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## The plant defensin RsAFP2 induces cell wall stress, septin mislocalization and accumulation of ceramides in *Candida albicans*

Karin Thevissen<sup>1,\*</sup>, Patricia de Mello Tavares<sup>2</sup>, Deming Xu<sup>3</sup>, Jill Blankenship<sup>4</sup>, Davy Vandebosch<sup>5</sup>, Jolanta Idkowiak-Baldys<sup>6</sup>, Gilmer Govaert<sup>1</sup>, Anna Bink<sup>1</sup>, Sonia Rozental<sup>7</sup>, Piet W.J. de Groot<sup>8</sup>, Talya R. Davis<sup>9</sup>, Carol A. Kumamoto<sup>9</sup>, Gabriele Vargas<sup>2</sup>, Leonardo Nimrichter<sup>2</sup>, Tom Coenye<sup>5</sup>, Aaron Mitchell<sup>4</sup>, Terry Roemer<sup>3</sup>, Yusuf A. Hannun<sup>6</sup>, and Bruno P.A. Cammue<sup>1</sup>

<sup>1</sup>Centre of Microbial and Plant Genetics (CMPG), Katholieke Universiteit Leuven, 3001 Heverlee, Belgium

<sup>2</sup>Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941590, Brazil

<sup>3</sup>Center of Fungal Genetics, Merck-Frosst Canada Ltd., Montreal, Quebec H9H 3L1, Canada

<sup>4</sup>Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213, USA

<sup>5</sup>Laboratorium voor Farmaceutische Microbiologie, Universiteit Gent, 9000 Gent, Belgium

<sup>6</sup>Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29403, USA

<sup>7</sup>Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941590, Brazil

<sup>8</sup>Biomedical Research, Albacete Science & Technology Park, University of Castilla – La Mancha, 02006 Albacete, Spain

<sup>9</sup>Department of Molecular Biology and Microbiology, Tufts University, Boston, MA 02111, USA

### Summary

The antifungal plant defensin RsAFP2 isolated from radish interacts with fungal glucosylceramides and induces apoptosis in *Candida albicans*. To further unravel the mechanism of RsAFP2 antifungal action and tolerance mechanisms, we screened a library of 2,868 heterozygous *C. albicans* deletion mutants and identified 30 RsAFP2-hypersensitive mutants. The most prominent group of RsAFP2 tolerance genes was involved in cell wall integrity and hyphal growth/septin ring formation. Consistent with these genetic data, we demonstrated that RsAFP2 interacts with the cell wall of *C. albicans*, which also contains glucosylceramides, and activates the cell wall integrity pathway. Moreover, we found that RsAFP2 induces mislocalization of septins and blocks the yeast-to-hypha transition in *C. albicans*. Increased ceramide levels have previously been shown to result in apoptosis and septin mislocalization. Therefore, ceramide levels in *C. albicans* membranes were analyzed following RsAFP2 treatment and, as expected, increased accumulation of phytoC24-ceramides in membranes of RsAFP2-treated *C. albicans* cells was detected. This is the first report on the interaction of a plant defensin with glucosylceramides in

\*To whom correspondence should be addressed: Karin Thevissen, CMPG, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium. Tel. +32 16 32 96 88; Fax. +32 16 32 19 66; Karin.thevissen@biw.kuleuven.be.

the fungal cell wall, causing cell wall stress, and on the effects of a defensin on septin localization and ceramide accumulation.

## Keywords

*Candida albicans*; plant defensin; mode of action; cell wall; septin; ceramide

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## Introduction

Plant defensins are small, basic, cysteine-rich peptides, which possess antifungal and in some cases also antibacterial activities (reviewed by Aerts *et al.*, 2008). They are postulated to be part of the innate immune system of plants, and provide protection against invading fungal and bacterial pathogens. Plant defensins are not only active against phytopathogenic fungi (e.g. *Fusarium culmorum* and *Botrytis cinerea*), but also against baker's yeast and human pathogenic fungi including *Candida albicans*. The latter is an opportunistic pathogen that can cause superficial and invasive infections in immunocompromised patients. Mortality associated with invasive *Candida* infection is high (Mavor *et al.*, 2005). Besides their anti-*Candida* activity, plant defensins are nontoxic to human cells (Thevissen *et al.*, 2007b; Tavares *et al.*, 2008), highlighting their therapeutic potential as novel antimycotics. We recently demonstrated that RsAFP2, a plant defensin isolated from seeds of radish (*Raphanus sativus*) (Terras *et al.*, 1992), was active in a prophylactic murine model of candidiasis, and was at least as effective as the frequently used drug fluconazole (Tavares *et al.*, 2008).

RsAFP2 interacts with the sphingolipid glucosylceramide (GlcCer) present in membranes and cell walls of susceptible fungal species, but not with structurally related GlcCer from humans, explaining their selective antifungal activity (Thevissen *et al.*, 2004). GlcCer is produced by most fungal pathogens (Barreto-Bergter *et al.*, 2004), and was recently described to be required for virulence in *C. albicans* (Noble *et al.*, 2010). The RsAFP2-GlcCer interaction leads to a subsequent permeabilization of the cell, Ca<sup>2+</sup> influx, and growth arrest (Thevissen *et al.*, 1996, Thevissen *et al.*, 1999). This RsAFP2-induced permeabilization is not mediated by pore-formation via direct insertion of RsAFP2 into the fungal membrane (Aerts *et al.*, 2007). But instead, it is the result of the induction of specific signaling pathway(s), involving the induction of reactive oxygen species (ROS) and programmed cell death or apoptosis, with concomitant activation of caspases (Aerts *et al.*, 2009). Strains with membranes lacking GlcCer, either due to a non-functional glucosylceramide synthase (*GCS/HSX11*) gene or due to the absence of *GCS* (as in *Saccharomyces cerevisiae* or *Candida glabrata*) (Leipelt *et al.*, 2001), are resistant to RsAFP2-induced membrane permeabilization and cell death (Thevissen *et al.*, 2004). This points to GlcCer as a prerequisite for RsAFP2 antifungal activity. Moreover, strains with a membrane composed of GlcCer with a structurally altered ceramide moiety are also resistant to RsAFP2-induced cell death (Ferket *et al.*, 2003; Park *et al.*, 2005), emphasizing the importance of the ceramide part of GlcCer in this process.

In the present study, we tested RsAFP2 in a *C. albicans* fitness test (CaFT) to further unravel its mechanism of action (MOA). This CaFT assay relies on chemically-induced haploinsufficiency by treating a collection of *C. albicans* heterozygotes (currently consisting of approx. 5,400 heterozygotes, covering ~90% of the *C. albicans* genome) with sublethal concentrations of an antifungal agent, and subsequent identification of fitness variations of the treated heterozygotes (Xu *et al.*, 2007). The *C. albicans* heterozygotes displaying fitness variations upon treatment with sublethal RsAFP2 concentrations could be grouped in three classes. Two classes represented RsAFP2-hypersensitive heterozygotes involved in cell wall

(glucan synthesis) or bud/septin formation, and one class represented RsAFP2-resistant heterozygotes involved in sphingolipid/ceramide biosynthesis. Consistent with these data, we demonstrated that RsAFP2 interacts primarily with the cell wall of *C. albicans*, and induces cell wall abnormalities and aberrant budding. As cell wall defects affect septin localization in *C. albicans* (Blankenship *et al.*, 2010), we further investigated a putative effect of RsAFP2 on the yeast-to-hypha transition, on septin localization itself, and on ceramide accumulation in *C. albicans*.

## Experimental procedures

### Strains and chemicals

The following yeast strains were used: *C. albicans* strain 78 (Tavares *et al.*, 2008), *C. albicans* CAI4 (Ura-) (Fonzi and Irwin, 1993), the homozygous  $\Delta gcs$  (homozygous deletion of *HSX11/orf19.4592*) *C. albicans* (Ura-) (Leipelt *et al.*, 2001), and a previously described *C. glabrata* isolate (Tavares *et al.*, 2008). Media used were YPD (1% Yeast Extract, Difco; 2% Peptone, Difco; 2% dextrose) or potato dextrose broth/yeast peptone dextrose (PDB/YPD 80/20 with PDB = 2.4% PDB, Sigma, St. Louis, MO, US; supplemented with 50 mM HEPES, pH 7.0). Inhibitor 235236 (Toenjes *et al.*, 2005) was obtained from ChemBridge (San Diego, CA, USA). Caspofungin was obtained from Merck Sharpe & Dohme (Whitehouse Station, NY, USA) as the pharmaceutical formulation Cancidas (containing caspofungin acetate). RsAFP2 was isolated from radish seed as described (Terras *et al.*, 1992).

### Statistical analysis

Statistical analysis was performed using unpaired student *t* test; differences were considered significant if  $p < 0.05$ .

### *C. albicans* fitness test

The *C. albicans* fitness test (CaFT) was performed as described (Xu *et al.*, 2007). *C. albicans* heterozygous mutants were treated with 10  $\mu\text{g/ml}$ , 13  $\mu\text{g/ml}$  or 16  $\mu\text{g/ml}$  RsAFP2 in YPD/PDB. The CaFT results were analyzed by hierarchical clustering with a cut-off value as indicated in the figure legend.

### Transmission electron microscopy (TEM)

Morphological changes caused by RsAFP2 treatment were evaluated by TEM. Strain 78 of *C. albicans* ( $10^5$  yeast cells) was treated with 50  $\mu\text{g/ml}$  RsAFP2 in PDB/YPD for 16 h, the cells were fixed and prepared for TEM as described (Franzen *et al.*, 2006). Ultrathin sections were examined with a Zeiss 900 transmission electron microscope.

