cerevisiae* exposure to the peptide. In order to establish the involvement of these molecules in fungal death we characterized the sensitivity and phenotype of deletion mutants in the master regulator of response to oxidative stress *YAP1*, and in the previously identified *ARG1*.

## 2. Materials and methods

### 2.1. Peptide and chemicals

PAF26 (amino acid sequence RKKWFW) was purchased at >90% purity from Genscript Corporation (USA) and used as described previously [25]. Dihydrorhodamine 123 (DHR123) and 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) were from Invitrogen (USA), and nitro-L-arginine methyl ester (L-NAME) from Sigma–Aldrich (USA).

### 2.2. Saccharomyces cerevisiae strains

The *S. cerevisiae* strains used were BY4741 (MATa; *his*3Δ1; *leu*2Δ0; *met*15 Δ0; *ura*3Δ0) and the corresponding isogenic deletion strains Y01750 (Δ*arg1*) and Y00569 (Δ*yap1*) from the public EUROSCARF collection (http://web.unifrankfurt.de/fb15/mikro/euroscarf).

### 2.3. Fluorescence microscopy

In all the experiments of this study *S. cerevisiae* cells were grown in YPD medium to exponential phase ($OD_{600}$ 0.4–0.5) at 30 °C with shaking [25]. For microscopy, 1 mL of $2.5 \times 10^7$ cells/mL were sedimented and treated in 100 μL of sterile water with 0.5 mM $H_2O_2$, 30 μM PAF26 or $H_2O$ (control) for 30 min, washed twice, resuspended in 100 μL of sterile water containing 40 μM DHR123 and incubated for 30 min. After treatments, cells were washed and fluorescence was examined and photographed with an epifluorescence microscope E90i (Nikon, Japan).

### 2.4. Flow cytometry

$2.5 \times 10^7$ cells were treated in 100 μL of sterile water containing either 30 μM PAF26, 2 mM $H_2O_2$ or 0.1 mM Menadione (MD) for 1 h at 30 °C. After treatment, cells were washed twice and incubated in 100 μL water with either 40 μM DHR123 to assess the intracellular level of ROS or 30 μM DAF-FM for NO, for 30 min at 30 °C and subsequently diluted to 1 mL in sterile water [26]. Flow cytometry analysis (20,000 cells/sample) was performed using an EPICS XL-MCL cytometer (Beckman-Coulter, USA).

### 2.5. Antifungal assays

For cell viability assays, cultures were adjusted to $10^7$ cells/ml in 20% YPD containing 40 μg/ml chloramphenicol, and serial 5-fold dilutions of cells were treated with peptide as described [25]. Aliquots (5 μl) of each sample were dotted onto peptide-free YPD agar plates and incubated at 30 °C for 2 days to determine viability. In some experiments, $10^7$ cells/ml in 20% YPD were pre-treated with 2 mM $H_2O_2$ for 1 h, washed, resuspended in 20% YPD and treated with peptide. Additional experiments were carried out in which cell dilutions were dotted onto YPD plates amended with $H_2O_2$. For growth inhibition assays, $5 \times 10^4$ BY4741 cells/mL were grown at 30 °C using a POLARstar Omega (BMG Labtech, Germany) microtiter plate reader and incubator.

## 3. Results and discussion

### 3.1. PAF26 induces an increase in the accumulation of intracellular ROS and NO in S. cerevisiae

PAF26 is a cell-penetrating peptide that enters *S. cerevisiae* cells at subinhibitory concentrations (around 5 μM) and inhibits growth with $IC_{50}$ around 30 μM [25]. We explored ROS production in yeast treated with PAF26 in order to elucidate whether oxidative stress is associated to peptide antimicrobial activity. Intracellular ROS in *S. cerevisiae* BY4741 treated with PAF26 were determined by using the ROS sensitive fluorescent dye DHR-123, as a general indicator of cellular ROS levels [12,26]. Microscopy observations of cells treated with 30 μM PAF26 showed a fluorescence signal higher than control cells, and even higher than cells treated with sublethal concentrations of the ROS generator oxygen peroxide (0.5 mM $H_2O_2$) (Fig. 1A). In order to confirm and also quantify ROS production, we carried out flow cytometry experiments. Our results showed that the subpopulation of positive cells with signal intensity over a threshold value increased from 49.2% in the control to 82.1% in the presence of PAF26, while the mean signal intensity increased from 3.2 to 6.5 (Fig. 1B). Additional controls of cells treated with the superoxide-generating agent menadione (MD) or $H_2O_2$ also displayed similar increases of fluorescence (Fig. 1B). These experiments demonstrate that the activity of PAF26 was associated with intracellular accumulation of ROS in *S. cerevisiae*, as shown with other AMP [12–14,18].

