

The biological activity of the wine anthocyanins delphinidin and petunidin is mediated through Msn2 and Msn4 in *Saccharomyces cerevisiae*

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Abstract

Polyphenols are considered to be responsible for some of the health benefits derived from the consumption of red wine. These protective effects might probably be explained in the context of the xenohormesis theory that considers plant metabolites as interspecific chemical signals. However, the complexity of the polyphenolic constituents of different wines makes it difficult to clarify the specific contribution of polyphenols to such effects. In the present work, we fractionated the polyphenols of a red wine and evaluated the effect of each polyphenolic fraction on the growth pattern of the yeast *Saccharomyces cerevisiae*. We observed a different contribution of the phenolic fractions to the xenohormetic response of *S. cerevisiae*, the fractions that were enriched with red pigments being the most protective against oxidative insults. Moreover, we found that red wine phenolic fractions exert their biological activity through the activation of the Yap1 and Msn2 stress-responsive regulators. Above all, the anthocyanins delphinidin 3-glucoside and petunidin 3-glucoside were found to improve significantly the growth rate of *S. cerevisiae* in an Msn2-, Msn4-dependent manner, indicating that the stress regulators Msn2 and Msn4 participate in the xenohormetic activity of the wine polyphenols delphinidin and petunidin.

Introduction

Red wine polyphenols have been historically considered as potent antioxidants, with a number of benefits for human health (Sun *et al.*, 2002; Corder *et al.*, 2006). Epidemiological studies have suggested that a moderate consumption of red wine is correlated with a reduction in the risk of cardiovascular disease and carcinogenesis (German & Walzem, 2000).

Polyphenols are plant secondary metabolites with diverse physiological functions, such as plant color, antiparasitic functions, and antioxidant protection (Stevenson & Hurst, 2007). Polyphenols also exert important functions through the activation of cellular antioxidant enzymes and signalling pathways (García-Alonso *et al.*, 2004; Stevenson & Hurst, 2007). In addition, plant polyphenols have been recently associated with xenohormesis, a hypothesis that proposes

the plant stress signalling molecules as interspecific chemicals that are able to regulate different physiological processes in ways that confer health benefits (Howitz & Sinclair, 2008). Therefore, polyphenols might perform important functions in mammals through the modulation of stress-response pathways rather than acting as direct antioxidant molecules. However, the molecular mechanisms underlying such biological activities have not yet been fully elucidated.

The unicellular yeast *Saccharomyces cerevisiae* is an excellent eukaryotic model for the study of the molecular mechanisms of novel drugs and natural compounds, including polyphenols. Thus, studies with yeast have served to uncover novel genes and regulatory mechanisms involved in important processes such as eukaryotic cell division, proliferation, and cell death (Mager & Winderickx, 2005; Lisa-Santamaría *et al.*, 2009). Also, the use of genome-wide screenings with collections of yeast deletion mutants has

helped to identify novel targets of different chemicals and pharmaceutical compounds (Tucker & Fields, 2004). In particular, *S. cerevisiae* has been used extensively for the study of oxidative stress, which has been proposed to be a key regulator of cellular physiology and is associated with several human pathologies, including cancer, neurological disorders, and cardiovascular diseases (Ikner & Shiozaki, 2005; Temple *et al.*, 2005). These studies have provided important insights into the mechanisms of cellular sensing and responses to reactive oxygen species and other forms of stress.

In *S. cerevisiae*, Yap1 and Msn2/Msn4 have been suggested to be the most important stress-responsive transcription factors, participating in the sensing of oxidative and nutritional stress (Temple *et al.*, 2005). The transcriptional activity of both Yap1 and Msn2/Msn4 is mainly modulated through their nuclear accumulation. Yap1 is activated by multistep disulfide bonding in the nuclear export signal at the C-terminus. Thus, oxidized Yap1 does not bind to the nuclear exportin Crm1, which constitutively determines its cytosolic localization, resulting in the nuclear accumulation of Yap1 and the subsequent transcriptional activation of target genes (Okazaki *et al.*, 2007). In contrast, the nuclear localization of Msn2/Msn4 is regulated by phosphorylation. Both the Ras/PKA and the TOR pathways are involved in the modulation of the nuclear translocation of Msn2/Msn4 (Medvedik *et al.*, 2007). Low levels of protein kinase A activity lead to the nuclear import of Msn2, whereas an increase in PKA activity promotes nuclear export (Gorner *et al.*, 1998; Lee *et al.*, 2008). Additionally, the inhibition of the TOR signalling pathway triggers dephosphorylation, and hence the nuclear accumulation of Msn2 (Wei *et al.*, 2008). Msn2/Msn4 has been proposed recently as a link between caloric restriction and increased lifespan through the transcriptional activation of sirtuins and antioxidant enzymes (Medvedik *et al.*, 2007).