### Immunofluorescence and FACS analysis

The localization of RsAFP2 in treated *C. albicans* cultures was analyzed using a polyclonal antibody preparation from rabbits immunized with RsAFP2 (François *et al.*, 2002). *C. albicans* (strains CAI4 and 78, and  $\Delta gcs$  (negative control)) and *S. cerevisiae* were treated with 50 RsAFP2 in PDB/YPD for 3h and fixed with 4% paraformaldehyde in PBS. Heat-inactivated (autoclaving) RsAFP2 was used as a control. The cells were washed and incubated with anti-RsAFP2 rabbit serum (1:200) for 1 h at room temperature. To block non-specific direct binding of rabbit antibodies to *C. albicans*, the serum preparation was incubated with  $10^7$  paraformaldehyde-fixed *C. albicans* cells for 2 h at room temperature, before exposure to peptide-treated cells. Different dilutions of serum were tested, and controls included cells that were not treated with RsAFP2. After washing with PBS, the cells were incubated with a fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG for 1 h

at room temperature. Then, cells were incubated for 15 min with a 10 µg/ml solution of Uvitex 2B to detect chitin at the fungal cell wall (Polysciences Inc., Warrington, PA, US). Cells were observed using an Observer Z1 (Zeiss, Germany) fluorescence microscope. Images were acquired with a Color View AxioCam MRm digital camera. Epifluorescent or deconvolved z-stacks were analyzed with AxioVision software (Zeiss). Samples were also analyzed by flow cytometry to determine the percentage of RsAFP2 positive cells. Fluorescent cells were measured by FACSCalibur flow cytometer (BD Biosciences), and 10,000 events were analyzed with winMDI software (NHI).

### Cell wall glucan and mannan

For total cell wall glucan (and mannan) determination, cell walls were isolated (De Groot *et al.*, 2004), and wall carbohydrates were hydrolyzed to monomers using sulfuric acid hydrolysis as described (Pardini *et al.*, 2006). Amounts of glucose (and mannose) in the samples were determined by HPLC (Agilent series 1200, Bio-Rad), using an Aminex HPX-87H precolumn coupled with two Aminex HPX-87H ion exclusion columns of 300 mm by 7.8 mm, thermostatically controlled at 65°C (Agilent G1316A). Sugars were detected by a G1314B variable-wavelength detector (Agilent) set to 210 nm and a refractive index detector (Agilent G1362A) in series, and compared to glucose and mannose standards. Given values are the mean of duplicate measurements from two independent experiments.

### Determination of glucosylceramide (GlcCer)

Cell wall and membrane fractions of *C. albicans* CAI4 and strain 78 were collected for GlcCer quantitation according to a previously established protocol (Fontaine *et al.*, 2003). Briefly, cells were washed with distilled water and then mechanically disrupted with glass beads (0.5–0.75 mm diameter; 1:1 v/v) in lysis buffer (in 200 mM Tris-HCl, 20 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride) using a cell homogenizer (B. Braun Biotech International). Ten cycles of 1 minute, alternating with intervals of 1 minute on ice, were used. Cell disruption was visualized microscopically. The cell wall fraction was collected after centrifugation of the lysate at 10,000 × *g* for 10 min at 4°C. The total membrane content was obtained after ultracentrifugation of the supernatant at 125,000 × *g* for 1 h at 4°C. GlcCer extraction and quantification was performed according to a method routinely used in our laboratory (Barreto-Bergter *et al.*, 2004). Pellets were then extracted with mixtures of successively chloroform/methanol 2:1, 1:1 and 1:2 (v/v) at room temperature. The crude lipids were pooled, dried under vacuum, and partitioned as described (Folch *et al.*, 1957). The lipids recovered from the lower phase were analyzed on high-performance thin layer chromatography (HPTLC) plates developed with chloroform/methanol/water 65:25:4 (v/v/v). A GlcCer fraction purified from *C. neoformans* was used as standard (Rodrigues *et al.*, 2000). The spots were visualized by transient reactivity of the bands with iodine vapor and by charring with orcinol/H<sub>2</sub>SO<sub>4</sub>. Quantification of GSL was performed using Scion Image software (2000 Scion Corporation, NIH).

### Intracellular RsAFP2 accumulation

Overnight *C. albicans* cultures in YPD ( $2 \times 10^8$  cells/ml) were washed and resuspended in PDB/YPD at  $2 \times 10^7$  cells/ml. 30 µg/ml RsAFP2 was added to 500 µl of these cultures. After 2.5 h of incubation at 30°C with shaking, 20 µl of the cultures was used for determination of the number of colony forming units, whereafter the spent medium of the cultures was collected. The cell pellet was washed three times with PBS, and the wash fractions were pooled and added to the supernatant. Five hundred µl 20% acetonitrile/80% PBS was added to the cell pellet, whereafter the cells were lysed using a Fastprep (BIO101/Savant, Toronto) reciprocal shaker, followed by centrifugation (5 min at 3000 rpm). The cell pellet was washed 2 times with 500 µl PBS and aliquots were used for cell counting in a Thoma counting chamber (Marienfeld GmbH & Co, Germany). The cell pellet was resuspended in

300  $\mu$ l acetonitrile/water 20/80 (V/V), after which the RsAFP2 concentration in both supernatant and cell lysates was determined using HPLC (Agilent Series 1100, Agilent Technologies, Santa Clara, CA, US). Values were normalised to the number of cells. UV signals ( $\lambda = 214$  nm) were monitored and peaks were integrated using the Agilent Chemstation software (Agilent Technologies). Separation of RsAFP2 was performed on a Microsorb-MV 300 C8 (250 $\times$ 4.6mm) column with a particle size of 5  $\mu$ m (Varian Medical Systems, Palo Alto, CA, US) (equilibrated with acetonitrile/water 20/80 (V/V)). The column was eluted with a linear gradient of 20 to 100% acetonitrile at 1 ml/min. Experiments were repeated at least three times, each measured in duplo.

### Antifungal activity assays

*C. albicans* CAI4 cultures ( $2\times 10^7$  cells/ml) were incubated with various concentrations of RsAFP2 or water in the presence or absence of 2 or 4 ng/ml caspofungin in PDB/YPD for 2.5h. Percentage survival was calculated as described previously (Aerts *et al.*, 2009). To assess growth of the *C. albicans* cultures in the absence or presence of different RsAFP2 concentrations, the cultures were incubated in an automated OD plate reader (Bioscreen C, Thermo Fisher Scientific Inc., Waltham, MA, US).

### Mkc1p phosphorylation assay

A single colony of *Candida albicans* (strain CAI4) was inoculated into 15mL PDB/YPD supplemented with uridine and grown overnight at room temperature. Cells were collected by centrifugation, resuspended in 40mL fresh media (PDB/YPD + uridine) and allowed to grow for 4 hours at 30°C. Cells were then treated with RsAFP2 (50  $\mu$ g/ml), Nikkomycin Z (10  $\mu$ g/mL), or water as a control for 1 hour. Cells were collected over ice and total protein was extracted as described previously (Kumamoto 2005). A total of 50  $\mu$ g (phospho-Mkc1p) or 10  $\mu$ g (actin) total protein was loaded per sample on an 8.5% (Mkc1p) or 10% (actin) SDS-PAGE. Gels were transferred onto 0.2 $\mu$ m nitrocellulose membranes using standard protocols and probed with anti-dually phosphorylated p42/44 MAPK rabbit monoclonal antibody (Cell Signaling CS-4370) or with rabbit anti-actin (Sigma A5060). Alexa 647-conjugated mouse anti-rabbit (Jackson Labs 211-605-109) was used as a secondary antibody, and signal was detected and quantified using a fluorescence imager. The experiment was performed twice.

### Yeast-to-hypha transition assay using solid phase cytometry (SPC)

The fraction of hyphae in *C. albicans* cultures, taking only viable cells into account, was determined as described (Nailis *et al.*, 2009). *C. albicans* CAI4 cultures, grown overnight in YPD ( $2\times 10^8$  cells/ml), were diluted in YPD supplemented with 10% foetal calf serum (FCS) ( $8\times 10^4$  cells/ml), and incubated with different concentrations of RsAFP2 or inhibitor 235236 for 4 h at 37°C. Cell suspensions (30  $\mu$ l) were diluted in 1 ml sterile and particle free 0.9% (w/v) NaCl. Further processing of the diluted suspensions and analysis of the fluorescent signals was performed as previously described (Nailis *et al.*, 2009). To test whether the inhibition of yeast-to-hypha transition after RsAFP2 treatment can be cured, *C. albicans* CAI4 cultures were treated with 0 and 50  $\mu$ g/ml RsAFP2 for 2h, washed three times with physiological saline, and further incubated for 2h at 37°C in YPD supplemented with 10% FCS. Data are means  $\pm$  SEM of at least sextuple measurements, experiments have been repeated at least twice.