Studies suggest that intracellular ROS production and oxidative stress in yeast might be linked to production of RNS from NO [26,27]. The production of ROS and NO in *C. albicans* treated with the plant defensin PvD1 has been recently reported [15]. In this latter study, however, the microscopy observations that support NO production showed very faint signals. In our experiments, microscopy records of NO production were not conclusive due to very low signal intensity (data not show). We used the NO indicator DAF-FM [26] coupled to flow cytometry to quantify NO production. This methodology allowed accurate detection of NO and confirmed increased NO production in cells treated with MD or $H_2O_2$, similarly to previous reports [26,27], and most importantly demonstrated NO generation in PAF26 treated cells (Fig. 1C). The overall increase of NO production in any of the three treatments (and measured as increase of signal intensity or number of positive cells), did not seem to be as important as ROS production. Collectively, these findings demonstrate that endogenous ROS and NO production are induced by PAF26 in *S. cerevisiae*. Further experiments were conducted to clarify the role of ROS and NO in the mode of action of PAF26.

### 3.2. The S. cerevisiae deletion mutant in the oxidative stress regulator YAP1 shows increased survival after PAF26 treatment

We screened a total of 11 gene deletion mutants from the EUROSCARF collection to further study the role of oxidative stress in PAF26 antifungal mechanism, including mutants of *SOD1*, *SOD2*, *TRX1*, *TRX2*, *MSN2* or *SKN7*. For most of them no significant

