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The antifungal properties of a 2S albumin-homologous protein from passion fruit seeds involve plasma membrane permeabilization and ultrastructural alterations in yeast cells

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Abstract

Different types of antimicrobial proteins were purified from plant seeds, including chitinases, β-1,3-glucanases, defensins, thionins, lipid transfer proteins and 2S albumins. It has become clear that these groups of proteins play an important role in the protection of plants from microbial infection. Recent results from our laboratory have shown that the defense-related proteins from passion fruit seeds, named Pf1 and Pf2 (which show sequence homology with 2S albumins), inhibit fungal growth and glucose-stimulated acidification of the medium by *Saccharomyces cerevisiae* cells. The aim of this study was to determine whether 2S albumins from passion fruit seeds induce plasma membrane permeabilization and cause morphological alterations in yeast cells. Initially, we used an assay based on the uptake of SYTOX Green, an organic compound that fluoresces upon interaction with nucleic acids and penetrates cells with compromised plasma membranes, to investigate membrane permeabilization in *S. cerevisiae* cells. When viewed with a confocal laser microscope, *S. cervisiae* cells showed strong SYTOX Green fluorescence in the cytosol, especially in the nuclei. 2S albumins also inhibited glucose-stimulated acidification of the medium by *S. cerevisiae* cells, which indicates a probable impairment of fungal metabolism. The microscopical analysis of the yeast cells treated with 2S albumins demonstrated several morphological alterations in cell shape, cell surface, cell wall and bud formation, as well as in the organization of intracellular organelles.

Keywords: 2S albumins; Antifungal protein; Fungal cell wall; Membrane permeabilization; *Saccharomyces cerevisiae*

1. Introduction

In addition to the well-known plant antifungal proteins like chitinases, β-1,3-glucanases, thionins, chitin-binding lectins and ribosome-inactivating proteins [1], a number of new types of protein that are capable of inhibiting fungal growth, in vitro, are emerging. In this situation, plant pathogenesis-related (PR) proteins and antimicrobial peptides are considered to be components of plant host defenses [2,3]. Many of these components have antimicrobial activity in vitro, and over-expression in plants leads to some degree of protection against disease symptoms. Osmotin, for example, a tobacco pathogenesis-related protein of family 5 (PR-5), has antifungal activity in vitro and in vivo [4,5]. Interest in the mechanism of action of PR-5 proteins, including osmotin, is heightened by their structural and functional similarities with antimicrobial peptides that are considered to be components of the related innate immune response of plants and animals [6,7]. Thus, the above list can be also completed with the family of 2S albumins from seeds.

2S albumins are a low molecular weight mix of storage proteins rich in glutamine with similar physical–chemical properties present in monocotyledonous and dicotyledonous seeds [8]. Aside from sunflower 2S albumins, the majority of

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these proteins are composed of two different subunits linked by disulfide bridges that derive from a single precursor molecule [9]. Some 2S albumins can additionally play a physiological role in plant defense [10]. As an example, 2S albumins from radish seeds are shown to be efficient in inhibiting the growth of fungi and bacteria, displaying synergistic activity with thionins of either wheat or barley origin, promoting the permeabilization of the plasma membrane of phytopathogens [11,12]. More recently, two novel proteins, showing homology with 2S albumin, designated CW-1 and CW-4 with strong antifungal activities against *Fusarium graminearum* (CW-1) and *Phytophthora infestans* (CW-4) were purified and characterized from *Malva parviflora* seeds [13,14].

We are systematically investigating the association of 2S albumin with plant defense against pathogens. Recently, two novel proteins, also showing homology with 2S albumins, were purified from passion fruit seeds (*Passiflora edulis f. flavicarpa*). These proteins were named Pf1 and Pf2 and are composed of two different subunits. The molecular masses of these proteins obtained by MALDI-TOF spectrometry corresponded to 12,088 Daltons for Pf1 and 11,930 Daltons for Pf2 [15]. The S2 fractions containing both proteins, Pf1 and Pf2, inhibited the growth, in an in vitro assay, of the phytopathogenic fungi *Fusarium oxysporum*, *Fusarium solani* and the yeast *Saccharomyces cerevisiae* and strongly inhibited glucose-stimulated acidification of the medium by *F. oxysporum* in a dose-dependent manner [15]. In this study, we report the plasma membrane permeabilization and ultrastructural alterations, in yeast cells, induced by an antifungal protein from passion fruit seeds that shows sequence homology to 2S albumins, thus suggesting a protective role for these proteins that could be associated with defense mechanisms in plant seeds.