### Septin localization assay

The *SEP7*-GFP allele was transformed into *C. albicans* CAI4 (resulting in JRB247) as described previously (Blankenship *et al.*, 2010). An overnight culture of JRB247 was diluted 100 fold in YPD/PDB, and allowed to grow to an OD600 of  $\sim$ 1. This culture was split, and

one half was treated with 50 µg/ml RsAFP2, while the other half was mock treated. After 2 h of incubation, 200 µl of these cultures were pipeted onto glass bottom dishes (MatTek Corporation, Ashland, MA) coated with concanavalin A. The dishes were washed twice with SC and cells were resuspended in SC for immediate visualization. For the washout experiment, cells were treated as above, however, treated cells were imaged 1.5 h after treatment with RsAFP2. The remaining cells were washed 3 times with fresh YPD/PDB, allowed to recover for 2 h in fresh YPD/PDB, and then imaged. Approximately 220 yeast cells for each experimental condition were visualized with a Zeiss Axio Observer Z.1 fluorescence microscope and a 100X NA 1.4 objective. Fluorescent images were acquired with an exposure time of 750 ms using a Coolsnap HQ2 (Photometrics) camera and Axiovision (Zeiss) software. DIC images are representative of a single image slice. GFP images were created by merging seven Z-stack slices (0.5 µm depth) in NIH ImageJ. DIC/GFP overlay images were created on ImageJ. Bud-neck width and the diameter of mother cells was measured using the Axiovision software. Because *C. albicans* cells normally are not totally round, two measurements were taken for the diameter: one across the length of the cell and the other approximately 90° from this measurement across the width of the cell. These measurements were averaged and a neck width/cell diameter ratio was calculated each cell independently.

### RT-PCR expression analysis

Following treatment with RsAFP2 (50 µg/ml) for 2.5 h at 30°C, cells were collected and washed with physiological saline. Cell disruption, RNA purification, DNase treatment, and RT-PCR were performed as described (Nailis *et al.*, 2006). Primers were developed for *GIN4* (forward: AGTGGTACGCAGTGGGTCCAAA – reverse: TGTTGCACCAGCGCCAGGAT). After testing their specificity, real-time PCR (CFX96 Real Time System, Bio-Rad, Nazareth, Belgium) was performed using a Mesa Green qPCR kit (Eurogentec, Seraing, Belgium). The expression levels of *GIN4* in both conditions were normalized using four reference genes (*RPP2B*, *RIP*, *PMA1* and *LSC2*) (Nailis *et al.*, 2006). Experiments were repeated three times.

### Sphingolipidomics

Following treatment with RsAFP2 (25 µg/ml) for 5h at 30°C, analysis of sphingolipid metabolites of total membrane preparations of *C. albicans* was performed as described previously using a sphingolipidomics approach (Bielawski *et al.*, 2006). Data represent duplicates of two independent experiments.

## Results

### *C. albicans* fitness test profiling of RsAFP2

RsAFP2 was tested in the CaFT at three concentrations (10, 13, and 16 µg/ml, IC51, 53 and 69, respectively). As the profile bore no relatedness to over 4,000 antifungal agents tested in the same assay (data not shown), we selected strains whose absolute values of the normalized *z*-scores (positive indicating RsAFP2-hypersensitivity, negative RsAFP2-resistance (Xu *et al.*, 2007)) were  $\geq 3.0$  in at least two experiments. Of the 50 selected strains, 28 were hypersensitive to RsAFP2, and 22 resistant (Fig. 1A; Table S1). At least three groups of genes with clear functional connections were represented in these strains, including 1) those involved in cell wall structure, especially glucan synthesis: *orf19.2843/RHO1*, encoding the regulatory subunit of the  $\beta$ -1,3-glucan synthase; *orf19.906/ROM2*, encoding the GTP/GDP exchange factor of Rho1p and Rho2p; *orf19.2334/BIG1*, encoding a modulator of Rho1p, *orf19.5867/WSC1*, encoding a mechanosensor protein of the PKC-MPK signaling pathway; *orf19.2765/PGA62* encoding a GPI-anchored protein potentially contributing to cell wall stability and structure; a strain containing two heterozygous

deletions of both *RHO1* and *orf19.5058/SMI1*; and *orf19.1989/DCW1*, encoding a GPI-anchored mannosidase required for cell wall biosynthesis during bud formation. With the exception of *PGA62*, all heterozygotes were RsAFP2-hypersensitive; 2) those involved in bud/septin ring formation: *orf19.663/GIN4*, encoding a protein kinase required for bud and septin ring formation, represented by two independently constructed heterozygotes; and *orf19.3174/CDC24*, encoding a GTP/GDP exchange factor for Cdc42p, required for cellular polarity. All heterozygotes were RsAFP2-hypersensitive; 3) those involved in sphingolipid/ceramide biosynthesis: *orf194592/HSX11*, encoding glucosylceramide synthase, GCS; *orf19.1514/VPS74*, encoding a protein required for Golgi localization of GCS; *orf19.4769/IPT1*, encoding inositol phosphoryl transferase, involved in the synthesis of mannosylinositolphosphoryl-containing sphingolipids; and *orf1919.7354/LAC1*, encoding a ceramide synthase component. All heterozygotes were RsAFP2-resistant (Fig. 1A). The resistance of heterozygous deletions of *HSX11*, *VPS74* and *LAC1* reiterates the previous conclusion that the antifungal activity of RsAFP2 is mediated via its interaction with GlcCer (Thevisen *et al.*, 2004), thus validating our approach of using the fitness test to study its MOA. Note that 11 genes identified in the CaFT RsAFP2 profile correspond to proteins that are located in the ER and/or Golgi apparatus (Fig. 1A), which is not surprising as sphingolipids and ceramides are synthesized in the ER and Golgi apparatus (Funato and Riezman, 2001). Within groups 1) and 2), various genes encode proteins that are regulated by or are part of G-protein signaling systems, namely *RHO1*, *ROM2*, *BIG1*, *WSC1*, *CDC24* and *SMI1* (Fig. 1A). In *S. cerevisiae*, the corresponding proteins have been implicated in governing tolerance against or signaling of cell wall stress. Recently, cell wall integrity and septin localization were found to be linked processes (Blankenship *et al.*, 2010). The hypersensitivity of heterozygotes for genes involved in these two related processes in the RsAFP2 CaFT further suggests that RsAFP2, upon interaction with GlcCer, perturbs the fungal cell wall integrity and/or activates cell wall stress signaling pathways. Next, we compared the data of the RsAFP2 CaFT with CaFT data obtained with other antifungal compounds that affect sphingolipid/ceramide biosynthesis (Fig. 1B), namely the mycotoxins lipoxamycins and fumonisin and the cyclic lipodepsipeptide phomafungin (Mandala *et al.*, 1994; Cowart and Obeid, 2007; Herath *et al.*, 2009). The CaFT profiles of these antifungal compounds were distinct from that of RsAFP2 (Fig. 1B), pointing to a unique MOA of RsAFP2.

To validate the above genetic data, we assessed the effect of RsAFP2 on *C. albicans* cell wall morphology and integrity and on septin localization.

### **RsAFP2 interacts with the cell wall**

First, we assessed whether RsAFP2 induces morphological changes in *C. albicans*. Cells were incubated with 50 µg/ml RsAFP2 for 16h and analysed by transmission electron microscopy (TEM) (Fig. 2). Incubation of *C. albicans* with 25–50 µg/ml RsAFP2 for 5–24 h typically resulted in 45% to 65% survival as compared to the control treatment (water). RsAFP2 induced clear alterations in cell wall morphology and septum formation. Whereas control cells displayed a normal ultrastructural profile with a continuous cytoplasmic membrane, a compact cell wall with an external fibrillar layer and short-sized bud necks (Fig. 2A, panels A and E), RsAFP2-treated *C. albicans* cells were characterized by an aberrant budding profile with large bud neck size (Fig. 2A, panel B), altered cell shape with abnormal cell wall structure (Fig. 2A, panels C and F); and by the absence of the outermost wall layer at some loci (Fig. 2A, panel D). All the above phenotypes appeared in more than 90% of the RsAFP2-treated cells. Similar observations were made on other *Candida* species including *C. krusei* and *C. parapsilosis* (data not shown). These data point to the induction of cell wall alterations and aberrant septin formation in *C. albicans* by RsAFP2. However, it remains uncertain whether the fungal cell wall is the primary target of RsAFP2, or whether

cell wall disruption is an indirect effect. Moreover, it can not be ruled out that the above described alterations result from factors other than RsAFP2-induced cell wall stress, implying that RsAFP2 may act on multiple targets.

Second, using immunofluorescence microscopy we determined whether RsAFP2 interacts with the *C. albicans* cell wall (Fig. 3). *C. albicans* were incubated with 50 µg/ml RsAFP2, whereafter an anti-RsAFP2 serum was added. *C. albicans*  $\Delta gcs$ , *C. glabrata* and *S. cerevisiae*, which do not produce GlcCer and are RsAFP2-resistant, were used as negative controls (Leipelt *et al.*, 2001; Tavares *et al.*, 2008). We found that RsAFP2 interacts with the cell wall of *C. albicans* CAI4 and induces cell death in a process that requires the presence of GlcCer, as RsAFP2 was not observed to be associated with cell walls of RsAFP2-resistant *C. glabrata* and *S. cerevisiae* cells. In case of incubation of *C. albicans*  $\Delta gcs$  cells with RsAFP2, only a minor fraction of RsAFP2 was found to be associated to the cell surface as compared to *C. albicans* CAI4 cells. FACS analysis of *C. albicans* CAI4 or  $\Delta gcs$  cells upon incubation with 50 µg/ml RsAFP2 showed that 44.4% of the CAI4 cells reacted positive for cell surface RsAFP2, whereas only 14.2% of  $\Delta gcs$  cells reacted RsAFP2 positive. As incubation of *C. albicans* CAI4 cells with 25–50 µg/ml RsAFP2 for 5–24 h typically results in 45% to 65% survival as compared to the control treatment (water), the percentage of RsAFP2-reactive cells (i.e. 44.4%) is in the same range as the percentage of RsAFP2-killed cells. Incubation of *C. albicans* CAI4 with heat-inactivated RsAFP2 did not result in any cell surface-associated defensin.