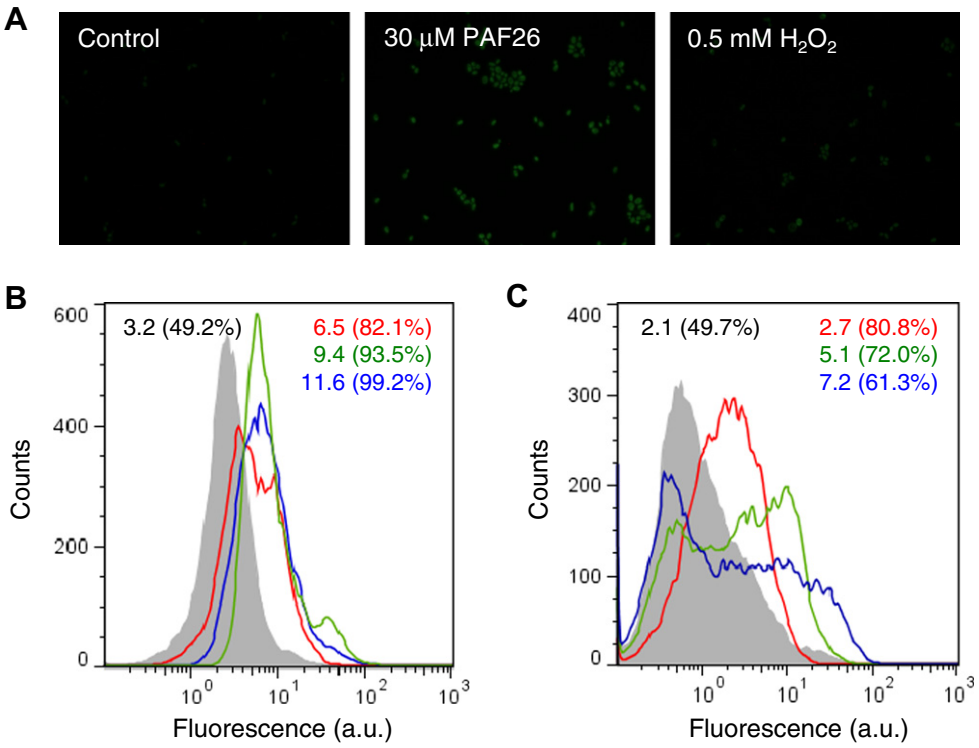


**Fig. 1.** Endogenous ROS and NO accumulation in *S. cerevisiae* after PAF26 treatment. (A) Fluorescence microscopy of *S. cerevisiae* BY4741 stained with DHR-123 after treatment with either water (control), 30 μM PAF26 or 0.5 mM $H_2O_2$. (B) and (C) Histograms of flow cytometry analyses of production of ROS determined by DHR-123 labeling (B), or of NO by DAF-FM labeling (C), in *S. cerevisiae* BY4741 treated with 30 μM PAF26 (red line), 2 mM $H_2O_2$ (green line) or 0.1 mM MD (blue line). Histograms of control untreated cells are shown in gray. The numbers indicate for each record the mean fluorescence signal and in parentheses the percentage of positive cells above the fluorescence threshold value of the mean fluorescence of the corresponding untreated control.

differences of sensitivity were found with the parental strain (data not show). The most significant phenotype was found for Δ*yap1* (EUROSCARF strain Y00569) (Fig. 2). YAP1p is a bZIP transcription factor and is considered a major regulator of the defense response to oxidative stress [28]. It is known that *YAP1* deletion strains are hypersensitive to $H_2O_2$ [29]. Accordingly, serial dilutions of CFU from Δ*yap1* did not grow on YPD plates containing 4 mM $H_2O_2$ (Fig. 2A). Unexpectedly, a 24 h treatment with PAF26 followed by plating onto YPD plates showed a resistance phenotype of Δ*yap1* as compared to the parental BY4741. As shown in Fig. 2B, BY4741 had a marked reduced viability after PAF26 treatment (see Fig. 2B mid panel) as reported previously [25], while the mutant only had a minor reduction of viability (Fig. 2B, compare with the control left panel). Treatment with PAF26 followed by plating onto $H_2O_2$ plates had an additive effect and strongly reduced the viability of both the parental and the mutant strain (Fig. 2B, right panel).

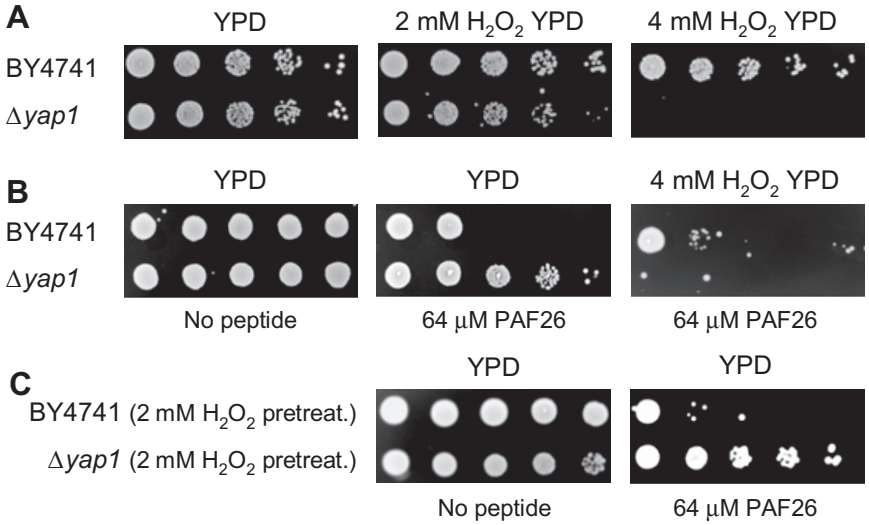


**Fig. 2.** Sensitivity of *S. cerevisiae* BY4741 and Δ*yap1* to PAF26 and $H_2O_2$. (A) Increased sensitivity of Δ*yap1* to $H_2O_2$. Serial dilutions of cells were applied onto YPD plates amended or not with $H_2O_2$ as indicated. (B) Increased resistance of Δ*yap1* to PAF26. Serial dilutions of cells were either control-treated or treated with 64 μM PAF26 for 24 h, and applied onto YPD plates amended or not with $H_2O_2$ as indicated. (C) Pre-treatment with $H_2O_2$ does not cross-protect against PAF26. Serial dilutions of cells were pre-treated with 2 mM $H_2O_2$ for 1 h, and subsequently either control-treated or treated with 64 μM PAF26 for 24 h, and applied onto YPD plates.