The principal aim of this study is to analyze the biological activity of red wine anthocyanins in the context of xenohormesis. Here, we evaluated the protective effect of fractionated red wine polyphenols in a set of deletion mutants from the knockout collection of *S. cerevisiae*. We also examined the ability of both the anthocyanin-enriched fractions and also that of the anthocyanins delphinidin 3-glucoside (Dp3g) and petunidin 3-glucoside (Pt3g) to induce a hormetic response through Msn2, Msn4 in *S. cerevisiae*.

Materials and methods

Winemaking

Vitis vinifera cv. Tempranillo fresh grapes from the Spanish Rioja Appellation were processed in the 2005 vintage by

Bodegas RODA (Haro, La Rioja, Spain). The maceration and fermentation steps were carried out in 1200-L steel tanks. Postfermentative maceration was maintained for 6 days, after which wine samples were taken.

Sample fractionation

Samples (180 mL) of wine were fractionated by gel permeation chromatography with a Toyopearl HW-40S column, using the method described by Alcalde-Eon *et al.* (2006). Nine fractions were obtained, which were subsequently freeze-dried and weighed. Solutions of 0.5 mg mL^{-1} in acidic water (HCl, pH 0.5) were prepared for further chromatographic analysis of phenolic compounds.

HPLC–diode array detector (DAD)–MS analysis

Analysis of anthocyanins and flavonols

Solutions corresponding to the nine fractions were filtered through a $0.45 \mu\text{m}$ Millex[®] syringe-driven filter unit (Millipore Corporation) and analyzed using the HPLC–DAD–MS technique. This was performed on a Hewlett-Packard 1100 series liquid chromatograph. An AQUAC18[®] reverse phase, $5 \mu\text{m}$, $150 \text{ mm} \times 4.6 \text{ mm}$ column (Phenomenex, Torrance, CA) thermostatted at 35°C was used for the simultaneous separation of anthocyanins and flavonols. The solvents used were (1) an aqueous solution (0.1%) of trifluoroacetic acid and (2) 100% HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B for 3 min; from 10% to 15% B for 12 min; isocratic 15% B for 5 min; from 15% to 18% B for 5 min; from 18% to 30% B for 20 min; and from 30% to 35% B for 5 min, at a flow rate of 0.5 mL min^{-1} . Detection was carried out at 520 nm (anthocyanins) and 360 nm (flavonols). Spectra were recorded in the 220–600 nm range.

Analysis of flavanols and phenolic acids

With a view to eliminating the anthocyanins, 2 mL of each sample was placed in Oasis MCX cartridges conditioned previously with 2 mL of methanol and 2 mL of water. After a wash in 4 mL of ultrapure water, the flavan-3-ols and phenolic acids were eluted with 8 mL of methanol, the anthocyanins and the flavonols being retained in the cartridge. A small volume of water was added to the eluate and concentrated under vacuum at a temperature of $< 30^\circ\text{C}$ until complete elimination of the methanol. The volume of the aqueous residue was adjusted to 500 μL with ultrapure water (MilliQ), filtered, and analyzed using an HPLC–DAD–MS system with a Waters Spherisorb[®] S3 ODS-2 reverse phase, $3 \mu\text{m}$, $150 \text{ mm} \times 4.6 \text{ mm}$ column (Waters, Ireland) thermostatted at 25°C . The mobile phase was (1) 2.5% acetic acid, (2) acetic acid/acetonitrile

(90:10, v:v), and (3) HPLC-grade acetonitrile. The elution gradient established ranged from 0% to 100% B for 5 min; from 0% to 15% C for 25 min; from 15% to 50% C for 5 min; and isocratic 50% C for 5 min, at a flow rate of 0.5 mL min⁻¹. Detection was carried out at 280 nm (flavonols) and 330 nm (phenolic acids). Spectra were recorded from 220 to 600 nm.