Consistent with a predominant localization of RsAFP2 in the outer cell surface layers, we found that  $43.0 \pm 0.1\%$  of GlcCer, the RsAFP2 target sphingolipid, is located in the *C. albicans* cell wall of strain 78, for strain CAI4 this value is  $41.0 \pm 0.1\%$ . Next, internalization of RsAFP2 by *C. albicans* cells was analyzed by quantitative HPLC analysis (François *et al.*, 2009; Bink *et al.*, 2010) of intracellular RsAFP2 as well as the fraction of RsAFP2 in the supernatant of cell cultures of *C. albicans* CAI4 and the  $\Delta gcs$  strain upon RsAFP2 treatment. Treatment of *C. albicans* CAI4 cells with 30 µg/ml RsAFP2 resulted in a very minor intracellular uptake of the plant defensin as only  $0.74 \pm 0.49\%$  of the total amount of added RsAFP2 could be retrieved intracellularly. More than 95% RsAFP2 could be detected in the supernatant. In case of RsAFP2-treated  $\Delta gcs$  cells,  $1.43 \pm 0.19\%$  RsAFP2 could be detected intracellularly. These data indicate that RsAFP2 does not need to be taken up intracellularly in *C. albicans* to exert its antifungal activity, which is consistent with the above presented microscopic data.

Our data of the genetic CaFT screen pointed to an important role for  $\beta$ -1,3-glucan synthesis in RsAFP2 tolerance. Therefore, the effect of RsAFP2 on the amount and structure of cell wall glucan was investigated. *C. albicans* CAI4 cells were treated with 0 or 50 µg/ml RsAFP2, after which polysaccharides in isolated cell walls were hydrolyzed with sulphuric acid followed by HPLC analysis of the resulting monosaccharides. These analyses showed that the relative amounts of glucan (and mannan) in the cell wall were unaltered upon RsAFP2 treatment for 2.5h or 16h. We measured  $48 \pm 2\%$  glucose and  $24 \pm 1\%$  mannan after 2.5h treatment with RsAFP2, and  $53 \pm 4\%$  glucose and  $27 \pm 1\%$  mannan for the mock incubation. After 16h treatment,  $51 \pm 4\%$  glucose and  $27 \pm 2\%$  mannan was present in cell wall of cells treated with RsAFP2 versus  $46 \pm 2\%$  glucose and  $24 \pm 1\%$  in corresponding control cell walls.

### **RsAFP2 activates the cell wall integrity (CWI) pathway via Mkc1p activation**

As we found that RsAFP2 interacts with the *C. albicans* cell wall and that various *C. albicans* heterozygotes for genes involved in the CWI are RsAFP2 hypersensitive, we investigated a putative activation of the CWI pathway by RsAFP2. To this end, we assessed the activation of Mkc1p as downstream interacting partner of Pkc1p by the determination of



the degree of Mkc1p phosphorylation using anti-dually phosphorylated p42/44 MAPK rabbit monoclonal antibodies on immunoblots of cell extracts (Kumamoto, 2005). Exponentially grown *C. albicans* cells were treated with 50 µg/ml RsAFP2, 10 µg/ml Nikkomycin Z (positive control), a chitin synthase inhibitor that causes cell wall stress and activation of Mkc1p (Kumamoto, 2005), or water for one hour. Dually phosphorylated Mkc1p in cell extracts was detected by immunoblotting with antibody specific for the dually phosphorylated form of p42/44 Map kinases. A 1.5-fold increase in dually phosphorylated Mkc1p in response to RsAFP2 and a 2.3-fold increase in response to Nikkomycin Z were observed. These data point to the activation of the CWI pathway by RsAFP2, governing RsAFP2 tolerance.

### **RsAFP2 induces septin mislocalization and impairs the yeast-to-hypha transition in *C. albicans***

Septin localization upon RsAFP2 treatment was studied by inserting a *SEP7*-GFP tagged allele in *C. albicans* CAI4 (Blankenship *et al.*, 2010), which was visualised using fluorescence microscopy. Thirty-five percent of the RsAFP2-treated cells showed abnormal septin localization (Fig. 5A; Fig. S1): 13.6% showed diffuse septin bands at the neck; 5.9% were characterized by septin spots in the daughter cell; and 3.1% had side-by-side necks. Moreover, 1.4% of the cells were characterized by septin that was localized around the periphery of the bud, and 3.2% of the cells had medium-sized buds with an additional bud at the distal end, both with septins at the mother-daughter interface. Septin localization within 7.7% of the RsAFP2-treated cells was otherwise abnormal. The GFP-tagged Sep7 septin also allowed us to determine neck sizes of budding *C. albicans* cells (Fig. 5B). Upon treatment of the yeast cells with 50 µg/ml RsAFP2 for 2h, we observed a 1.5-fold increase of the average neck size (2.72 µm, n=244, with a ratio of bud neck/mother cell diameter of 0.4) as compared to untreated cells (1.72 µm, n=99,  $p < 0.001$ , bud neck/mother cell ratio of 0.32). Thus, RsAFP2 induced a general mislocalization of the septin ring. The RsAFP2-induced septin localization defects do not appear to be similar to the earlier reported ectopic septin localization caused by caspofungin (Blankenship *et al.*, 2010). However, *C. albicans* cells treated with low levels of caspofungin for longer periods of time actually look similar to RsAFP2-treated cells (data not shown). These data indicate that the observed phenotypic differences between RsAFP2 and caspofungin may be due to dosage effects.

Recently, it was shown that abnormal septin localization may be associated with inhibition of the yeast-to-hypha transition in *C. albicans* (Toenjes *et al.*, 2009). As Gin4, a S/T kinase that is involved in hyphal growth, was also identified in the RsAFP2 CaFT assay (Fig. 1A), we assessed whether RsAFP2 affects the yeast-to-hypha transition of *C. albicans* in fetal calf serum (FCS)-containing medium. *C. albicans* CAI4 cells were incubated with different concentrations of RsAFP2 in YPD in the presence of 10% FCS and the fraction of cells developing hyphae was determined using solid phase cytometry (Nailis *et al.*, 2009). The applied RsAFP2 doses in YPD + 10% FCS had no effect on growth of the *C. albicans* cultures as assessed by Bioscreen analysis (data not shown). As a positive control, we used inhibitor 235236, which has been shown to block yeast-to-hypha transition in *C. albicans* (Toenjes *et al.*, 2005). Treatment of *C. albicans* cultures with RsAFP2 reduced the fraction of hyphae in a dose-dependent manner (Fig. 6A). In the presence of water (mock treatment),  $64.1 \pm 2.1\%$  of the *C. albicans* cell population showed hyphal growth in YPD supplemented with 10% FCS upon incubation for 4 h. The fraction of cells forming hyphae dropped to  $18.2 \pm 3.4\%$  in the presence of 50 µg/ml (10 µM) RsAFP2, and to  $28.5 \pm 1.5\%$  in the presence of 10 µg/ml (30 µM) inhibitor 235236. Therefore, we conclude that RsAFP2 impairs the yeast-to-hypha transition in *C. albicans*. In a curing experiment, septin localization returned to normal upon removal of RsAFP2. The average bud neck/cell diameter ratio for treated cells was  $0.42 \pm 0.01$ , and for RsAFP2-washed out cells this was

0.31±0.01. Similarly, no significant differences in the fraction of hyphae were observed between RsAFP2- and water-treated *C. albicans* cell populations upon curing ( $39.0 \pm 2.6\%$  versus  $44.3 \pm 3.5\%$ ). This indicates that the RsAFP2-induced septin mislocalization and the block of the yeast-to-hypha transition is transient. As *C. albicans* cells that are trapped in the yeast form have been reported to be less susceptible to caspofungin (Wheeler *et al.*, 2008), we studied a possible antagonism between RsAFP2 and caspofungin. Co-incubation of *C. albicans* cells with a mild RsAFP2 dose (5 µg/ml, resulting in 100% viability of the yeast culture) and caspofungin resulted in significantly increased survival rates of *C. albicans* cells, as compared to treatment with caspofungin alone (Fig. 6B), supporting the idea that the two antifungals have antagonistic effects. The applied RsAFP2 doses in PDB/YPD had no effect on growth of the *C. albicans* cultures as assessed by Bioscreen analysis (data not shown). Whereas loss of core septins leads to increased caspofungin sensitivity (Blankenship *et al.*, 2010), RsAFP2-treatment results in increased caspofungin tolerance. We hypothesize that septin dispersal is part of the natural defense/tolerance against cell wall stress. Hence, loss of a core septin (as in septin mutant cells), may obstruct this protection, resulting in hypersensitivity to caspofungin. Upon co-incubation of cells with mild RsAFP2 doses, however, the core septins are still present and, according to the above hypothesis, the cell can deal with the cell wall stress imposed by caspofungin.

To further elucidate the role for Gin4 in governing tolerance to RsAFP2 in *C. albicans*, expression of *GIN4* in RsAFP2-treated *C. albicans* cultures was assessed. A slight but significant induction of *GIN4* expression (1.4-fold, standard deviation 0.2-fold,  $p=0.05$ ) was observed in RsAFP2-treated cells compared to untreated cells.