YAP1p activation results in up-regulation of genes involved in oxidative stress response such as superoxide dismutase, catalases or thioredoxins [28,29]. Gene deletion of some of these oxidative stress related genes increase susceptibility to oxidizing compounds. Nevertheless, no induction of any of these genes upon PAF26 treatment was found previously [25], and deletion of a selection of them caused no altered sensitivity to PAF26 (this study, data not shown). The transcriptomic data initially indicated induction of *YAP1* after PAF26 treatment [25], although this induction could not be confirmed by qRT-PCR during this study (data not shown). Activation of YAP1p is mostly accomplished by oxidation of cysteine residues and relocation to the cell nucleus, and not by induction of gene expression [30,31].

The deletion of *YAP1* causes hypersensitivity to oxidizing agents and antifungal compounds that act by causing oxidative stress [29], in marked contrast to the increased resistance to PAF26. It is remarkable that Δ*yap1* is highly sensitive to more than 20 antimicrobial compounds (SGD, www.yeastgenome.org/), while no antimicrobial agent has been reported to which it shows increased resistance as does to PAF26.

Cross-protection experiments indicated distinct cytotoxicity pathways for ROS and PAF26. Pre-treatment with a sublethal dose of 2 mM $H_2O_2$ (Fig. 2A) that results in increased ROS (Fig. 1B) did not protect BY4741 cells against PAF26 antifungal effect, and neither changed the resistance phenotype of Δ*yap1* (Fig. 2C). Altogether, these results suggest that the effect of PAF26 towards *S. cerevisiae* is independent from that of $H_2O_2$, and presumably other ROS. Similarly, *C. albicans* treated with the AMP histatin 5 produced ROS, while sub-lethal pre-treatment to oxidative stress did not change susceptibility to the peptide [32].

### 3.3. NO production after PAF26 treatment was blocked in S. cerevisiae deletion mutants of genes YAP1 and ARG1

We next quantified ROS after peptide treatment in Δ*yap1* and observed a high ROS level (7.7 mean intensity and 80.1% of positive cells), similarly to that found in the parental strain (Fig. 3A). Fluorescence microscopy showed no obvious DHR-123 signal difference between Δ*yap1* and the parental strain (data not shown). The ROS response to PAF26 observed in Δ*yap1* was comparable to the response to either $H_2O_2$ or MD treatments (data not shown). In contrast, no ROS generation was induced in a *C. albicans* Δ*gcs* mutant resistant to the plant defensin RsAFP2, thus supporting the involvement of ROS in antimicrobial action [16].

Recently, we observed that the arginine metabolism *S. cerevisiae* genes *ARG1*, *ARG3*, *ARG5,6*, and *ARG7* were induced by PAF26, and the corresponding gene deletion mutants were highly resistant to peptide killing [25]. We investigated ROS production in the deletion mutant of *ARG1* (strain Y01750). As occurred with BY4741 and Δ*yap1*, ROS production in Δ*arg1* was increased by PAF26 (Fig. 3A), and also by MD and $H_2O_2$ treatments (data not shown). Therefore, the resistance phenotype of the two mutants analyzed did not correlate with significant differences in ROS production upon peptide treatment. Considering the data presented in this study on the (i) absence of susceptibility to PAF26 of the *YAP1* mutant, (ii) additive effects of $H_2O_2$ and PAF26, (iii) no cross-protection between $H_2O_2$ and PAF26, and (iv) no correlation between ROS production and resistant phenotypes, we conclude that the generation ROS is not the primary killing mechanism of PAF26 to *S. cerevisiae*.

We next determined NO production in mutants Δ*yap1* and Δ*arg1*, and found that NO did not increase in any of them in response to either PAF26 (Fig. 3B) or MD/$H_2O_2$ treatments (data not show), unlike the parental strain BY4741. However, the behavior was qualitatively different in each mutant. Very low basal levels of NO were observed in Δ*arg1*, as concluded from the low fluorescence signal in the absence of any treatment (mean value of 0.5 as compared with 3.2 in the parental), and moreover NO did not change with treatments. On the other hand, Δ*yap1* had basal levels of NO similar to BY4741; nevertheless the induction of NO upon treatments was blocked. Our data show a correlation between NO generation after treatments and resistance phenotype in the mutants, suggesting that endogenous NO may be involved in the killing mechanism of PAF26.