2. Materials and methods

2.1. Biological materials

Passion fruit seeds (*Passiflora edulis* Sims. *f. flavicarpa* Deg.) were commercially available in local markets (Campos dos Goytacazes, RJ, Brazil). *Saccharomyces cerevisiae* (1038) was obtained from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The yeast was maintained on Agar Sabouraud (1% peptone, 2% glucose and 1.7% agar–agar).

2.2. Purification of the Pf1 and Pf2 antifungal proteins

The extraction and purification of antifungal proteins (Pf1 and Pf2) from passion fruit seeds was performed as described by Agizzio et al. [15] with some minor modifications. Seed flour (100 g) was extracted for 2 h (at 4 °C) with 500 mL of extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 1.5% EDTA), pH 5.4. Precipitate formed at between 0 and 70% relative ammonium sulfate saturation was re-dissolved in distilled water and heated at 80 °C for 15 min. The resulting suspension was clarified by centrifugation and the supernatant was extensively dialyzed against distilled water. The dialyzed solutions were recovered by freeze-drying (F/0–70) and submitted to further purification by chromatographic methods. A DEAE-Sepharose column was used in instead of the Sepharose G-50 and employed for further separation of proteins from the F/0–70 fraction. The column (40 cm × 3 cm) was equilibrated and developed with 0.1 M Tris–HCl (pH 8.0). The basic fraction containing both (Pf1 and Pf2) 2S albumins, named D1, was pooled, dialyzed against water, freeze dried, submitted to cation-exchange CM Sepharose chromatography (column of 1.5 cm × 50 cm) equilibrated with 0.025 M phosphate buffer (pH 6.0), eluted in a step-wise manner using 0.1 and 0.2 M of NaCl and dialyzed against distilled water. The purification of Pf1 and Pf2 proteins was confirmed by C4 reserve-phase (RP) chromatography in HPLC as described by Agizzio et al. [15].

2.3. Gel electrophoresis

SDS–Tricine-gel electrophoresis was performed according to the method of Schagger and Von Jagow [16].

2.4. Inhibition of the glucose-stimulated acidification of the medium by S. cerevisiae yeast

The Sabouraud broth (100 mL) containing the *S. cerevisiae* culture was shaken for 16 h at 30 °C with good aeration. The cells (A_{660} = 0.2) were pelleted by centrifugation at 3000 × *g* (5 min, 4 °C), followed by two water-washing steps. The antifungal activity of the D1 fraction (containing Pf1 and Pf2 2S albumins) and purified Pf2 were determined by incubation of *S. cerevisiae* cells (1×10^7) with 5 mL of 10 mM Tris–HCl, pH 6.0. Fractions were added to the final concentrations of 100, 250 and 500 μg mL^{-1} for D1 and 100 and 250 μg mL^{-1} for Pf2. After an incubation time (20 min), a 0.5 M glucose solution was added to a final concentration of 0.1 M. pH measurements were taken at each subsequent minute for the next 30 min. Negative (instead of peptide fraction, BSA was added at 500 μg mL^{-1}) and positive (1000 U nistatin added) controls were run in order to evaluate the influence of peptides on H^+ extrusion by yeast cells.

The results of inhibition of the glucose-stimulated acidification are shown by average values and standard deviations of triplicate for each experiment.

2.5. SYTOX Green uptake assay

Plasma membrane permeabilization was measured by SYTOX Green uptake, as described previously by Thevisen et al. [17] with some modifications. *S. cerevisiae* cells were incubated in the presence of D1 fraction (containing Pf1 and Pf2 2S albumins) and purified Pf2, both at the concentration of 100 μg mL^{-1}. After 1 h of incubation, the cells were pelleted by centrifugation and washed in 10 mM Tris–HCl, pH 6.0. One hundred-microlitrealiquots of the suspension of yeast cells were incubated with 0.2 μM SYTOX Green in 24-well microplates for 30 min at 25 °C with periodic agitation. The cells were observed in a DIC microscope (Axiophoto Zeiss)

equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm). Negative (no peptide fraction added and addition of BSA at 100 μg mL^{-1}) and positive (1000 U nistatin added) controls were also run in order to evaluate the membrane permeabilization.

2.6. Scanning electron microscopy

For scanning electron microscopy, the yeast cells, grown for 60 h in Sabouraud broth in the presence or absence of 2S albumin fractions (D1 and Pf2, both at 100 μg mL^{-1}), were fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde, 2.0% formaldehyde in 0.1 M phosphate buffer, pH 7.3. Subsequently, the materials were rinsed three times with the above buffer, post-fixed for 30 min at room temperature with 1.0% osmium tetroxide diluted in the same buffer and rinsed with distilled water. After this procedure, the fungi were dehydrated in acetone, critical point dried in CO_2, covered with 20 nm gold and observed in a Zeiss 962 scanning electron microscope. Cell growth control without addition of peptides was also determined.