### **RsAFP2 induces ceramide accumulation in *C. albicans* membranes**

As accumulation of ceramides, the sphingolipid backbones, following aureobasidin treatment has been shown to induce septin mislocalization in membranes of the Basidiomycete *Ustilago maydis* (Cánovas and Pérez-Martín, 2009), we determined the effects of RsAFP2 on sphingolipid species in *C. albicans* CAI4. Sphingolipid metabolites, such as  $\alpha$ -hydroxy-phytoceramides, dihydro- and phytoceramides, dihydro- and phytosphingosine, as well as the corresponding sphingoid base phosphates were analyzed in the total cell membrane fraction of *C. albicans* treated with RsAFP2 by mass spectrometry analysis. Treatment of *C. albicans* with RsAFP2 led to a significant increase in the most abundant ceramides, namely C-24phytoceramides and ( $\alpha$ -hydroxy)C-24phytoceramide (Fig. 7). Levels of the other ceramide species or sphingolipid metabolites in *C. albicans* membranes were not significantly altered upon RsAFP2 treatment.

### **Discussion**

To uncover tolerance mechanisms of *C. albicans* against the plant defensin RsAFP2 and gain more insight in its mode of action, we screened a collection of heterozygous *C. albicans* mutants for altered RsAFP2 sensitivity. Most of the altered RsAFP2-tolerance genes belong to a limited set of functional classes and define specific areas of cellular biology. Most prominent were genes involved in cell wall integrity and hyphal growth/septin ring formation (*WSC1*, *ROM2*, *BIG1*, *DCW1*, *PGA62*, *RHO1*, *CDC24*, *GIN4*). These processes are functionally connected; an intact septin ring is required for normal cell wall synthesis, and a normal cell wall structure is required for the formation of a septin ring. Moreover, septins are important for cell survival and are mislocalized upon cell wall stress (Blankenship *et al.*, 2010). Other groups of RsAFP2-tolerance genes were involved in protein and vesicle transport, and in the regulation of transcription. The latter probably represents general stress tolerance mechanisms, as was described previously (Thevissen *et al.*, 2007a; Parsons *et al.*, 2004; Thorpe *et al.*, 2004). More specifically, several RsAFP2-tolerance genes encode components of small GTPase signalling transduction pathways. The

small GTPase Rho1 is activated by cell wall alterations, which can be sensed through the mechanosensor Wsc1 in the plasma membrane, via its GTP/GDP exchange factor (GEF) Rom2. The small GTPase Cdc42 is activated by various internal and external signals including calcium influx via its GEF Cdc24. RsAFP2 induces calcium influx in susceptible fungi (Thevisen *et al.*, 1996). Once activated, Cdc42 regulates the organization of the actin cytoskeleton and the septins, and interacts with components of the exocytic machinery (Park *et al.*, 1997). Both Cdc42 and Rho1 pathways have been shown to interact with each other, regulating exocytosis, septin organization, and cell wall synthesis (Drees *et al.*, 2001).

To validate the obtained genetic data, we have focused on the effects of RsAFP2 on cell wall morphology and integrity, and on septin localization in *C. albicans*. We demonstrated that RsAFP2 interacts primarily with the cell wall of *C. albicans* in a GlcCer-dependent way, and that RsAFP2 does not need to be taken up intracellularly in *C. albicans* to exert its antifungal activity. The extracellular action of RsAFP2 is in contrast to the intracellular accumulation of other antifungals, like miconazole and BAR0329, a fungicidal piperazine-carboxamide (François *et al.*, 2009; Bink *et al.*, 2010). Using the quantitative HPLC technique, we previously demonstrated a significant intracellular accumulation of miconazole ( $97.4 \pm 1.5\%$  of total amount miconazole added) and BAR0329 ( $38.6 \pm 5.6\%$  of total amount BAR0329 added) in yeast cells. The reason for the primary interaction of RsAFP2 with the *C. albicans* cell wall could be attributed to the presence of GlcCer in its cell wall. This observation is in line with a previous report describing the presence of GlcCer ligands in the cell wall of the fungal pathogen *Cryptococcus neoformans* (Rodrigues *et al.*, 2000).

RsAFP2 is the first example of an antifungal plant defensin that acts via the extracellular side of fungal cells. Previously, it has been shown that a plant defensin from flowers of *Nicotiana glauca*, NaD1, is taken up and localized to the cytoplasm of susceptible fungi (van der Weerden *et al.*, 2008). Very recently, NaD1 was shown to permeabilize fungal cells via a novel mechanism, which required the presence of the fungal cell wall (van der Weerden *et al.*, 2010). In the latter study, the authors hypothesized that an as yet unidentified NaD1-receptor may be located in the proteinaceous layer of the cell wall. An intracellular accumulation was also demonstrated for a plant defensin from *Pisum sativum* (PsD1). This defensin localizes to the nucleus of *Neurospora crassa* where it interacts with the cell cycle control protein Cyclin F (Lobo *et al.*, 2007). Consistent with our observation that the plant defensin RsAFP2 interacts with the fungal cell wall, recent papers reported the interaction between antibacterial human, invertebrate and fungal defensins and the bacterial cell wall precursor Lipid II (Schmitt *et al.*, 2010; Schneider *et al.*, 2010; Sass *et al.*, 2010), suggesting similarities in the mode of antifungal or antibacterial action of defensins from different kingdoms.

We further demonstrated that RsAFP2 activates the MAPK cell wall integrity pathway, a signal transduction pathway known to be activated by cell wall stress or membrane perturbing conditions in *C. albicans* (Kumamoto, 2005). These data are consistent with a previous report on the hypersensitivity of a *mgv1*-deletion mutant of *Fusarium graminearum* to RsAFP2 and other defensins like MsDef1 from *Medicago sativa* (Ramamoorthy *et al.*, 2007). Mgv1 is a MAP kinase that is homologous to the *S. cerevisiae* Slt2 kinase, which is involved in regulating the maintenance of cell wall integrity (Hou *et al.*, 2002) and is activated upon treatment of the fungus with MsDef1 (Ramamoorthy *et al.*, 2007).

Consistent with the reported link between cell wall integrity and septin localization (Blankenship *et al.*, 2010), we observed septin mislocalization in *C. albicans* following RsAFP2 treatment. Septins are an evolutionarily conserved family of filament-forming proteins that act in cell division, polarity determination, vesicle trafficking, and cytoskeletal dynamics (Sudbery, 2001). Except for ER and cell wall stress (Babour *et al.*, 2010;

Blankenship *et al.*, 2010), no other stress has been shown to affect septin mislocalization, indicating that septin dispersal is not a general response to stress, nor is it a common hallmark of dying cells. Hence, the observed effect of RsAFP2 on septin mislocalization seems a unique feature of this antimicrobial compound, also reflected by its CaFT.

As RsAFP2 treatment results in septin mislocalization and septins are required for polarized growth of filamentous fungi (Boyce *et al.*, 2005), this observation can also explain the previously observed hyperbranching of the filamentous fungus *Fusarium culmorum*, induced by RsAFP2 (Terras *et al.*, 1992). It has been shown recently that abnormal septin localization may be associated with inhibition of the yeast-to-hypha transition in *C. albicans* (Toenjes *et al.*, 2009), and RsAFP2 blocks this transition in *C. albicans*. As the S/T kinase Gin4, which phosphorylates the septin Cdc11 during hyphal growth, is part of the tolerance mechanism of *C. albicans* against RsAFP2, this may be a response to the blocking of the yeast-to-hypha transition induced by RsAFP2. Moreover, a lethal accumulation of ceramides in fungal membranes can induce septin mislocalization (Cánovas and Pérez-Martín, 2009). Through sphingolipidomics analysis, we demonstrated that RsAFP2 induces accumulation of long chain C24-phytoceramides.