### 3.4. Inhibition of NO production partially protects S. cerevisiae from PAF26 killing

Dual roles have been described for NO in yeast cells. When exogenously applied at high concentrations it has a cytotoxic function used in defense mechanisms of higher organisms. The production and toxicity of ROS and NO are related in yeast [26]. NO toxicity is dependent on oxygen and ROS, although YAP1p played only a minor role in the induction of responses to RNS [33,34]. Conversely, $H_2O_2$-induced apoptotic yeasts also synthesize NO (and RNS), which partly accounts for $H_2O_2$-mediated cell death [27]. Endogenous NO is also a signaling molecule and mediates adaptation to stress in yeast [35,36]. Despite its importance, the regulation of endogenous NO production in fungi is unclear. In plants and animals NO synthase (NOS) uses arginine to produce NO and citrulline. No gene has been identified that would code for an orthologous of NOS in *S. cerevisiae*, despite a NOS-like activity that uses arginine and is sensitive to NOS inhibitors has been characterized, and immunoreactive proteins detected [27,35,36]. It has been proposed that MD induces toxicity in *S. cerevisiae* by triggering endogenous NO production from arginine [26]; moreover, MD toxicity was blocked by pre-treatment with the NOS inhibitor L-NAME. Similarly, we carried out growth experiments of BY4741 in the presence of inhibitory concentrations of PAF26 after pre-treatment with L-NAME, and observed that this NOS inhibitor partially protected cells from PAF26 antifungal action (Fig. 4). This different experimental approach further indicates that endogenous NO biosynthesis is involved in the antifungal activity of PAF26.

We previously hypothesized that the induction of arginine metabolism *ARG* genes by PAF26 might be related to accumulation of toxic metabolites in the cell [25]. The results described here confirm that NO basal biosynthesis depends on the *ARG1* gene product (Fig. 3B), likely through arginine metabolism, and that NO production correlates with peptide toxicity, providing an explanation for the high resistance to PAF26 of the *ARG* mutants.

### 3.5. Concluding remarks

We have demonstrated ROS and NO production in *S. cerevisiae* upon treatment with the cell-penetrating antimicrobial hexapeptide PAF26. Our data indicate that although the induction of both reactive species might be linked, it is the NO induction, and not the ROS, the one having a primary role in peptide toxicity.

An open question remains on the involvement of YAP1p in the killing mechanism of PAF26, on one hand, and induced NO production, on the other, although it is very likely that both aspects are somehow related. YAP1p has been proposed to regulate nitrosative stress responses to exogenous NO application [37,38]. The high resistance phenotype of Δ*yap1* is in marked contrast with its increased susceptibility to other antifungals. Diverse modes of *YAP1* activation have been described through ROS or thiol-reactive compounds, whose involvement depends on the oxidant agent and affect different subsets of genes [30,39,40]. The effect of YAP1p on PAF26 toxicity and endogenous NO production might relate to a yet undiscovered biological function of this transcription factor and future research will aim to characterize it.