MS conditions

Mass analyses were performed using a FinniganTM LCQ ion-trap instrument (Thermoquest, San Jose, CA) equipped with an electrospray ionization (ESI) interface. The LC system was connected to the probe of the mass spectrometer *via* the UV cell outlet. Both the sheath gas and the auxiliary gas were a mixture of nitrogen and helium. The sheath gas flow was 1.2 L min⁻¹, and the auxiliary gas flow was 6 L min⁻¹. The capillary voltage was 26 V for anthocyanins and flavonols; 11 V for flavanols; and 10 V for phenolic acids and flavanols. The source voltage was 4.5 kV for anthocyanins, flavonols, and flavanols, and 2.5 kV for phenolic acids. The capillary temperature was 195 °C. Spectra were recorded in the positive-ion mode between 120 and 1500 *m/z*. The mass spectrometer was programmed to perform a series of three consecutive scans: a full mass; an MS² scan of the most abundant ion in the full mass; and an MS³ of the most abundant ion in the MS². The normalized collision energy was 45%.

Quantification

For quantitative analyses, calibration curves were obtained using standards of anthocyanin 3-glucosides (Dp3g, cyanidin 3-glucoside, Pt3g, peonidin 3-glucoside, and malvidin 3-glucoside); flavonols (myricetin, quercetin, and kaempferol); flavanols [(+)-catechin, (-)-epigallocatechin, the dimer B2, and the trimer epicatechin-4,8-epicatechin-4,8-catechin]; and phenolic acids (3,4-dihydroxybenzoic acid and 4-hydroxycinnamic acid) (Gonzalez-Manzano *et al.*, 2006). Anthocyanins were purchased from Polyphenols Labs (Sandnes, Norway). Myricetin, kaempferol, and (-)-epigallocatechin were purchased from Extrasynthèse (Genay, France). Quercetin, (+)-catechin, 3,4-dihydroxybenzoic acid, and 4-hydroxycinnamic acid were purchased from Sigma (Steinheim, Germany). Dimer and trimer procyanidins were obtained at our laboratory.

Yeast strains and growth conditions

The nonessential haploid *MATa* yeast deletion strains derived from the parent strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were from Euroscarf (Frankfurt, Germany; <http://web.uni-frankfurt.de/fb15/mikro/euroscarf/in>

[dex.html](#)). Double mutants $\Delta msn2$, $\Delta msn4$ and $\Delta msn2$, $\Delta pnc1$ were obtained using a PCR-based gene-replacement strategy (see Supporting Information, Table S1 for primer sequences) using a loxP-NatMX-loxP replacement cassette flanked by recombinogenic sequences for the corresponding target gene (Guldener *et al.*, 1996). This method enables to eliminate and subsequently reuse the selection marker by expressing a Cre recombinase as described (Guldener *et al.*, 1996). The triple mutant $\Delta msn2$, $\Delta msn4$, $\Delta pnc1$ was obtained using the above strategy in a $\Delta msn2$, $\Delta pnc1$ genetic background.

Cells were routinely grown at 28 °C in either a standard rich YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose) or a synthetic complete (SC) medium lacking leucine, containing 2% glucose as the carbon source. Growth on liquid cultures was monitored spectrophotometrically at OD_{600 nm}. The antibiotics G418 (150 mg L⁻¹; Gibco-BRL) and cloNAT (100 mg L⁻¹; Werner Bioagents) were added when indicated.

Phenotypic screening of *S. cerevisiae* deletion mutants

A subset of 44 mutant strains in either the BY4741 or the BY4743 background was screened for their ability to grow in the presence or absence of nine red wine fractions (Table S2). Solutions containing 0.2 mg mL⁻¹ of each lyophilized fraction were prepared in a wine-like solution [12% ethanol (v/v) containing 5 g L⁻¹ of tartaric acid buffered with 1 M NaOH at pH 3.6]. All assays, including the negative controls without polyphenols, contained the same volume of wine-like solution.

The concentration of the phenolic fractions was set at 150 µg mL⁻¹, which has been shown to be effective in previous studies (Garcia-Alonso *et al.*, 2004). The chemically undefined F1 fraction was excluded from the analysis. The analyses were repeated four times and some variability was observed between the experiments.