2.7. Transmission electron microscopy

For transmission electron microscopy, the yeast cells, grown for 60 h in Sabouraud broth in the presence or absence of 2S albumin fractions (D1 and Pf2, both at 100 μg mL^{-1}), were fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde (vol/vol) and 4% paraformaldehyde (vol/vol) in 50 mM cacodylate buffer (pH 7.2). After fixation, the materials were washed, post-fixed in 1% (wt/vol) osmium tetroxide in corresponding buffer for 1 h at room temperature. The samples were dehydrated in a graded acetone series (30%, 50%, 70%, 90% and 100% (vol/vol)) and embedded in Epon resin (Polybeded). Ultrathin sections (0.1 μm) 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.

3. Results and discussion

3.1. Purification of the Pf1 and Pf2 antifungal proteins

Pf1 and Pf2 antifungal proteins from passion fruit seeds were purified using a combination of anion (DEAE) and cation (CM) exchange chromatography. The proteins precipitated with ammonium sulphate (F/0–70) were initially separated by means of anion exchange chromatography in DEAE Sepharose. The basic fraction, containing both protein Pf1 and Pf2, was named D1 and was further fractionated by cation exchange chromatography. Pf1 and Pf2, were eluted separately in 0.1 and 0.2 M NaCl, respectively as described by Agizzio et al. [15] (data not show). The purification was confirmed by HPLC in C4 phase-reverse column (data not shown). The analysis of the proteins Pf1 and Pf2 showed the same profile of Pf1 and Pf2

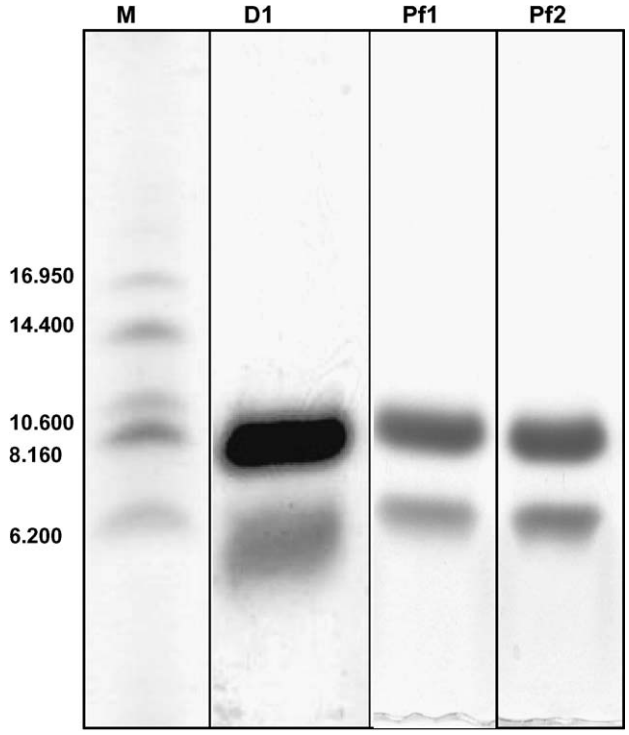

Fig. 1. SDS–Tricine-gel electrophoresis of purified 2S albumins from passion fruit seeds: D1 fraction (containing Pf1 and Pf2 proteins) from anion-exchange chromatography eluted with 1 M NaCl; Pf1 protein from cation-exchange chromatography eluted with 0.1 M NaCl; Pf2 fraction from cation-exchange chromatography eluted with 0.2 M NaCl. Side numbers in lane (M) refer to molecular mass (Da) markers.

obtained by Agizzio et al. [15] (Fig. 1). Pf1 and Pf2 obtained from cation-exchange chromatography, showed the presence of two major bands in SDS–Tricine-gel electrophoresis after treatment with β-mercapto ethanol. These bands represent the light and heavy chains with apparent molecular weights of 3,500 and 8,000 Daltons, respectively (Fig. 1). The analysis of the proteins of the D1 fraction showed the presence of both Pf1 and Pf2 proteins in this fraction, also with apparent molecular weights of 3,500 and 8,000 Daltons (Fig. 1). The values for the subunits lie in the range of molecular masses found by several authors for these proteins isolated from other plants, but differ from those of the 2S albumins, named CW-1 and CW-4, which are composed of two different subunits of 5,000 and 3,000 Da [13,14].

