Molecular Characterization of Propolis-Induced Cell Death in Saccharomyces cerevisiae

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Propolis, a natural product of plant resins, is used by the bees to seal holes in their honeycombs and protect the hive entrance. However, propolis has also been used in folk medicine for centuries. Here, we apply the power of Saccharomyces cerevisiae as a model organism for studies of genetics, cell biology, and genomics to determine how propolis affects fungi at the cellular level. Propolis is able to induce an apoptosis cell death response. However, increased exposure to propolis provides a corresponding increase in the necrosis response. We showed that cytochrome c but not endonuclease G (Nuc1p) is involved in propolis-mediated cell death in S. cerevisiae. We also observed that the metacaspase YCA1 gene is important for propolis-mediated cell death. To elucidate the gene functions that may be required for propolis sensitivity in eukaryotes, the full collection of about 4,800 haploid S. cerevisiae deletion strains was screened for propolis sensitivity. We were able to identify 138 deletion strains that have different degrees of propolis sensitivity compared to the corresponding wild-type strains. Systems biology revealed enrichment for genes involved in the mitochondrial electron transport chain, vacuolar acidification, negative regulation of transcription from RNA polymerase II promoter, regulation of macroautophagy associated with protein targeting to vacuoles, and cellular response to starvation. Validation studies indicated that propolis sensitivity is dependent on the mitochondrial function and that vacuolar acidification and autophagy are important for yeast cell death caused by propolis.
the cellular level. First, we evaluate how propolis can affect S. cerevisiae cell survival by assessing several genetic determinants involved in apoptosis and/or necrosis in this organism. As a complementary step, we have used the yeast nonessential gene deletion library to investigate possible cell targets for propolis.

MATERIALS AND METHODS

Strains, media, and culture methods. The following S. cerevisiae strains were used: BY4742 (MATa his3Δ1 leu2Δ2 lys2Δ2 ura3Δ0) (10), BY4741A and BY4742 rho0-5 (both MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [rho0-5] (this study)), YJL208C/NUI (MATa his3Δ1 leu2Δ0 met15Δ200 ura3Δ0 [rho0-5]) (Invitrogen), YJR048W/CYC1 (MATa his3Δ1 leu2Δ0 met15Δ200 ura3Δ0 GFP-cyc1) (Invitrogen), and ΔCYC1 (MATa his3Δ1 leu2Δ0 met15Δ200 yca1Δ lanCMV4) (49). The following media were used: the complete medium YPD agar (2% [wt/vol] Bacto yeast extract, 2% [wt/vol] peptone, and 2% [wt/vol] agar), minimal medium SC agar (0.7% [wt/vol] yeast nitrogen base [DIFCO], 2% [wt/vol] glucose, 0.1g/liter L-leucine, 0.1g/liter L-tryptophan, 0.05g/liter L-histidine, 2% [wt/vol] agar), and YPD, YPGal, and SC liquid media with the same composition as above but without agar. Rosella strains were constructed by the transformation of c-Rosella plasmid (67) into the BY4742 strain. Transformants were selected in SC agar minimal medium.

Preparation of the propolis standardized extract. Three batches of propolis standardized extract (PSE), 14004/10, 14401/10, and 010/08, were produced by Apis Flora Company (PEPF-AF; Ribeirão Preto, SP, Brazil). The extracts were standardized using a propolis blend composed of raw material obtained from several sites of Brazil (A. A. Beretta-Silva, A. C. Meda, and M. E. T. Ferreira, 1 February 2005, Brazilian patent application PI 0405483), as the states of Minas Gerais, São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul. Propolis powder in a blender, and its particle size was standardized using a 42-mesh sieve. It was then extracted using hydroalcoholic solution (7:3), with dynamic maceration followed by a percolation process and finally by filtration. The PSE obtained represents 11% [wt/vol] of dry residue and chemical composition standardized qualitatively and quantitatively by reverse-phase high-pressure liquid chromatography (RP-HPLC) by analyzing the following compounds: caffeic, p-coumaric, and cinnamic acids; isosakuranetin; and artepilllin C (ARC) (see Table S1 in the supplemental material).