It remains to be elucidated which enzymes are responsible for the RsAFP2-induced ceramide accumulation in membranes of susceptible fungi and which signaling pathway is regulating this process. The ceramide accumulation might result from *de novo* ceramide synthesis, through activation of a ceramide synthase enzyme, and/or via breakdown of glucosylceramides, the target of RsAFP2. However, as GlcCer in *C. albicans* are mainly composed of C18 fatty acids, in the latter case the question remains how C18-ceramides are converted to long chain C24-ceramides. Consistent with the first hypothesis, we found the *orf19.7354* (orthologue of *S. cerevisiae* *LAC1*) heterozygous *C. albicans* deletion mutant to be RsAFP2 resistant (Fig. 1). Lac1 is a ceramide synthase component, involved in the synthesis of ceramide from C24/26(acyl)-coenzyme A and dihydrosphingosine or phytosphingosine.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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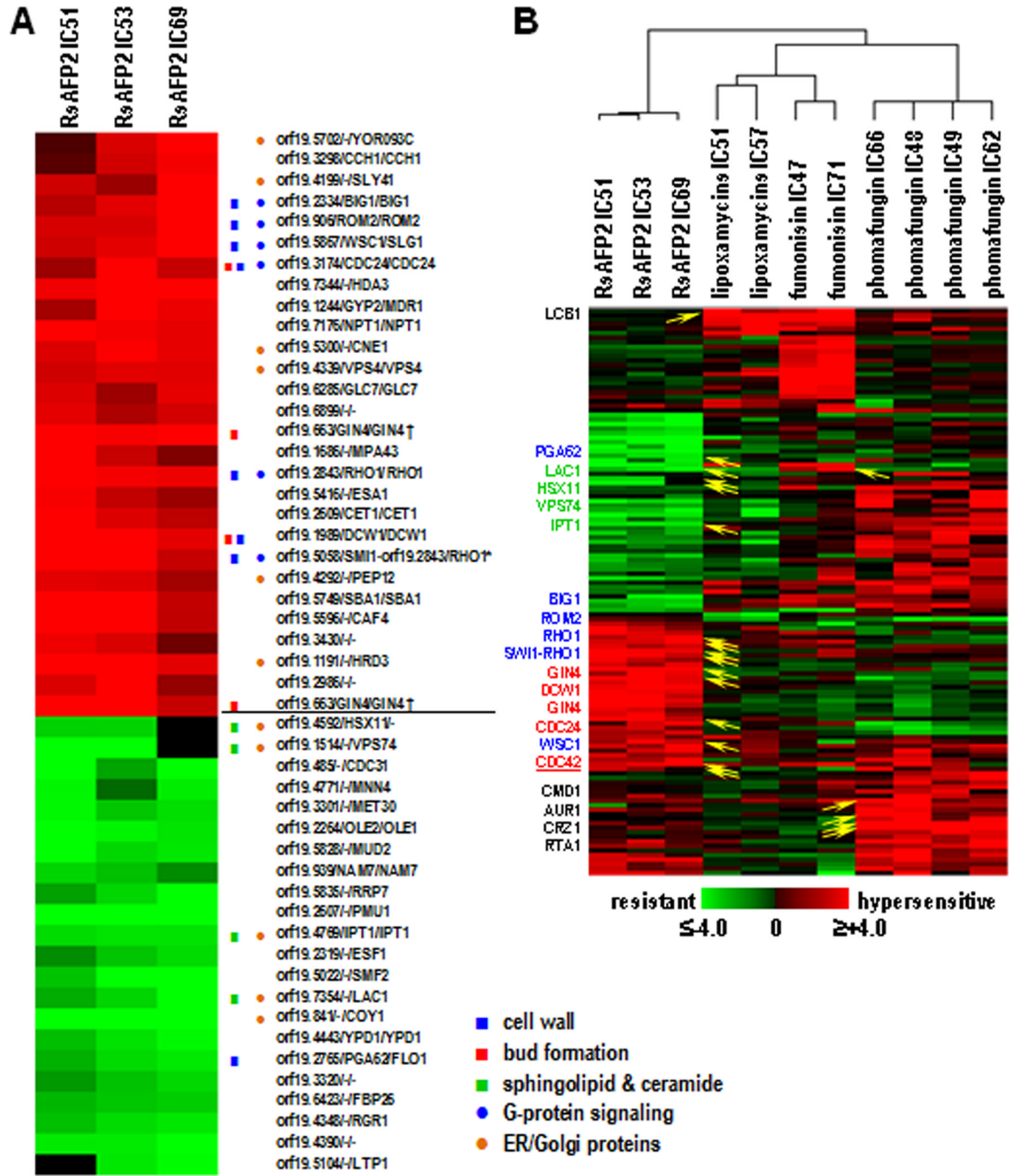
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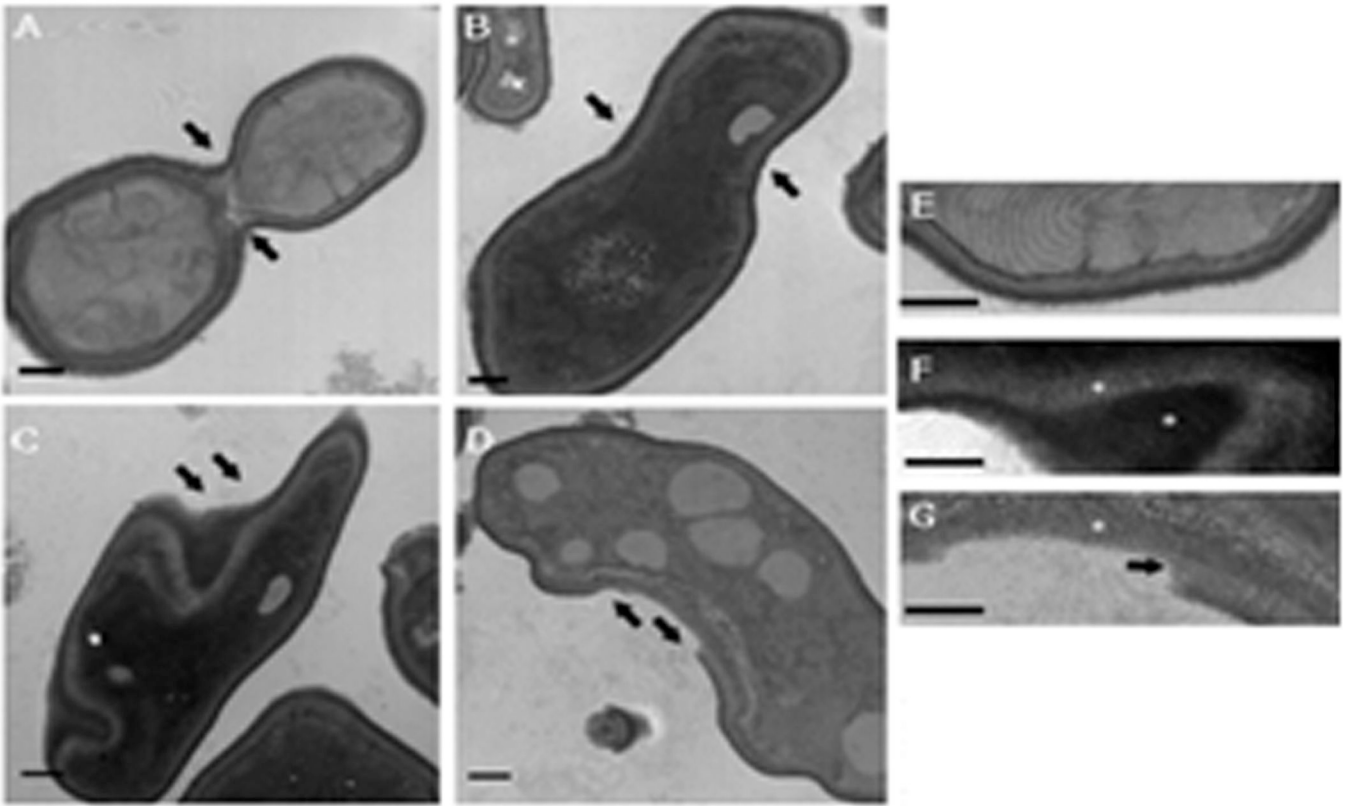


**Fig. 1. CaFT profile of RsAFP2**

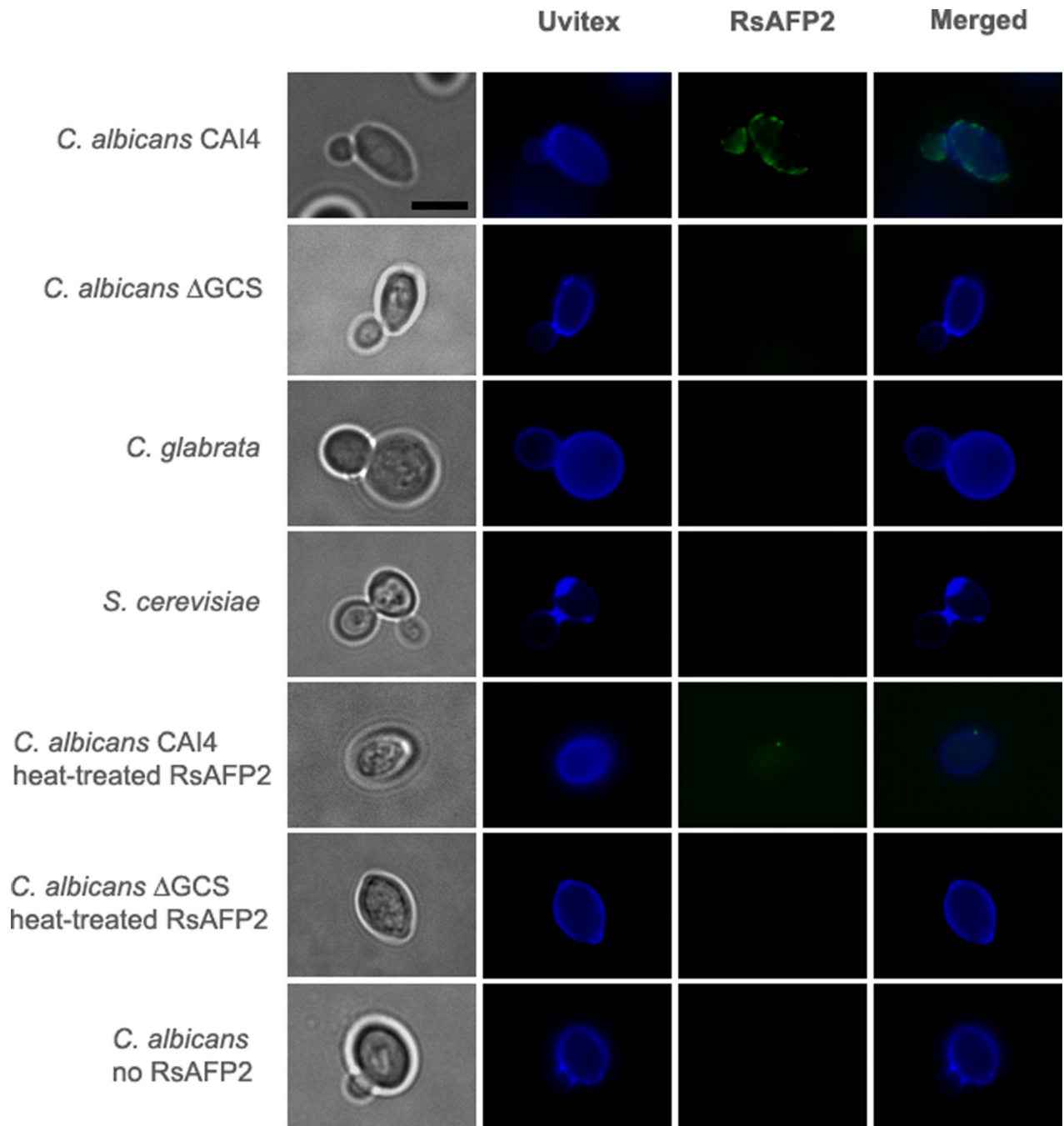
A, The plant defensin was tested at three different concentrations (10, 13 and 16 µg/ml, equivalent IC51, IC53 and IC69) in the CaFT using YPD/PDB medium. In the hierarchical clustering, heterozygotes with significant fitness variations were selected based on the absolute values of their z-scores (positive indicating hypersensitivity, and negative resistance) being  $\geq 3$  in at least two experiments. Gene annotations were adopted from the *Candida* Genome Database in following order: orf19 designation, *C. albicans* gene name, *S. cerevisiae* ortholog/homolog. Highlighted are those described in the text, with the functional group indicated. B, Comparison of the CaFT profiles of RsAFP2 and other antifungal natural products that affect sphingolipid/ceramide biosynthesis, by hierarchical clustering.