3.2. Inhibition of the glucose-stimulated acidification of the medium and plasma membrane permeabilization

Our group recently purified two proteins from passion fruit seeds, referred to as Pf1 and Pf2. The S2 fraction, containing both proteins, exhibits a considerable antifungal activity against several fungi including, C*olletotrichum lindemuthianum*, *C. musae*, *Fusarium oxysporum* and *S. cerevisiae* and induced various hyphal morphological alterations in these fungi [15]. In this same study, the fraction containing both proteins also inhibited the glucose-stimulated acidification of the medium by *F. oxysporum* by up to 40%, furthermore this inhibition

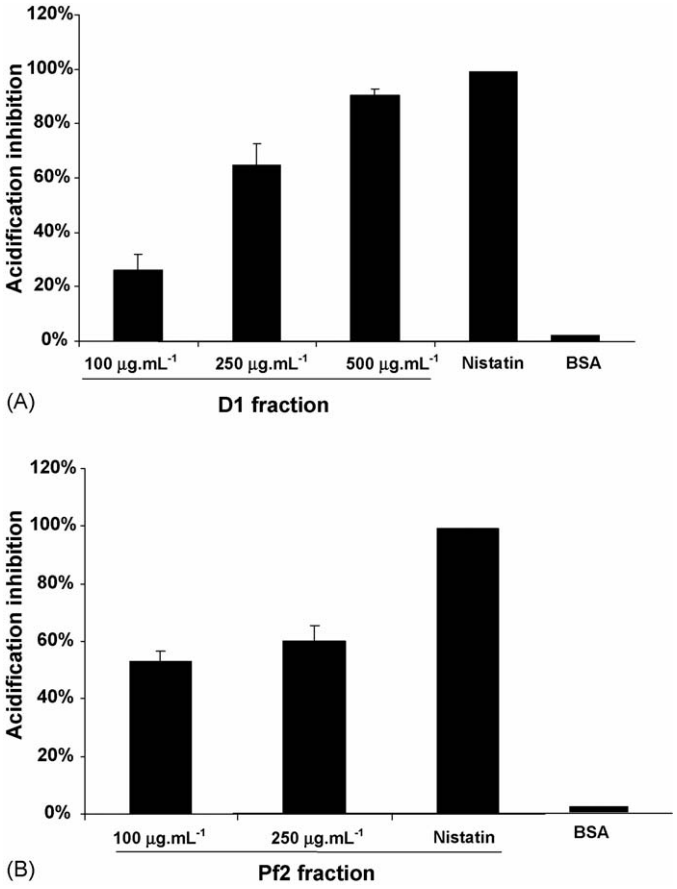

Fig. 2. The effect of D1 fraction (containing Pf1 and Pf2 protein) (A) and purified Pf2 protein (B) on the glucose-dependent acidification of the medium by *Saccharomyces cerevisiae* cells. Glucose was added after 20 min of cell incubation with the 2S albumin fractions.

correlated well with the inhibition of fungal growth. In this study, we investigated whether the D1 fraction (containing Pf1 and Pf2 proteins) and purified Pf2 could interfere with fungal H^+-ATPase. For this, we monitored the glucose-stimulated acidification, a phenomenon dependent on the activity of H^+-ATPase, of the incubation medium by *S. cerevisiae* cells in the presence of various concentrations of D1 fraction and Pf2 protein. As shown in Fig. 2A, D1 fraction, at the concentrations of 100, 250, and 500 $\mu g\ mL^{-1}$, was able to inhibit this acidification by 27%, 62% and 85%, respectively. Pf2 protein was also able to inhibit the acidification by 50% and 60%, at the concentrations of 100, and 250 $\mu g\ mL^{-1}$ respectively (Fig. 2B).

The ability of the D1 fraction and Pf2 protein to permeabilize the plasma membrane of *S. cerevisiae* cells was also examined. SYTOX Green permeabilization was assessed after 60 h of growth in the presence of proteins (D1 fraction and Pf2) and 30 min after the addition of SYTOX Green. When observed with a fluorescence microscope, *S. cerevisiae* cells showed strong SYTOX Green fluorescence either in the presence of D1 (Fig. 3A and B) fraction, Pf2 protein (Fig. 3C and D) and positive control nistatin (Fig. 3E and F), as compared to negative controls where cells were grown without peptides (Fig. 3G and H) and in the presence of BSA (Fig. 3I and J). Therefore, it is possible that the inhibition of the glucose-stimulated acidification of the incubation medium by *S. cerevisiae* cells, observed after the addition of 2S albumin-containing fractions is a consequence of plasma membrane permeabilization, which leads to the dissipation of the H^+ gradient across the membrane, thus impairing the function of H^+-ATPase. Other different plant proteins have also been found to permeabilize membranes and to modulate ion flux across membranes and have since become popular models for understanding how ion channel proteins function [18–21].