Screening of the yeast deletion library. For the screening of the yeast deletion library (84; http://www-sequence.stanford.edu/group/yeast_deletion_project) a set of approximately 4,800 haploid deletions in nonessential genes of S. cerevisiae were screened for propolis sensitivity. Mutants were inoculated from stock cultures in 96-well master plates at −80°C and were grown at 30°C in YPD medium containing 200 g/ml geneticin (G418; Sigma, St. Louis, MO) and stored at −80°C. The yeast cells were grown in 50 ml of fresh YPD medium for Rosella containing 0.125% (vol/vol) alcoholic propolis extract; the treatments were carried out for 15, 10, and 90 min, respectively, at 30°C with mechanical shaking (200 rpm). A positive control for Rosella staining was performed by growing cells for 16 h at 30°C in YPD medium and transferring them to starvation medium (0.17% [wt/vol] yeast extract, 0.1% [wt/vol] KH2PO4, 0.01% [wt/vol] CaCl2, 0.005% [wt/vol] FeC12, 0.07% [wt/vol] MgCl2, 0.05% [wt/vol] NaCl, 2% [wt/vol] glucose) to induce autophagy. After 4 h of incubation at 30°C, protophore carbonyl cyanide m-chlorophenylhydrazone (CCCP, at 10 μM) was added in pH 7.5 buffer for 10 min. Incubation of starved cells before imaging in pH 7.5 buffer containing the protophore CCCP resulted in an increase in green fluorescence emission in the vacuole (67). For positive control to apoptosis in the NUC1 microfluid, we used 0.4 mM H2O2 for 2 h (13). Cells were washed with phosphate-buffered saline (PBS) and incubated for 5 min in a solution supplemented with 100 ng/ml of 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO). After incubation, cells were washed with PBS buffer for 10 min at room temperature, and then the slides were mounted. Slides were viewed with a Carl Zeiss (Jena, Germany) microscope using a 100× magnification oil immersion objective lens (EC Plan-Neofluar; numerical aperture, 1.3). In order to verify the accumulation of reactive oxygen species (ROS), cells were stained with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes), and to visualize the DNA strand breaks were demonstrated by TUNEL with an In situ Cell Death Detection Kit, Fluorescin, from Roche. This procedure was according to Madco et al. and Ribeiro et al. (47, 65) with some modifications. Yeast cells were fixed with 3.7% (vol/vol) formaldehyde for 30 min at room temperature and washed three times with PBS, and cell walls were digested with 15 U/ml lyticase, from Arthrobacter luteus (crude 100,000 U; 362 units/mg: Sigma) at 37°C for 60 min. Ten microliters of the cell suspensions was applied to a microscope slide and allowed to dry for 30 min at 37°C. The slides were rinsed with PBS, incubated in permeabilization solution (0.1% [vol/vol] Triton X-100 and 0.1% [wt/vol] sodium citrate) for 2 min on ice, and rinsed twice with PBS.
a positive control the endonuclease DNase I (Sigma) was applied to the cells on microscope slides. The slides were placed in a humidified box for 1 h at 37°C and then washed twice in PBS. Slides were subsequently incubated with 10 μl of TUNEL reaction mixture, containing terminal deoxynucleotidyltransferase and fluorescein isothiocyanate (FITC)-dUTP, for 60 min at 37°C. They were rinsed three times with PBS and incubated for 5 min in a solution supplemented with 100 ng/ml of DAPI (Sigma Chemical, St. Louis, MO). After incubation with the dye, they were washed with PBS buffer for 10 min at room temperature and then rinsed in distilled water, mounted, and visualized in a confocal microscope.

**Annexin and PI assays.** Phosphatidylserine exposure was detected by an annexin-V-Fluos staining kit (Roche), as described by Madeo et al. (48) with some modifications. Cells were harvested and washed with sorbitol buffer (1.2 M sorbitol, 0.5 mM MgCl₂, 35 mM KH₂PO₄, pH 6.8). Cell walls were digested with 15 U of lyticase (Sigma) in sorbitol buffer for about 30 min at 37°C. Cells were then washed twice with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) containing 1.2 M sorbitol (binding-sorbitol buffer). To 38-μl cell suspensions in binding-sorbitol buffer, 2 μl of annexin V (Roche) and 2 μl of a propidium iodide (PI) working solution (50 μg/ml) were added, and the mixture was incubated for 15 min at room temperature. The cells were then washed and resuspended in binding-sorbitol buffer. Finally, the slides were mounted with the cell suspensions. Microscope settings and image acquisitions were as described above except fluorescein microscopy. For quantitative assessment of annexin V-PI staining, at least 100 yeast cells were counted per sample.

**Negative regulation of transcription from RNA polymerase II promoter by glucose**

| Biological process                  | GO identification no. | P value | Corrected P value | k<sub>c</sub> | f<sub>d</sub> |
|------------------------------------|------------------------|---------|-------------------|--------------|--------------|
| Cell division                      | 51301                  | 2.95 × 10⁻⁴ | 2.56 × 10⁻¹⁴       | 36           | 323          |
| Cell budding                       | 7114                   | 1.31 × 10⁻¹³| 5.68 × 10⁻¹⁴       | 19           | 82           |
| Asexual reproduction               | 19954                  | 4.16 × 10⁻¹³| 1.20 × 10⁻¹⁰       | 19           | 87           |
| Reproduction of a single-celled organism | 32505              | 6.45 × 10⁻¹³| 1.40 × 10⁻¹⁰       | 19           | 89           |
| Establishment of cell polarity     | 30468                  | 1.23 × 10⁻¹²| 1.67 × 10⁻¹⁰       | 20           | 104          |
| Mitochondrial electron transport, ubiquinol to cytochrome<sup>c</sup> | 6122 | 2.79 × 10⁻¹¹ | 2.11 × 10⁻⁹        | 8            | 11           |
| Cytokinesis                        | 16288                  | 2.91 × 10⁻¹¹| 2.11 × 10⁻⁹        | 19           | 109          |
| Cell cycle                         | 7049                   | 2.13 × 10⁻⁷  | 1.05 × 10⁻⁷        | 40           | 566          |
| Electron transport chain           | 22900                  | 8.55 × 10⁻⁹  | 1.61 × 10⁻⁵        | 8            | 31           |
| pH reduction                       | 45585                  | 2.63 × 10⁻⁵  | 3.08 × 10⁻⁴        | 6            | 24           |
| Vacuolar acidification             | 7035                   | 2.63 × 10⁻⁵  | 3.08 × 10⁻⁴        | 6            | 24           |
| Negative regulation of transcription from RNA polymerase II promoter | 433 | 3.30 × 10⁻⁴ | 2.63 × 10⁻³        | 3            | 6            |