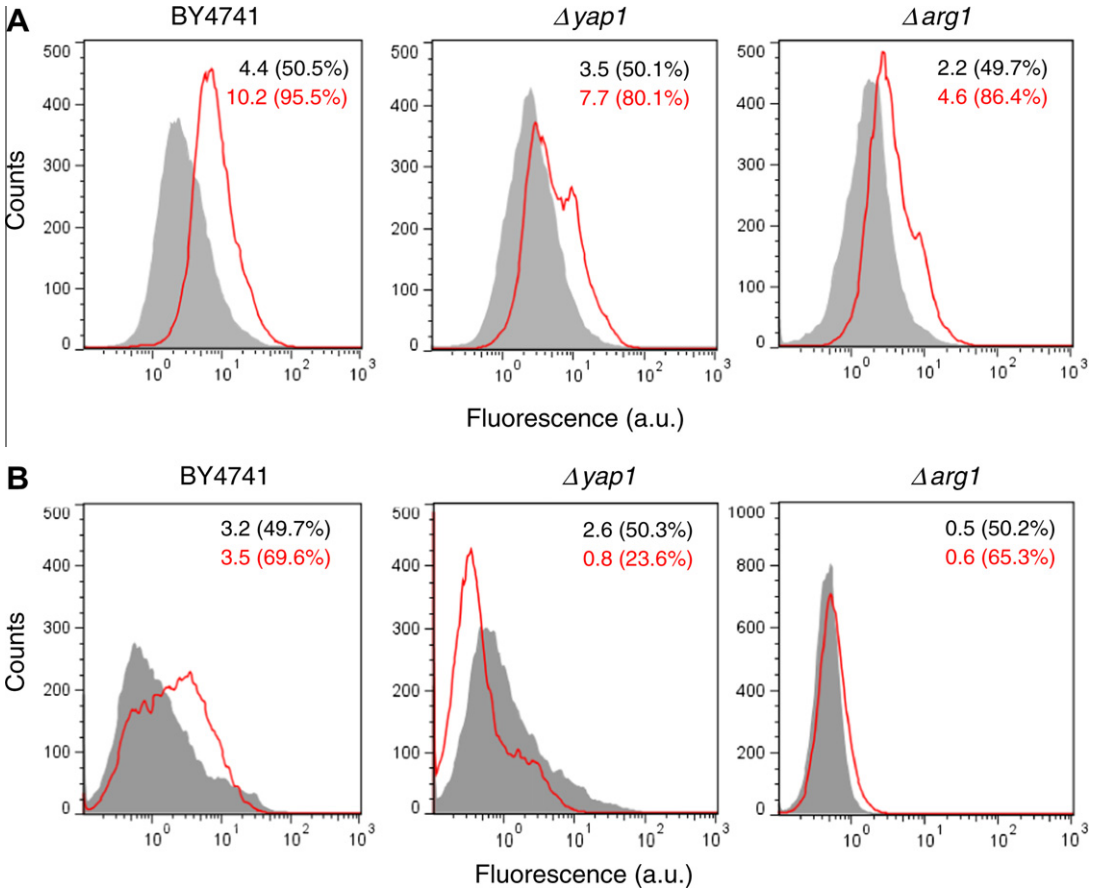


**Fig. 3.** Endogenous ROS and NO accumulation in *S. cerevisiae* BY4741, Δ*yap1* and Δ*arg1* after PAF26 treatment. (A) Flow cytometry analyses of ROS production. (B) Flow cytometry analyses of NO production. Other details as in Fig. 1.

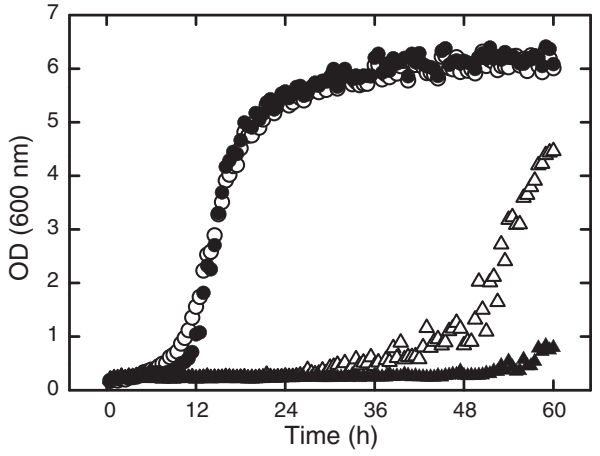


**Fig. 4.** Effect of L-NAME on growth inhibition of *S. cerevisiae* caused by PAF26. Growth curves of *S. cerevisiae* BY4741 in the absence (circles) or presence (triangles) of 32 μM PAF26, after pre-treatment with either 200 mM L-NAME (white symbols) or $H_2O$ (black symbols).

## Acknowledgments

We acknowledge the technical assistance of M. José Pascual. Work was funded by Grants BIO2009-12919 (MICINN, Spain) and ACOMP/2011/258 (Generalitat Valenciana). MG is funded by the JAE program (CSIC).