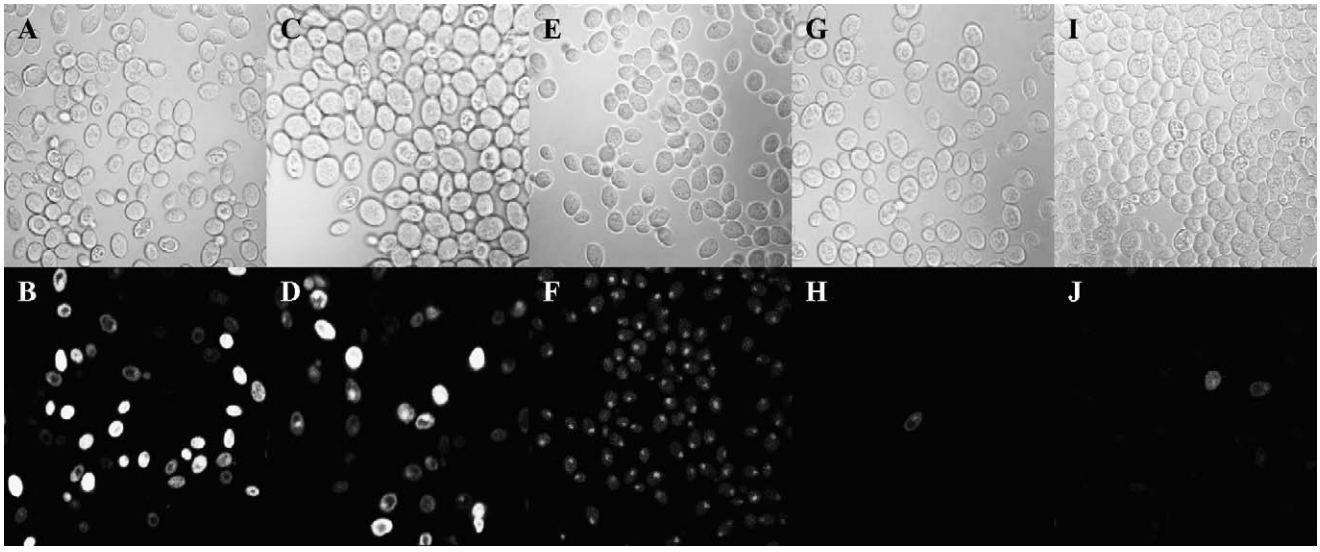

Fig. 3. Fluorescence microscopy of *S. cerevisiae* cells treated with SYTOX Green. (A) yeast cells treated with proteins from D1 fraction (100 $\mu g\ mL^{-1}$) viewed by DIC; (B) yeast cells treated with proteins from D1 fraction viewed by fluorescence. (C) yeast cells treated with proteins from Pf2 protein (100 $\mu g\ mL^{-1}$) viewed by DIC; (D) Yeast cells treated with proteins from Pf2 protein viewed by fluorescence. (E) positive control treated with nistatin viewed by DIC; (F) positive control treated with nistatin (1000 U) viewed by fluorescence. (G) control cells (without protein addition) viewed by DIC; (H) control cells (without protein addition) viewed by fluorescence. (I) negative control treated with BSA (100 $\mu g\ mL^{-1}$) viewed by DIC and (J) negative control treated with BSA viewed by fluorescence.

Thevissen et al. [17], for example demonstrated that when the fungi *Neurospora crassa* and *Fusarium culmorum* were treated with the plant defensins, *Rs*-AFP_2 and *Dm*-AMP_1, an ion flux across the fungal plasma membrane was also observed. The antifungal activity of the osmotin has been also suspected to involve specific target component(s) on the fungal plasma membrane. Osmotin was also able to inhibit, in vitro, the growth of a number of unrelated pathogens. Yun et al. [22] showed that osmotin either induced spore lysis, inhibited spore germination or reduced germling viability in several fungal species that exhibited some degree of sensitivity in hyphal growth inhibition tests. The species-specific growth inhibition was correlated with the ability of osmotin to dissipate the fungal membrane pH gradient. Genetic experiments have also shown that the susceptibility of microbes to osmotin and also antimicrobial peptides is regulated by proteins that control the composition of the cell wall [22–24].

At present, the information on the mechanism of action of antifungal proteins, especially 2S albumins, is very limited. Scanning electron microscopy and the measurement K^+ leakage from fungal hyphae demonstrate that 2S albumins probably have a similar mode of action comparable to that of thionins, which also cause permeabilization of the hyphal plasma membrane for different ions [11]. In addition, another group of antimicrobial proteins, namely vicilins (7S storage proteins), have been shown to inhibit glucose-dependent acidification by yeast cells and interfere with the germination of spores or conidia of phytopathogenic fungi [25,26]. It has been recently suggested that the 2S albumin-related antifungal proteins might have a broader spectrum against potential pathogens and exist in different biochemical forms.