<sup>a</sup> P values were calculated by the hypergeometric distribution of one ontology class visualized in the network.
<sup>b</sup> Calculated values based on P values obtained after FDR was applied.
<sup>c</sup> Total number of proteins found in the network which belong to a gene ontology.
<sup>d</sup> Total number of proteins that belong to a specific gene ontology.
are propidium iodide (PI) positive (PI
growth, respectively, by observing the number of cells which
in the exponential and stationary phases, i.e., 9 and 16 h of
are much more sensitive (Fig. 1B). In the next step, we inves-
while cells in the exponential phase (9 h; 2.0
C. albicans
was more sus-
ceptible to concentrations of propolis between 0.50 and 0.75%
the spectrophotometer determinations (data not shown). The
viability was affected. Since propolis is dissolved in ethanol
working propolis concentration and determining how yeast
dead effects of propolis could be due to a combination of chemical
and concentrations, we decided to investigate the cell
dead effects of propolis by concentrating our experiments
on alcoholic extracts of propolis.

The first series of experiments was related to verifying a
working propolis concentration and determining how yeast
viability was affected. Since propolis is dissolved in ethanol
(60% alcoholic extract), the control treatment was grown on
2.73% ethanol because 0.5% propolis has a final concentration
of about 2.73% ethanol (Fig. 1A). Tenfold dilutions of S.
cerevisiae
cells suggested that 0.125% propolis could be an adequate
choice as a subinhibitory concentration (Fig. 1A). All the
controls for further experiments using 0.125% propolis as a
treatment have 0.68% ethanol (what corresponds to 60%
alkoholic extract for 0.125% propolis). We were not able to
perform the determination of MICs by using microdilutions
because the color of the propolis is dark, which interferes in
the spectrophotometer determinations (data not shown). The
inhibitory effects of propolis on S.
cerevisiae
are not affected by
growth on lower oxygen concentrations, such as 1% O2 (data
not shown). We also observed that C. albicans
was more sus-
ceptible to concentrations of propolis between 0.50 and 0.75%
(Fig. 1A). However, C. parapsilosis and Aspergillus fumigatus
growth was not affected by the same concentrations of propolis
(data not shown). Yeast cells in the initial mid-exponential
(6 h; 1.2 \times 10^6 cells ml^-1) and stationary (16 h; 3.0 \times 10^6 cells
ml^-1) phases are more resistant to 0.125% propolis extracts
while cells in the exponential phase (9 h; 2.0 \times 10^7 cells
ml^-1) are much more sensitive (Fig. 1B). In the next step, we inves-
tigated what was the main mechanism of cell death in the cells
in the exponential and stationary phases, i.e., 9 and 16 h of
growth, respectively, by observing the number of cells which
are propidium iodide (PI) positive (PI^+ ) or annexin and
TUNEL positive (preliminary indications of necrosis or apop-
tosis, respectively).

Early apoptosis is characterized by an increased number of
annexin V-positive (A^- ) cells and PI negative (PI^- ) cells while
in late apoptosis (leading to secondary necrosis), there is an
increase in the number of A^- and PI^- cells. The primary
necrosis is characterized by an increased number of annexin
V-negative (A^- ) cells and PI^- cells. We have observed about
15% A^- and PI^- cells in the negative control (0.68% ethanol
[EtOH]) while 100% A^- and PI^- cells in the positive control
(acetic acid, pH 3.0, for 200 min). Upon 5 min of exposure to
0.125% propolis, cells grown for 9 and 16 h have an increased
number of A^- (about 30% both A^- and PI^- and 80% A^-),
suggesting early apoptosis (Fig. 1B to D). Upon 10 min of
exposure to 0.125% propolis, cells grown for 9 and 16 h have
an increased number of A^- and PI^- (about 80% for both
classes), suggesting late apoptosis, leading to secondary necro-
sis (Fig. 1B and C). However, the TUNEL-positive staining
remained constant for both 5 and 10 min of exposure to
0.125% propolis (about 80%) (see Fig. S3 in the supplemental
material).

There is an accumulation of mitochondrion-produced reactive
dioxide species (ROS) during yeast apoptosis (for reviews, see references 19, 58, and 62). To investigate the production of
ROS during propolis-induced cell death in S.
cerevisiae,
we used 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), a
cell-permeable ROS indicator that is nonfluorescent until acetate groups are removed (H2DCF) by intracellular esterases and oxidation occurs within the cell. Although H2DCF
can be oxidized by different ROS, it is mainly used to detect
superoxides. Yeast cells grown for 9 or 16 h were either un-
treated or exposed to 0.125% propolis for 5 and 10 min, incub-
ated with H2DCFDA, and examined by fluorescence micros-
copy (Fig. 2). The DCFDA-dependent green fluorescence was
detected in about 10% of the 9- or 16 h-grown cells not ex-
posed to propolis. Treatment of 9-h-grown cells with 0.125%
propolis for 5 and 10 min yielded 70 to 80% fluorescence while
treatment of 16-h-grown cells treated for 5 and 10 min pro-
duced 40 to 60% fluorescence (Fig. 2). These results indicate
that there is a great accumulation of ROS during cell death
induced by propolis.