## References

[1] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[2] R.E.W. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551–1557.
[3] J.F. Marcos, A. Muñoz, E. Pérez-Payá, S. Misra, B. López-García, Identification and rational design of novel antimicrobial peptides for plant protection, Ann. Rev. Phytopathol. 46 (2008) 273–301.
[4] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.
[5] N.Y. Yount, A.S. Bayer, Y.Q. Xiong, M.R. Yeaman, Advances in antimicrobial peptide immunobiology, Biopolymers 84 (2006) 435–458.
[6] J.F. Marcos, M. Gandía, Antimicrobial peptides: to membranes and beyond, Expert Opin. Drug Discov. 4 (2009) 659–671.
[7] P. Nicolas, Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides, FEBS J. 276 (2009) 6483–6496.
[8] S.T. Henriques, M.N. Melo, M.A.R.B. Castanho, Cell-penetrating peptides and antimicrobial peptides: how different are they? Biochem. J. 399 (2006) 1–7.
[9] M.L. Narasimhan, B. Damsz, M.A. Coca, J.I. Ibeas, D.J. Yun, J.M. Pardo, P.M. Hasegawa, R.A. Bressan, A plant defense response effector induces microbial apoptosis, Mol. Cell 8 (2001) 921–930.
[10] E. Leiter, H. Szappanos, C. Oberparleiter, L. Kaiserer, L. Csernoch, T. Pusztahelyi, T. Emri, I. Pócsi, W. Salvenmoser, F. Marx, Antifungal protein PAF severely affects the integrity of the plasma membrane of *Aspergillus nidulans* and induces an apoptosis-like phenotype, Antimicrob. Agents Chemother. 49 (2005) 2445–2453.
[11] C.O. Morton, S.C. dos Santos, P. Coote, An amphibian-derived, cationic, a-helical antimicrobial peptide kills yeast by caspase-independent but AIF-dependent programmed cell death, Mol. Microbiol. 65 (2007) 494–507.
[12] M.T. Andrés, M. Viejo-Díaz, J.F. Fierro, Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of $K^+$-channel-mediated $K^+$ efflux, Antimicrob. Agents Chemother. 52 (2008) 4081–4088.
[13] C. Park, D.G. Lee, Melittin induces apoptotic features in *Candida albicans*, Biochem. Biophys. Res. Commun. 394 (2010) 170–172.
[14] B. Hwang, J.S. Hwang, J. Lee, D.G. Lee, The antimicrobial peptide, psacotheasin induces reactive oxygen species and triggers apoptosis in *Candida albicans*, Biochem. Biophys. Res. Commun. 405 (2011) 267–271.