3.3. Ultrastructural alterations in yeast cells induced by 2S albumin-homologous proteins

Further tests to evaluate medium acidification and plasma membrane permeabilization were analyzed through scanning electron microscopy to verify possible alterations in yeast morphology, caused by D1 fraction and Pf2 protein. Photomicrographs of *S. cerevisiae* were taken after 60 h of yeast growth in the presence of 2S albumin fractions. Normal growth development was observed for control cells (Fig. 4A). *S. cerevisae* cultures treated with both D1 and Pf2 fraction exhibited notable growth inhibition as well as several morphological alterations, especially at the cell surface and in the cell wall. Alterations in the bud formation were also observed (Fig. 4B and C). At the used concentration (100 μg mL^{-1}), the treated cells lost their symmetrical appearance, they were completely deformed, clumped together and deep wrinkles were observed in the cells (Fig. 4B and C). Normal growth and appearance of the cells was observed in the control (Fig. 4A). TEM observations of *S. cerevisiae* cells showed normal ultrastructure development in control cells (Fig. 5A), however cells treated with D1 fraction (Fig. 5B–F) and Pf2 protein (Fig. 6A–C) exhibited cells with disappearing and shrinking cytosol, inclusions in the organization of the nucleus and other organelles, plasma membrane blebbing,

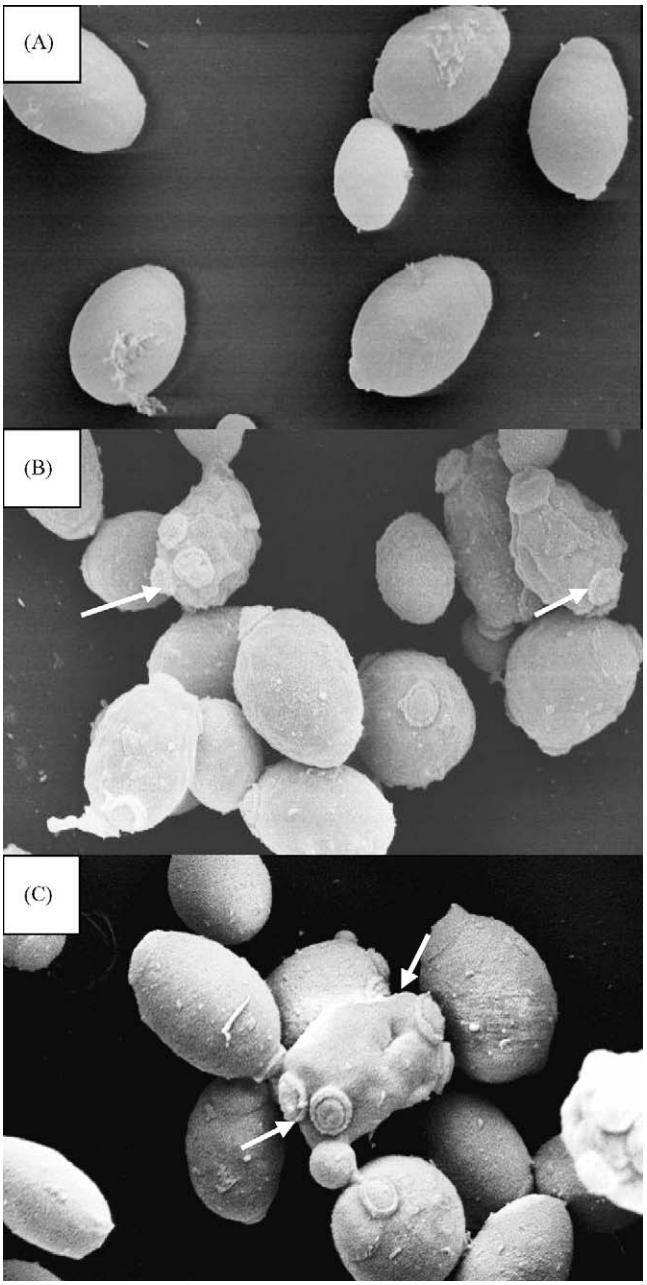

Fig. 4. Scanning electron microscopy of *S. cerevisiae* cells in the presence of 2S albumins from passion fruit seeds. (A) control (absence of 2S albumins); (B) presence of D1 fraction (100 μg mL^{-1}); (C) presence of Pf2 protein (100 μg mL^{-1}). Bars: (A) 5 μm; (B) 7 μm; (C) 25 μm. Arrows indicate the buds with malformation.