Caspases are members of a family known as cysteine pro-
teases that are actively involved in cell death in eukaryotes (for
a review, see reference 41). Although S.
cerevisiae
does not have caspases in its genome, a caspase-like protein named
YCA1, fitting into the type I category of metacaspases, was
identified and characterized previously (49, 54, 81). It has been
shown that YCA1 is involved in several cell death stimuli (for
a review, see reference 54). When yeast cells grown for 16 h
were exposed to 0.125% propolis for 5, 10, and 20 min, there

RESULTS

Propolis induces cell death in S. cerevisiae. Propolis is a
complex product derived from plant resins and bee saliva.
There are several chemical compounds present in this natural
product that could potentially be responsible for its antibiotic
properties. As a first step to understand whether individual
components of propolis could play such role, we fractionated
propolis extracts by HPLC and purified some of the most
abundant chemical compounds (see Fig. S1 and Table S1 in the
supplemental material). We have obtained similar fingerprints
for three different batches of propolis (see Fig. S2). We tested
S. cerevisiae
growth in the presence of four of these chemical
compounds (caffeic, p-coumaric, and cyaninic acids and iso-
sakuranetin). We applied 1, 2, 5, 10, 25, 50, and 100 μg/ml of
each compound and a mix of 100 μg/ml of each in microtiter
plate liquid cultures. None of these treatments was able to
inhibit the growth of S. cerevisiae
BY4742,
Candida albicans
CAI4, or Candida parapsilosis ATCC 2201 (data not shown).
Thus, taking into consideration the fact that the cell death
effects of propolis could be due to a combination of chemical
compounds and concentrations, we decided to investigate the
cell death effects of propolis by concentrating our experiments
on alcoholic extracts of propolis.

To perform the determination of MICs by using microdilutions
about 2.73% ethanol (Fig. 1A). Tenfold dilutions of S.
cerevisiae
cells suggested that 0.125% propolis could be an adequate
choice as a subinhibitory concentration (Fig. 1A). All the
controls for further experiments using 0.125% propolis as a

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because the color of the propolis is dark, which interferes in
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are not affected by
growth on lower oxygen concentrations, such as 1% O2 (data
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while cells in the exponential phase (9 h; 2.0 \times 10^7 cells
ml^-1) are much more sensitive (Fig. 1B). In the next step, we inves-
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in the exponential and stationary phases, i.e., 9 and 16 h of
growth, respectively, by observing the number of cells which
are propidium iodide (PI) positive (PI^+ ) or annexin and
TUNEL positive (preliminary indications of necrosis or apop-
tosis, respectively).

Early apoptosis is characterized by an increased number of
annexin V-positive (A^- ) cells and PI negative (PI^- ) cells while

was a reduction in the cell viability of 40, 70, and 95% in the wild-type strain while in the YCA1/H9004 strain, this reduction was about 10, 50, and 80% (Fig. 3). These results strongly indicate that the YCA1 gene is important for propolis-mediated cell death.

Cytochrome c is released during propolis-induced cell death. We have also investigated if other components of the cell death machinery are being activated during propolis-induced cell death in S. cerevisiae. We concentrated our attention on two of these putative determinants: CYC1 and NUC1.
In mammalian cells, at least two major apoptotic pathways have been described: (i) the intrinsic pathway that needs the involvement of the mitochondria, and (ii) the extrinsic pathway where mitochondria are bypassed and caspases are activated (for a review, see reference 21). One of the hallmarks of the intrinsic pathway is the release of apoptogenic factors such as cytochrome c to the cytosol and the consequent assembly organized by this protein of the high-molecular-weight complex, the mitochondrial apoptosome, that activates caspases (for a review, see reference 21). In *S. cerevisiae* cells undergoing an apoptotic process induced by acetic acid, translocation of cytochrome c to the cytosol was observed (45). We performed cell fractionation studies to determine if cytochrome c was translocated to the cytoplasm during exposure of yeast cells grown for 16-h (16-h yeast cells) to 0.125% propolis for 10 min (Fig. 4). When the yeast cells were not exposed to propolis, all the cytochrome c was located in the mitochondrial protein extracts while upon propolis addition, there was a significant presence of cytochrome c in the cytoplasm protein extracts (Fig. 4, right panel). There are corresponding protein amounts in each cell fraction extract, as shown by Coomassie staining (Fig. 4, left panel).