Heterozygotes were selected as described in *A*. Yellow arrows indicate the corresponding genes that are related to the Mode of Action (MOA) of compounds in question, with gene names given on the left. The three functional groups (reflecting the MOA of RsAFP2) described in the text are highlighted in color, with those in black reflecting MOAs of other natural products. Note that the same heat map scale is used in both *A* and *B*, with black indicating data not available (most likely) due to low hybridization signals (and thus excluded from z-score calculation).

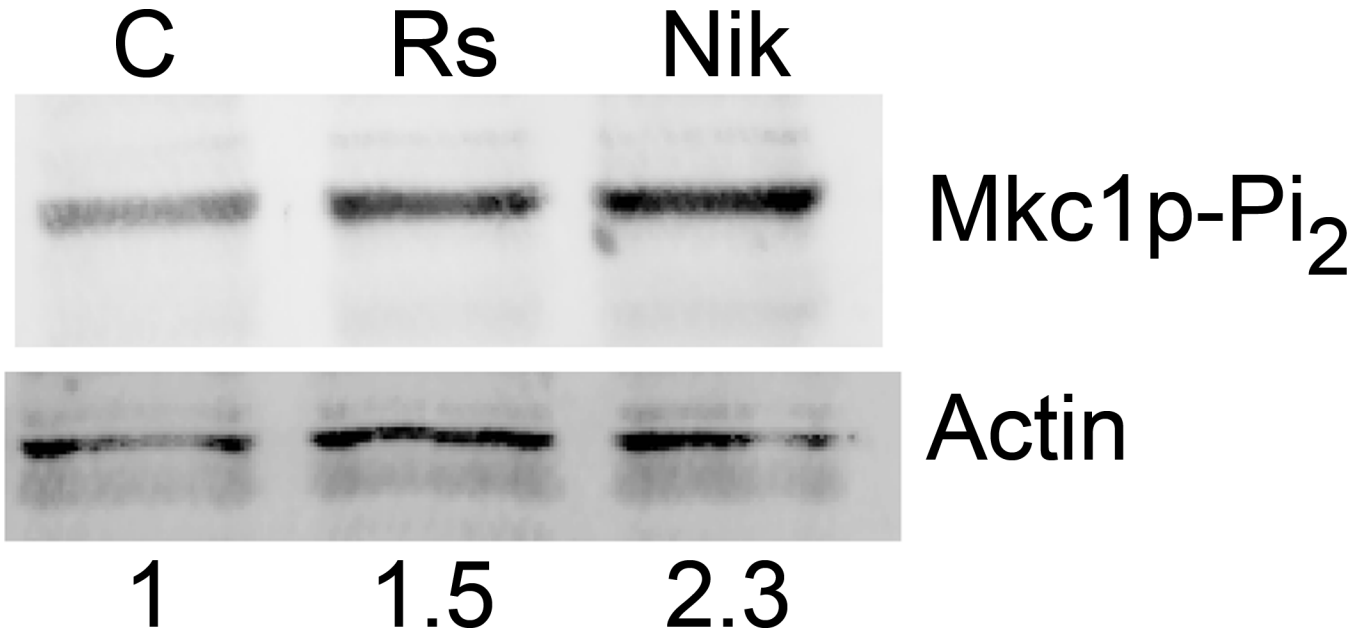


**Fig. 2. Effect of RsAFP2 on cell wall morphology in *C. albicans***  
Transmission electron micrographs (TEM) of *Candida albicans* strain 78 incubated with 50  $\mu\text{g/ml}$  RsAFP2 for 16 h at room temperature. Control cells have normal ultrastructural profiles with a continuous cytoplasmic membrane, a compact cell wall with an external fibrillar layer, and budding cells with short-sized necks (arrows in panel A). RsAFP2-treated cells show ultrastructural alterations, such as an aberrant budding profile with a large neck size of the buds (arrows in panel B), altered cell shape with abnormal cell wall structure (arrows in panel C), and absence of the outermost wall layer in some areas (arrows in panel D). Panels E–G represent large magnification images of B, C and D, respectively. Bars: 0.3  $\mu\text{m}$ .



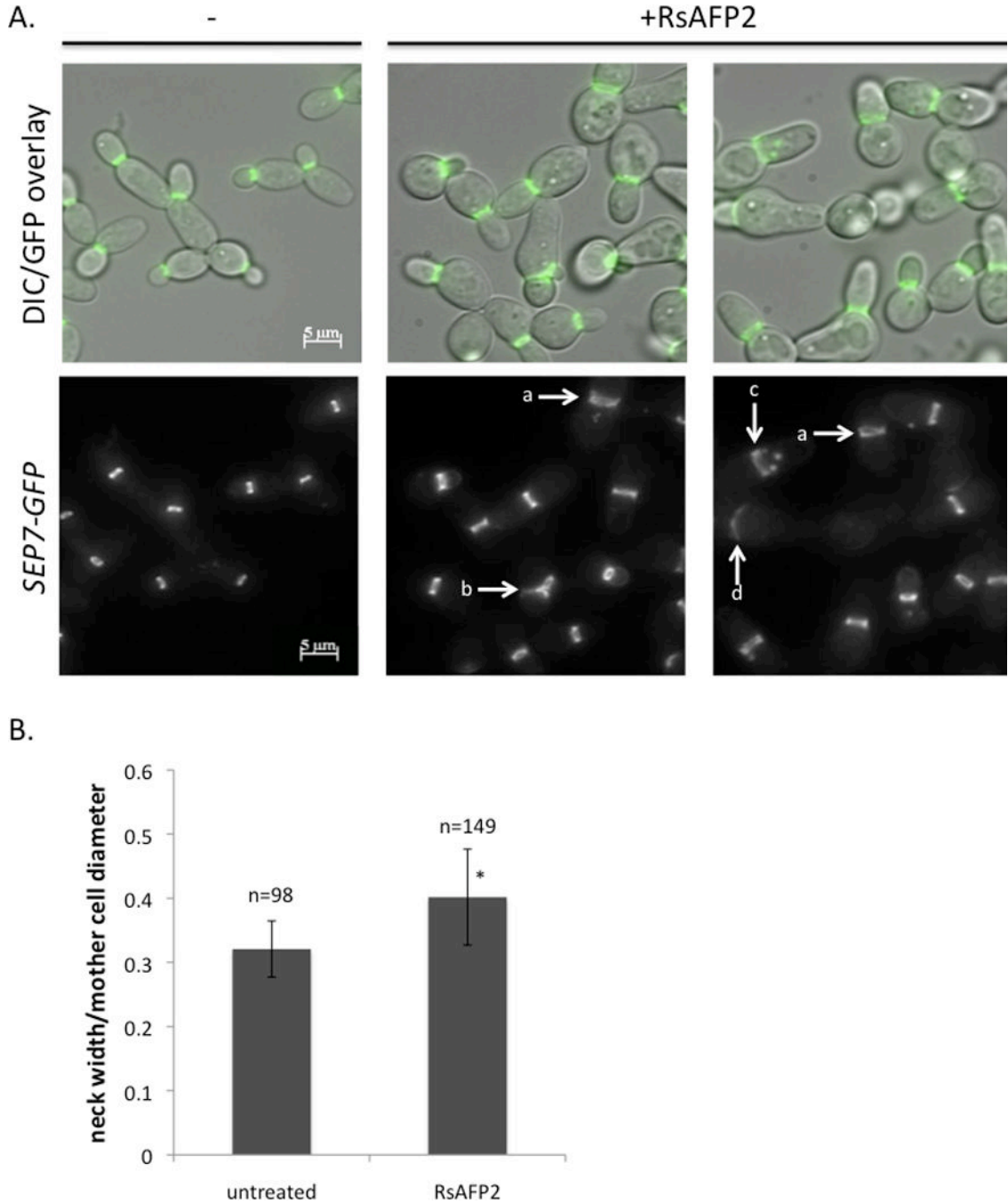
**Fig. 3. Localization of RsAFP2 at the fungal cell surface**

Epifluorescence microscopy followed by deconvolution images of *C. albicans* (CAI4 and  $\Delta$ *gcs* strains), *C. glabrata* and *S. cerevisiae* treated with 50  $\mu$ g/ml (native or heat-inactivated) RsAFP2 for 3 h and further incubated with Uvitex B2 (blue) and anti-RsAFP2 antibodies in combination with a FITC-labeled goat anti-rabbit IgG (green). Scale bars = 5  $\mu$ m.