[15] E.O. Mello, S.F.F. Ribeiro, A.O. Carvalho, I.S. Santos, M. Da Cunha, C. Santa-Catarina, V.M. Gomes, Antifungal activity of PvD1 defensin involves plasma membrane permeabilization, inhibition of medium acidification, and induction of ROS in fungi cells, Curr. Microbiol. 62 (2011) 1209–1217.
[16] A.M. Aerts, I.E.J.A. François, E.M.K. Meert, Q.T. Li, B.P.A. Cammue, K. Thevissen, The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*, J. Mol. Microbiol. Biotechnol. 13 (2007) 243–247.
[17] A. Lupetti, A. Paulusma-Annema, S. Senesi, M. Campa, J.T. van Dissel, P.H. Nibbering, Internal thiols and reactive oxygen species in candidacidal activity exerted by an N-terminal peptide of human lactoferrin, Antimicrob. Agents Chemother. 46 (2002) 1634–1639.
[18] I.K. Maurya, S. Pathak, M. Sharma, H. Sanwal, P. Chaudhary, S. Tupe, M. Deshpande, V.S. Chauhan, R. Prasad, Antifungal activity of novel synthetic peptides by accumulation of reactive oxygen species (ROS) and disruption of cell wall against *Candida albicans*, Peptides 32 (2011) 1732–1740.
[19] E.J. Helmerhorst, R.F. Troxler, F.G. Oppenheim, The human salivary peptide histatin 5 exerts its antifungal activity through the formation of reactive oxygen species, Proc. Natl. Acad. Sci. U S A 98 (2001) 14637–14642.
[20] E.C.I. Veerman, K. Nazmi, W. van Hof, J.G.M. Bolscher, A.L. den Hertog, A.V.N. Amerongen, Reactive oxygen species play no role in the candidacidal activity of the salivary antimicrobial peptide histatin 5, Biochem. J. 381 (2004) 447–452.
[21] F.C. Fang, Antimicrobial reactive oxygen and nitrogen species: concepts and controversies, Nat. Rev. Microbiol. 2 (2004) 820–832.
[22] B. López-García, E. Pérez-Payá, J.F. Marcos, Identification of novel hexapeptides bioactive against phytopathogenic fungi through screening of a synthetic peptide combinatorial library, Appl. Environ. Microbiol. 68 (2002) 2453–2460.
[23] B. López-García, W. Ubhayasekera, R.L. Gallo, J.F. Marcos, Parallel evaluation of antimicrobial peptides derived from the synthetic PAF26 and the human LL37, Biochem. Biophys. Res. Commun. 356 (2007) 107–113.
[24] A. Muñoz, B. López-García, J.F. Marcos, Studies on the mode of action of the antifungal hexapeptide PAF26, Antimicrob. Agents Chemother. 50 (2006) 3847–3855.
[25] B. López-García, M. Gandía, A. Muñoz, L. Carmona, J.F. Marcos, A genomic approach highlights common and diverse effects and determinants of susceptibility on the yeast *Saccharomyces cerevisiae* exposed to distinct antimicrobial peptides, BMC Microbiol. 10 (2010) 289.
[26] N.S. Osório, A. Carvalho, A.J. Almeida, S. Padilla-Lopez, C. Leao, J. Laranjinha, P. Ludovico, D.A. Pearce, F. Rodrigues, Nitric oxide signaling is disrupted in the yeast model for batten disease, Mol. Biol. Cell 18 (2007) 2755–2767.
[27] B. Almeida, S. Buttner, S. Ohlmeier, A. Silva, A. Mesquita, B. Sampaio-Marques, N.S. Osório, A. Kollau, B. Mayer, C. Leao, J. Laranjinha, F. Rodrigues, F. Madeo, P. Ludovico, NO-mediated apoptosis in yeast, J. Cell Sci. 120 (2007) 3279–3288.
[28] C. Rodrigues-Pousada, R.A. Menezes, C. Pimentel, The Yap family and its role in stress response, Yeast 27 (2010) 245–258.
[29] S. Kuge, N. Jones, Yap1 dependent activation of Trx2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides, EMBO J. 13 (1994) 655–664.
[30] A. Delaunay, A.D. Isnard, M.B. Toledano, $H_2O_2$ sensing through oxidation of the Yap1 transcription factor, EMBO J. 19 (2000) 5157–5166.
[31] M.J. Wood, G. Storz, N. Tjandra, Structural basis for redox regulation of Yap1 transcription factor localization, Nature 430 (2004) 917–921.
[32] S. Vylkova, W.S. Jang, W.S. Li, N. Nayyar, M. Edgerton, Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway, Eukaryot. Cell 6 (2007) 1876–1888.
[33] S. Horan, I. Bourges, B. Meunier, Transcriptional response to nitrosative stress in *Saccharomyces cerevisiae*, Yeast 23 (2006) 519–535.
[34] K.T. Chiang, C.H. Switzer, K.O. Akali, J.M. Fukuto, The role of oxygen and reduced oxygen species in nitric oxide-mediated cytotoxicity: studies in the yeast *Saccharomyces cerevisiae* model system, Toxicol. Appl. Pharmacol. 167 (2000) 30–36.
[35] T. Domitrovic, F.L. Palhano, C. Barja-Fidalgo, M. DeFreitas, M.T.D. Orlando, P.M.B. Fernandes, Role of nitric oxide in the response of *Saccharomyces cerevisiae* cells to heat shock and high hydrostatic pressure, FEMS Yeast Res. 3 (2003) 341–346.
[36] C. Kig, G. Temizkan, Nitric oxide as a signaling molecule in the fission yeast *Schizosaccharomyces pombe*, Protoplasma 238 (2009) 59–66.
[37] O.V. Lushchak, Y. Inoue, V.I. Lushchak, Regulatory protein *yap1* is involved in response of yeast *Saccharomyces cerevisiae* to nitrosative stress, Biochemistry (Moscow) 75 (2010) 629–635.
[38] A. Tillmann, N.A. Gow, A.J. Brown, Nitric oxide and nitrosative stress tolerance in yeast, Biochem. Soc. Trans. 39 (2011) 219–223.
[39] D. Azevedo, F. Tacnet, A. Delaunay, C. Rodrigues-Pousada, M.B. Toledano, Two redox centers within Yap1 for $H_2O_2$ and thiol-reactive chemicals signaling, Free Radic. Biol. Med. 35 (2003) 889–900.
[40] S.T. Coleman, E.A. Epping, S.M. Steggerda, W.S. Moye-Rowley, Yap1p activates gene transcription in an oxidant-specific fashion, Mol. Cell. Biol. 19 (1999) 8302–8313.