Endonuclease G (EndoG) has been described as a mitochondrial endonuclease that digests both DNA and RNA (12, 13, 61). Upon apoptosis induction, translocation of mammalian EndoG and its *S. cerevisiae* homologue NUC1 to the nucleus coincides with large-scale DNA fragmentation (12, 13, 41, 61). We have not observed increased sensitivity of NUC1Δ yeast cells to propolis (data not shown). Next, we examined if Nuc1p would translocate to the nucleus when yeast cells were exposed to propolis (Fig. 5). When 16-h yeast cells were not exposed to propolis, the Nuc1p-GFP showed a diffuse mitochondrial filament-like distribution, as confirmed by MitoTracker staining (Fig. 5, first row). In yeast cells exposed to hydrogen peroxide, part of the Nuc1p-GFP was translocated to the nucleus (DAPI staining), as previously shown by Büttner et al. (13), but it also accumulated in the mitochondria as indicated by MitoTracker staining (Fig. 5, first row). In yeast cells exposed to hydrogen peroxide, part of the Nuc1p-GFP was translocated to the nucleus (DAPI staining), as previously shown by Büttner et al. (13), but it also accumulated in the mitochondria as indicated by MitoTracker staining (Fig. 5, second row). In contrast, when yeast cells were exposed to 0.125% propolis for 15 min, about 100% of the yeast cells showed a Nuc1p-GFP dot-like distribution, suggesting mitochondrial fragmentation, as again confirmed by MitoTracker staining (Fig. 5, third row). Moreover, the Nuc1p-GFP did not translocate to the nuclei, as shown by DAPI staining (Fig. 5, third row). These results strongly indicate that cytochrome c, but not Nuc1p, is involved in propolis-mediated cell death in *S. cerevisiae*.
High-throughput screen of the yeast deletion library for propolis sensitivity. To elucidate the gene functions that may be required for propolis sensitivity in eukaryotes, the full collection of haploid *S. cerevisiae* deletion strains was screened for growth on YPD plates supplemented with 2.73% ethanol (as a control; 2.73% is the ethanol concentration used to dissolve 0.500% propolis) and 0.250 and 0.500% propolis for 5 days at 37°C. About approximately 4,800 different strains were involved in this screening, and after three confirming screenings, we were able to identify 138 deletion strains that have different

FIG. 4. Exposure of yeast cells to propolis induces cytochrome c release into the cytoplasm. Yeast cells were grown for 16 h at 30°C and left untreated or exposed to 0.125% propolis for 10 min at 30°C. The cells were harvested and fractionated (mitochondria and cytoplasm) as described in the Materials and Methods section, and proteins were run on a polyacrylamide gel. At left is a Coomassie-stained gel with the same amount of proteins that were transferred to a nitrocellulose filter (right panel). This membrane was probed with the antibody rabbit anti-cytochrome c.

![Coomassie-stained gel and immunoblot](image1)

FIG. 5. Nuc1p is not involved in propolis sensitivity. Yeast Nuc1-GFP cells were grown for 16 h at 30°C and left untreated or exposed to 0.125% propolis for 15 min, and cells were visualized by a fluorescence microscope. A positive control was made by incubating 16-h-old yeast cells with 0.4 mM H$_2$O$_2$ for 20 h at 30°C. Bar, 5 μm. DIC, differential interference contrast. Arrows indicate the nuclei.

![Fluorescence images](image2)
degrees of propolis sensitivity compared to the corresponding wild-type strain (see Table S2 in the supplemental material). The survival data obtained from yeast deletion strains prompted us to ask how the absence of the determined proteins of propolis-sensitive yeast strains affects different biological processes that lead to cell death. In this sense, a search for potential proteins and/or mechanisms and their associated biological processes that are affected by propolis exposure was initiated. To achieve this goal, different PPPI networks using yeast deletion data were retrieved from the STRING database. Shared proteins and subnetworks present in the major PPPI network (Fig. 6) were identified and retrieved using the Cytoscape-associated plug-in MCODE and subjected to a Gene Ontology (GO) analysis in order to obtain information about the nature and number of subnetworks belonging to the network and their associated biological processes. Results obtained from MCODE and GO analysis showed that the final PPPI network (Fig. 6A) contains 553 nodes and 3,651 connectors and is composed of six heavily interconnected clusters, each comprising different biological processes. GO analyses revealed that these biological processes can be classified into the following categories: (i) translation/energy derivation by oxidation of organic compounds/mitochondrial genome maintenance, (ii) transcription/chromosome organization and biogenesis/G1 phase of mitotic cell cycle; cluster 3, carboxylic acid metabolic process/protein targeting to peroxisome/respiration metabolism; cluster 4, establishment and/or maintenance of chromatin architecture/histone deacetylation; cluster 5, RNA catabolic process; cluster 6, cell division mechanisms; and unclustered nodes.

The distribution analysis of the proteins whose deletion increases the sensitivity of yeast strains to propolis indicated that both cluster 6 and unclustered protein subnetworks contain 61.73% of the total proteins observed in the deletion assay (Fig. 6B; see also Table S3 in the supplemental material). The other 38.27% proteins were found distributed among clusters 1 to 5 (Fig. 6B; see also Table S3), showing that cluster 6 and the unclustered protein subnetworks appear to be important for the pleiotropic responses observed when yeast cells are submitted to propolis treatment. When the proteins of the cluster 6 subnetwork (Fig. 7; see also Table S3) were subjected to GO analysis, the data indicated that the major biological processes found within this subnetwork were associated with cell division and reproduction, which includes the establishment of cell polarity, cell cycle, and budding (Table 1). Moreover, the proteins of the cluster 6 subnetwork appear to be important to the mitochondrial electron transport chain, vacuolar acidification, and negative regulation of transcription from RNA polymerase II promoter (Table 1). Interestingly, the GO analysis of the unclustered protein subnetworks (Fig. 7B; see also Table S3) showed that these proteins are important for the regulation of macroautophagy associated with protein targeting to vacuoles and the cellular response to starvation, among other processes (Table 2).