increased vacuolation and cell wall deformed. Not all of these features were observed in all of the cells, but they occurred quite frequently; these characteristics are very similar to those observed in cells undergoing apoptosis. Apoptosis or programmed cell death (PCD) is a distinct process in which a cell activates an intrinsic suicide program resulting in sequential changes that lead to cell death with the packaging of cellular constituents into membrane-bound pieces [27]. PR-5 proteins, like the antimicrobial peptides, have a predominant disulfide-stabilized antiparallel sheet structure and can cause membrane leakage in target microorganisms and artificial membranes

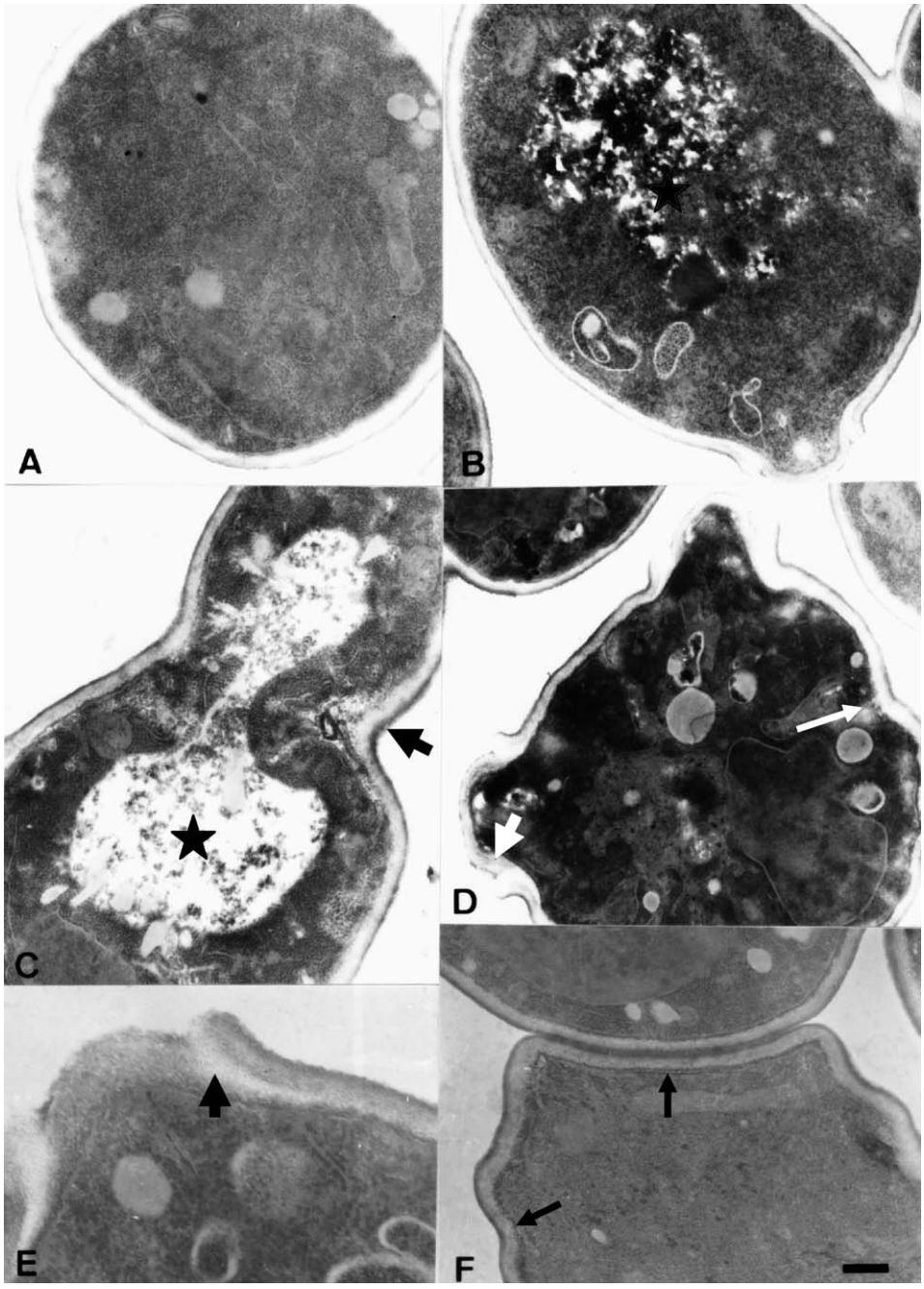

Fig. 5. Transmission electron microscopy of *S. cerevisiae* cells in the presence of 2S albumins from passion fruit seeds. (A) control (absence of 2S albumins); (B–F) presence of D1 fraction (100 μg mL^{-1}). Star indicates condensation and shrinkage of cytosol with increased vacuolation and loss of cytosol structure and contents; small arrows indicate cell and plasma membrane blebbing; large arrows indicate malformation of buds and imperfect cell wall. Bars: (A–D, F) 0.4 μm; (E) 0.15 μm.