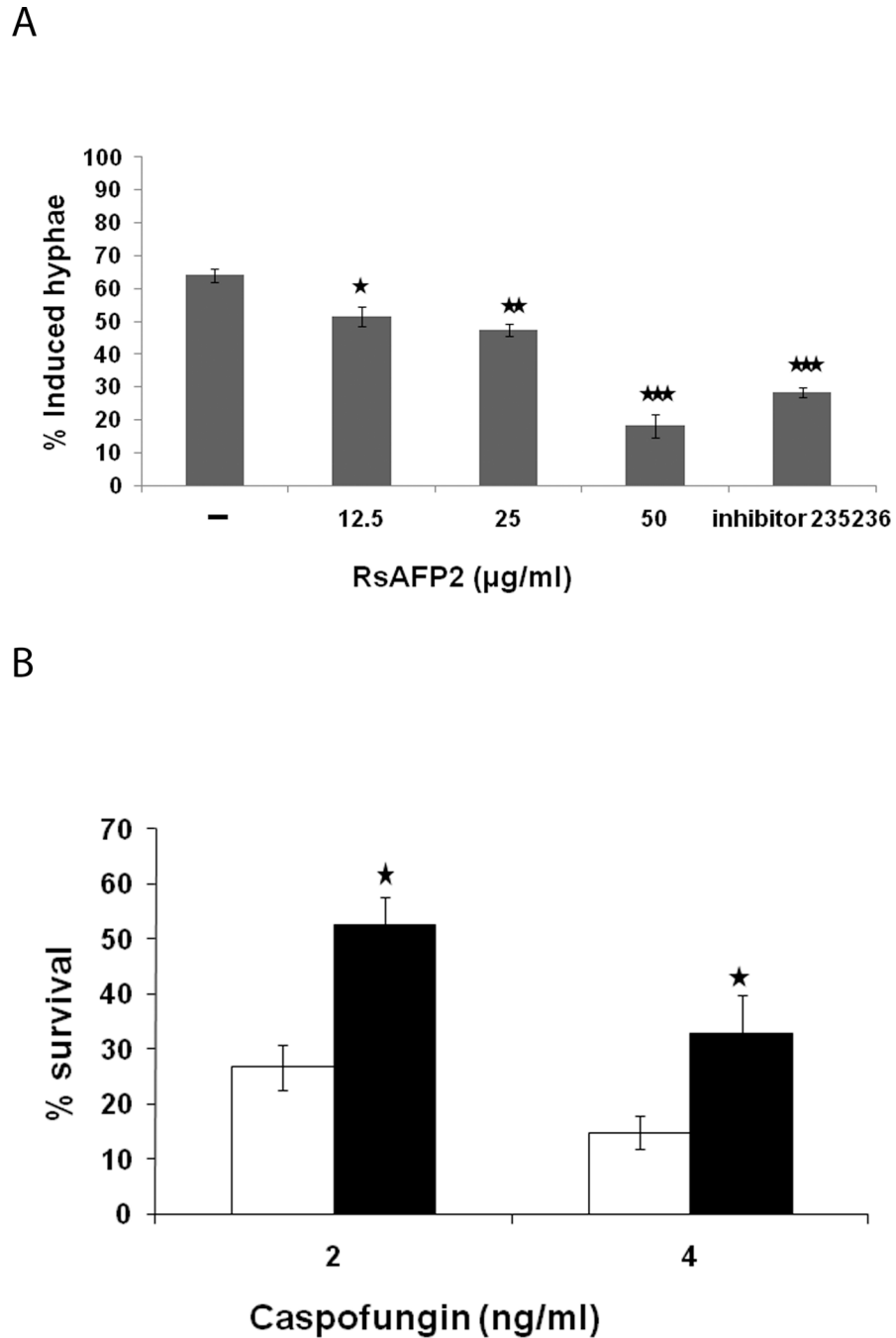
**Fig. 4. RsAFP2 activates the cell wall integrity pathway via Mkc1p**

*C. albicans* cells were treated with 50 µg/ml RsAFP2 (Rs), 10 µg/ml Nikkomycin (Nik) or water (control, C) for one hour. Protein was extracted and probed as described in experimental methods. Top panel, dually phosphorylated Mkc1p; bottom panel, actin. Numbers at bottom indicate amounts of dually phosphorylated Mkc1p normalized to actin levels and expressed relative to the water control. One representative experiment out of two is shown.



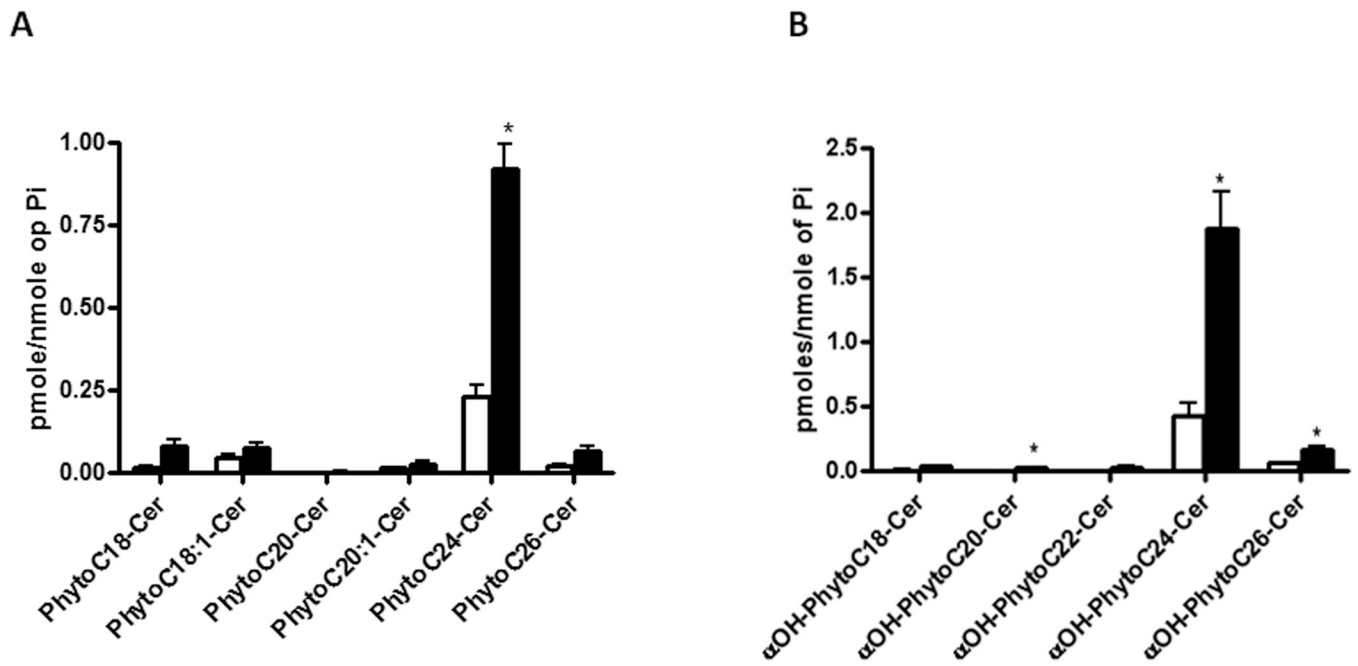
**Fig. 5. RsAFP2 affects bud neck size and septin localization**

**A.** A *SEP7-GFP*-tagged strain (JRB247) was imaged after treatment with 50  $\mu\text{g/ml}$  RsAFP2 in YPD/PDB for 2 h, and compared to an untreated control. The top panel represents the overlay of DIC (Differential Interference Contrast) and fluorescence microscopy images. Abnormal septin localization is indicated by arrows, including diffuse septin localization at the neck (a), side-by-side necks (b), septin spots in the daughter cell (c), and otherwise abnormal localization (d). **B.** The bud neck width/mother cell diameter ratio was measured in cells treated with 50  $\mu\text{g/ml}$  RsAFP2 and untreated (–) cells. Values are statistically significant ( $p=3.68 \times 10^{-22}$ ).



**Fig. 6. RsAFP2 impairs the yeast-to hyphae transition in *C. albicans***

**A**, Overnight grown *C. albicans* CAI4 cultures in YPD ( $2 \times 10^8$  cells/ml) were diluted in YPD/10%FCS ( $8 \times 10^4$  cells/ml) and incubated with different concentrations of RsAFP2 or 10 µg/ml inhibitor 235236. Using solid phase cytometry, the percentage of living yeast cells displaying hyphal morphology were determined. Data are means of triplicate measurements of one representative experiment out of three. **B**, Percentage survival (as determined by counting colony forming units) of caspofungin-treated cells in the absence (open bars) or presence of 5 µg/ml RsAFP2 (sublethal dose; black bars), normalized to that of the mock treated cells. 5 µg/ml RsAFP2 alone resulted in > 80% survival. Data represent mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Fig. 7. RsAFP2 induces accumulation of ceramide in *C. albicans* membranes**  
 Levels of *A*, phyto- and *B*,  $\alpha$ -hydroxy-phyto-ceramides in *C. albicans* CAI4 following treatment with water (open bars) or 25  $\mu$ g/ml RsAFP2 (black bars). \* $p < 0.05$ .