In the next two sections, we show validation data about the role played by clusters related to the mitochondrial electron transport chain and vacuolar acidification.
Petite strains are less sensitive to propolis. It has recently been shown that during necrosis in *S. cerevisiae*, there is an inhibition of mitochondrial function with a corresponding reduction of ATP production (for a review, see reference 19). In this paper, there are several indications that propolis induces a secondary necrosis cell death mechanism in *S. cerevisiae*. In addition, as demonstrated in the previous section, we found that when several genes related to energy derivation by oxidation of organic compounds, mitochondrial genome maintenance, and the mitochondrial electron transport chain are deleted, there is an increase in *S. cerevisiae* propolis sensitivity. We decided to investigate a possible correlation between propolis sensitivity, mitochondria and *S. cerevisiae* necrotic cell death by isolating petite strains of *S. cerevisiae* and checking their sensitivity to propolis. In order to isolate BY4742 *[^rho^0]/[^rho^-]* cells, the respiratory-competent strain BY4742 (10) was mutagenized with ethidium bromide, and the resultant petite respiratory-deficient colonies were crossed to *[^rho^-]/[^rho^0]* tester strains (80) to confirm the nature of the isolated petite mutant respiratory incapacity. Two of these petite strains, BY4741A *[^rho^-]/[^rho^-]* and BY4742 *[^rho^-]/[^rho^-]*, and the corresponding wild-type strain were exposed to 0.125% propolis for 20 min, and cell viability was determined (Fig. 8). The petite strains showed dramatic increased viability compared to the wild-type strain (compare 95 and 75% viability of BY4741A *[^rho^-]/[^rho^-]* and BY4742 *[^rho^-]/[^rho^-]* strains, respectively, against about 10% viability in the wild-type strain) (Fig. 8A). Accordingly, when cell protein extracts were fractionated, cytochrome *c* could not be observed in cytoplasmic protein extracts of petite cells exposed to propolis but only in mitochondrial protein extracts (Fig. 8B). We measured the effect of propolis on mitochondria isolated from yeast strain W303 by monitoring the oxygen consumption rate as a consequence of mitochondria NADH oxidase activity. In the presence of propolis the NADH oxidase activity is 1/10 of that observed before the addition of this compound, indicating its toxic effect on respiration (Fig. 8C). These results strongly suggest that *S. cerevisiae*

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**TABLE 2. Specific GO classes derived from PPPI of unclustered protein subnetworks**

| Biological process                  | GO identification no. | P value<sup>a</sup> | Corrected P value<sup>b</sup> | k<sup>c</sup> | l<sup>d</sup> |
|-------------------------------------|-----------------------|---------------------|-------------------------------|--------------|--------------|
| Macroautophagy                      | 34262                 | 7.44 × 10^-14       | 5.44 × 10^-11                 | 11           | 23           |
| CVT pathway                         | 32258                 | 9.36 × 10^-12       | 3.43 × 10^-9                  | 9            | 18           |
| Protein targeting to vacuole        | 6623                  | 7.37 × 10^-11       | 1.80 × 10^-8                  | 13           | 62           |
| Cellular response to starvation     | 9267                  | 3.03 × 10^-10       | 5.54 × 10^-7                  | 11           | 44           |
| Cellular response to stress         | 33554                 | 1.06 × 10^-9        | 5.09 × 10^-7                  | 11           | 49           |
| Cellular response to nutrient levels| 31669                 | 4.86 × 10^-9        | 5.09 × 10^-7                  | 11           | 56           |
| Response to starvation              | 42594                 | 1.80 × 10^-8        | 1.46 × 10^-6                  | 11           | 63           |
| Vacular transport                   | 7034                  | 5.05 × 10^-8        | 3.69 × 10^-6                  | 14           | 122          |
| Cell communication                  | 7154                  | 1.44 × 10^-6        | 6.21 × 10^-5                  | 20           | 317          |
| Regulation of protein polymerization| 32271                 | 1.76 × 10^-6        | 6.79 × 10^-5                  | 4            | 6            |

<sup>a</sup> P values calculated by the hypergeometric distribution of one ontology class visualized in the network.

<sup>b</sup> Calculated values based on *P* values obtained after FDR was applied.

<sup>c</sup> Total number of proteins found in the network which belong to a gene ontology.

<sup>d</sup> Total number of proteins that belong to a specific gene ontology.
Propolis sensitivity is dependent on the mitochondrial function.