[28,29]. It has been demonstrated that osmotin not only induces rapid cell death in yeast, but also there is specificity between the antifungal activity of a particular PR-5-family protein and the plasma membrane of its target cell [4,22,28]. It has also been demonstrated that cell wall components are important determinants of osmotin resistance and that differential resistance among yeast strains with plasma membranes targeted by osmotin is determined by variations in the architecture of the cell wall. Among the resistance determinants previously identified is a family of yeast cell wall localized proteins, collectively called PIR proteins [22,28]. In addition, the induction of apoptosis correlated with the intracellular accumulation of reactive oxygen species and was mediated by RAS2, but not RAS1 [7,30]. Interestingly results obtained by Crimi et al. [31] have also showed the effect of a plant lipid transfer protein (LTP) to initiate the apoptotic cascade at the mitochondrial level. The results in this work also are discussed in light of the possible role of lipid exchange in mammalian apoptosis.

In conclusion, our results indicate that the ability of antimicrobial plant proteins, such as 2S albumins, to induce microbial inhibition could be an important factor in determining

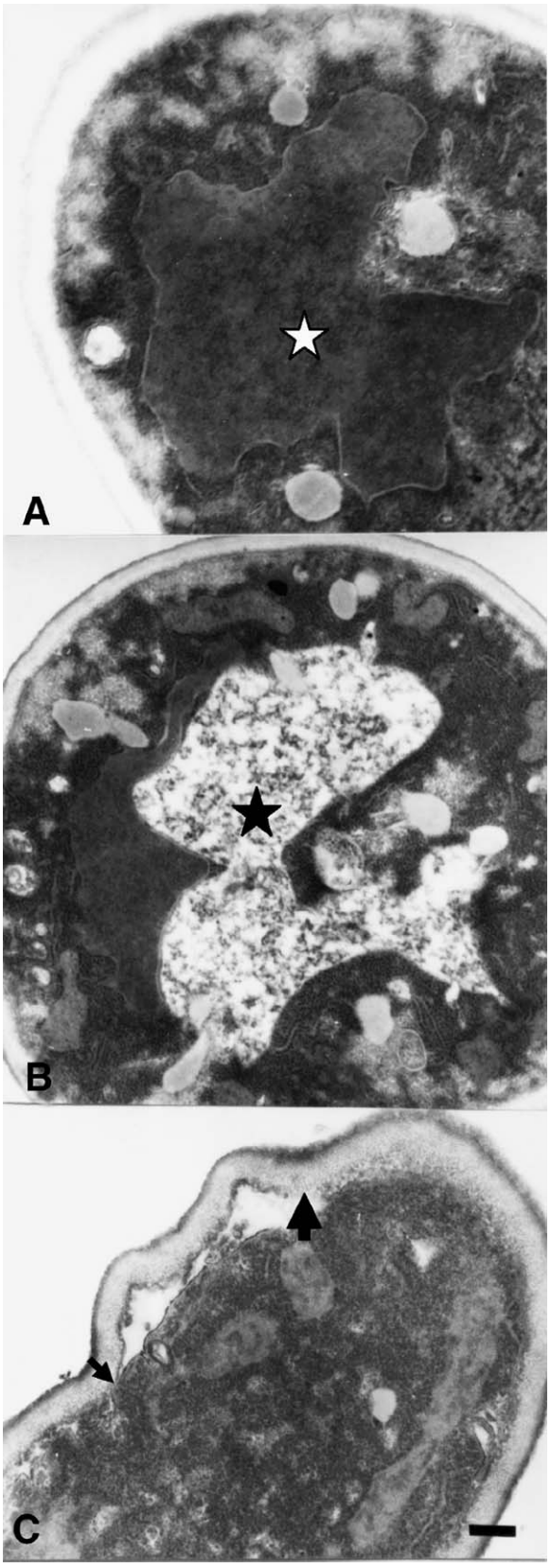

Fig. 6. Transmission electron microscopy of *S. cerevisiae* cells in the presence of 2S albumins from passion fruit seeds. (A–C) presence of Pf2 protein (100 μg mL^{-1}). Star indicates condensation and shrinkage of cytosol with increased vacuolation and loss of cytosol structure and contents; small arrows indicate cell and plasma membrane blebbing and imperfect cell wall; large arrows indicate malformation of buds. Bars: (A–C) 0.35 μm.

a pathogen's virulence and may, therefore, be targets for the design of new antifungal drugs.

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