A role for the vacuole and the vacuolar H⁺-ATPase in propolis sensitivity. Another set of genes important for propolis sensitivity observed in our screening were involved in vacuolar acidification and macroautophagy associated with protein targeting to vacuoles and cellular response to starvation (Table 2). Organelle acidification is implicated in protein sorting in the biosynthetic and endocytic pathways, proteolytic activation of zymogen precursors, and transmembrane transport of viral contents and toxins (for a review, see reference 32). In fungi, the lysosome-like vacuole also plays a role in storage of metabolic building blocks, calcium homeostasis, and osmotic control. Vacuolar acidification is essential for all these functions. The vacuolar proton-translocating ATPase (V-ATPase) is an essential enzyme to catalyze all these processes. Their primary role in eukaryotic cell is ATP-driven transport of protons from the cytosol into acidic organelles (32). When several genes (VPHI, VMA3, VMA4, VMA5, VMA11, and VMA22, RAV1, and SOP4) involved in the assembly of the yeast V-ATPase were deleted, the corresponding yeast deletion strains became more sensitive to propolis (Fig. 9A). Recently, Rosado et al. (67) developed a method for monitoring autophagy using Rosella, a biosensor comprised of a fast-maturing pH-stable red fluorescent protein fused to a pH-sensitive green fluorescent protein variant. Its mode of action relies upon differences in pH between different cellular compartments and the vacuole. Rosella is a genetically encoded dual-color emission biosensor, a product of a fusion of a relatively pH-insensitive red fluorescent protein fused to a pH-sensitive green fluorescent protein variant. Its mode of action relies upon differences in pH between different cellular compartments and the vacuole. Rosella is a genetically encoded dual-color emission biosensor, a product of a fusion of a relatively pH-insensitive red fluorescent protein fused to a pH-sensitive green fluorescent protein variant. Its mode of action relies upon differences in pH between different cellular compartments and the vacuole. Rosella is a genetically encoded dual-color emission biosensor, a product of a fusion of a relatively pH-insensitive red fluorescent protein fused to a pH-sensitive green fluorescent protein variant. Its mode of action relies upon differences in pH between different cellular compartments and the vacuole. Rosella is a genetically encoded dual-color emission biosensor, a product of a fusion of a relatively pH-insensitive red fluorescent protein fused to a pH-sensitive green fluorescent protein variant. Its mode of action relies upon differences in pH between different cellular compartments and the vacuole. Rosella is a genetically encoded dual-color emission biosensor, a product of a fusion of a relatively pH-insensitive red fluorescent protein fused to a pH-sensitive green fluorescent protein variant. Its mode of action relies upon differences in pH between different cellular compartments and the vacuole.
Cells were then transferred to starvation medium (without nitrogen) to induce autophagy. After a 4-h incubation, red fluorescence, but not green, was observed to accumulate in the vacuole of approximately 40% of the cells (Fig. 9B, second row). Then, the cells were exposed to 0.125% propolis, and after a 90-min incubation, a subtle phenotype of red fluorescence, but not green, was observed to accumulate in the vacuole of approximately 60% of wild-type cells (Fig. 9B, second and third rows).

Autophagy can be involved in the turnover of long-lived proteins and whole organelles (for example, mitochondria in mitophagy and the endoplasmic reticulum in reticulophagy) (51). Autophagy is also an important process during starvation; i.e., by the catabolism of macromolecules autophagy generates metabolic substrates (51). Autophagy has been followed by various biochemical and morphological methods (for reviews, see references 35 and 36). Expression of GFP-Atg8p in yeast can be used to follow the localization or accumulation of the preautophagosomal structure, autophagosomes, and autophagic bodies. We have observed that \textit{ATG1} (encoding a protein serine/threonine kinase required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting [CVT] pathway), \textit{ATG10} (encoding a conserved E2-like conjugating enzyme that mediates formation of the Atg12p-Atg5p conjugate, which is a critical step in autophagy), and \textit{ATG20} (encoding a nexin family member required for the CVT pathway and for endosomal sorting) deletion strains are more sensitive to propolis (for reviews about yeast genes involved in autophagy, see references 33 and 39) (Fig. 10A). We have used mRNA accumulation of \textit{ATG14} and \textit{ATG8} and monitoring GFP-Atg8 as assays to verify if propolis induces an increase in autophagy. Propolis induced \textit{ATG8} and \textit{ATG14} mRNA accumulation about 4 times and 14 times, respectively (5- and 10-min exposure to 0.125% propolis) (Fig. 10B). The GFP-Atg8 translocated to vacuolar-like structures after exposure to 0.125% propolis for 20 min (Fig. 10C).

Taken together, these data strongly indicate that vacuolar acidification and autophagy are important for yeast cell death caused by propolis.

DISCUSSION

The three main types of propolis around the world have different compositions: (i) caffeic acid phenethyl ester (CAPE)-based propolis in Europe, the Far East and New Zealand; (ii) artepillin C (ARC)-based Brazilian green propolis;
and (iii) Brazilian red propolis. Recently, a screen of 1,266 compounds with known pharmaceutical activities was performed, and 15 compounds that prolonged survival of *C. albicans*-infected nematodes and inhibited *in vivo* filamentation of *C. albicans* were identified (11). One of these compounds, CAPE, exhibited antifungal activity in a murine model of candidiasis (11). CAPE is a poplar-type propolis while Brazilian green propolis is a *Baccharis* type. We along with other investigators have been unable to identify any traces of CAPE in the Brazilian green propolis (75, 79; also data not shown). Here, we have investigated molecular targets involved in cell lethality caused by the Brazilian green propolis. We were not able to see any cell death effect when *S. cerevisiae* was exposed to high concentrations of four of the major compounds that are present in the green propolis. Thus, we decided to verify the effects of propolis on *S. cerevisiae* cell lethality by using alcoholic propolis extracts. Cell death in *S. cerevisiae* is frequently followed by indicative apoptotic and/or necrotic markers such as externalization of phosphatidylserine to the outer leaflet of the plasma membrane, chromatin condensation, the generation of reactive oxygen species, propidium iodide accumulation, HMGB1/Nhp6p localization, detection of plasma membrane ruptures, and complete disintegration of subcellular structures (14, 19). In addition, ROS accumulation, mitochondrial fragmentation, cytochrome *c* release, the metacaspase Yca1p, the apoptosis-inducing factor Aif1p, the endonuclease G Nuc1p, the serine protease OMI, cytoskeleton perturbations, and chromatin epigenetic modification have also been observed (79). Necrosis has also been described during yeast chronological aging (20).

We have noticed that apoptosis and necrosis markers are induced by propolis, but increased time exposure to propolis intensifies the number of cells with necrotic markers, such as PI and nucleo-cytosolic translocation of the Nhp6Ap-GFP. Our data indicate a dual role for propolis treatment as an agent that induces apoptosis and secondary necrosis. These effects are partially mediated by YCA1 metacaspase since the *YCA1*Δ mutant is more resistant to propolis. In *S. cerevisiae* overexpression of *YCA1* caused cell death while *YCA1* deletion protected against cell death caused by reactive oxygen species or chronological aging (49). Additionally, we have also observed that Nuc1p does not translocate to the nucleus upon propolis cell death induction. Interestingly, Büttner et al. (13) observed that apoptotic death mediated by Nuc1p does not require YCA1. More interestingly, simultaneous markers for apoptosis and necrosis have also been noticed during propolis cell death induction in *Trypanosoma cruzi* (55).

There are several conditions where mitochondria-produced ROS have been associated with yeast apoptosis (for reviews, see references 19, 59, and 62). Propolis can induce ROS formation, and it is more lethal when *S. cerevisiae* grows in the presence of glycerol and ethanol as carbon sources (data not shown), suggesting that respiration increases propolis lethality. Actually, we were able to demonstrate that propolis can inhibit respiration in *S. cerevisiae*. We have observed that propolis cell death induction stimulates the cytochrome *c* release and that also [rho0] cells are more tolerant to propolis. Cytochrome *c* is a mitochondrial protein with a function in the respiratory chain and an additional function as an activator of caspase-9 in the intrinsic pathway of mammalian apoptosis (40). Treatment of yeast cells with acetic acid leads to mitochondrial cytochrome *c* release (45). Disruption of cytochrome *c* partially prevents acetic acid-induced cell death, and accordingly [rho0] cells show resistance against acetic acid-induced cell death (45). However, it is not very clear, as it is in mammals, if cytochrome *c* release can lead to the formation of an apoptosome-like structure and activation of caspases. In contrast, Sripriya et al. (77) have observed loss of mitochondrial membrane potential and absence of cytochrome *c* release during necrotic cell death of *S. cerevisiae* induced by expression of a proteinaceous elicitor hairpin (Pss) from *Pseudomonas syringae*. However, the deficiency of functional mitochondrial DNA, and consequent inability to respire from petite mutants, conferred resistance to death.

We have applied the power of genomics by screening the full collection of haploid *S. cerevisiae* deletion strains to compre-
hend how propolis, a complex phytotherapeutic compound, affects cell metabolism. This strategy has already been extensively used for understanding how other compounds affect S. cerevisiae cell metabolism (8, 15, 18, 27, 71, 86). By applying systems biology, we observed that most of the proteins whose deletion increases the sensitivity of yeast strains to propolis are involved in cell division mechanisms, the mitochondrial electron transport chain, vacuolar acidification, regulation of macroautophagy associated with protein targeting to vacuoles, cellular response to starvation, and negative regulation of transcription from the RNA polymerase II promoter. We have investigated and validated in more detail the mitochondrial electron transport chain and vacuolar acidification pathways. We have shown that propolis induces vacuolar acidification and translocation of Atg8p to the vacuoles, one of the hallmarks of autophagy. In S. cerevisiae, the vacuole is very important for maintaining cellular homeostasis comprising the regulation of intracellular pH and degradation mainly during nutrient limitation of proteins and organelles by autophagy (for reviews, see references 2, 29, and 46). Cell death induced by acetic acid is increased in S. cerevisiae VPS gene deletion mutants (VPS genes are involved in homotypic vacuole fusion and vacuolar protein sorting and are essential for normal vacuolar function) (70). It has been observed that the intracellular pH was acidified in VPS mutant cells upon treatment with acetic acid (70). It is possible that the disturbance of the homeostatic pH control may trigger necrosis by release of pro-necrotic proteases, which would find an optimal pH for their enzymatic activity in the acidified cytosol (19).

Our work emphasizes the importance of S. cerevisiae as a model system to understand at a molecular level the mechanism of action of a phytotherapeutic compound. There is a multitude of studies describing the most diverse effects of propolis on different biological systems, ranging from cancer chemoprevention (78) to cariostatic activity on streptococci (46) and antimicrobial activity of propol isolated from Brazilian propolis (70). Antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens, Anaerocobe (82–85) and other diseases such as cancer, HIV/AIDS, and other infections (10). It is well documented that propolis is capable of inducing apoptosis and it has been recognized as an alternative therapeutic agent against fun-

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