Analyses of the wine fractions and standards were carried out in the presence or absence of 1 mM hydrogen peroxide (H₂O₂) or 0.5–1 mM *t*-BOOH. Screening was performed as described previously (Botet *et al.*, 2008). Briefly, strains were pinned from 96-well frozen stock plates using a stainless-steel 96-pin replicator (Nalgene Nunc International) into 96-well plates containing 150 µL of liquid YPD medium supplemented with G418 (150 mg L⁻¹; Gibco-BRL). When the cultures had reached the stationary phase (approximately 24 h), the plates were pin-replicated either onto liquid YPD 96-well plates containing a wine-like solution or YPD plates containing the wine fractions, or YPD plates containing both the wine fractions and the oxidants. The plates were incubated at 28 °C and growth was scored quantitatively every 12 h over a period of 4 days

by recording the $OD_{600\text{nm}}$, using a microplate reader spectrophotometer (model 550, Bio-Rad Laboratories). The effect of the red wine fractions on yeast growth was scored on the basis of the relative growth of each mutant in every treatment against that of the mutants on the control YPD plates.

Additionally, a number of mutants (Figs 2 and 4) were selected to analyze the effect of either wine fractions or pure anthocyanins on the growth rate in the presence or absence of oxidants. For these experiments, 2–5 mL cultures of either YPD or SC media supplemented with the indicated chemicals (i.e. polyphenols and oxidants) were used and the growth of the cultures were scored by recording $OD_{600\text{nm}}$ at the indicated time-points. All the experiments were carried out including the corresponding volumes of the wine-like solution in the negative controls to exclude the effect of ethanol in the results.

Yap1 and Msn2 nuclear translocation analyses

The *S. cerevisiae* strains used in this analysis were $\Delta yap1$ and $\Delta msn2$ transformed with either YCplac111/YAP1-green fluorescent protein (GFP) or YCplac111/MSN2-GFP, respectively. The plasmids for YAP1-GFP and MSN2-GFP were a gift from Y. Inoue and C. Schüller, respectively (Gorner *et al.*, 1998; Maeta *et al.*, 2007). In this assay, we examined the intracellular localization of both MSN2-GFP and YAP1-GFP after the addition of different phenolic compounds to the media: either the F5 red wine fraction or the F6 red wine fraction, or Dp3g or Pt3g (concentrations ranging from 12.5 to 100 $\mu\text{g mL}^{-1}$). The localization of the GFP-fusion proteins in yeast cells was observed at different times using a Nikon Eclipse 90i fluorescence microscope. The excitation and emission wavelengths were 480 and 530 nm, respectively.

Transcription analysis by quantitative real-time PCR

Quantitative real-time PCR was performed with a LightCycler 480 real-time PCR instrument (Roche), using

SYBR green I master mix (Roche) and following the manufacturer's instructions. Total RNA samples were obtained as described previously (Jimenez *et al.*, 2007) and the cDNA samples were prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Primer sequences are indicated in Table S1. All real-time PCR reactions were performed in duplicate and in at least two independent experiments. Quantitative analyses were carried out using the LightCycler 480 software.

Results

Phenolic composition of the wine fractions

A red wine was fractionated into nine different fractions (F1–F9) as described above; the phenolic compositions of the resulting fractions are shown in Table 1. While fractions F1, F2, F8, and F9 contained very low amounts of phenolic compounds (0.3–4.3 $\mu\text{g mL}^{-1}$), the F5 and F6 fractions had a significant proportion of phenolics, with > 200 and 100 $\mu\text{g mL}^{-1}$, respectively. Additionally, > 50% of the phenolic compounds present in the F5 and F6 fractions were identified as red pigments.

Thirty-seven red pigments were identified among the wine fractions, including monoglucosylated anthocyanins, monoglucosylated anthocyanins esterified with acetic acid, coumaric acid or caffeic acid, and other anthocyanin-derived pigments (pyranoanthocyanins and direct flavanol–anthocyanin condensation pigments). Monoglucosylated anthocyanins were represented in all fractions (Table 2), but the F5 and F6 fractions contained the highest amounts of these compounds. In the F5 fraction, Dp3g and Pt3g represented 33% and 42%, respectively. In the F6 fraction, Dp3g was the most abundant anthocyanin, accounting for 72% of all the monoglucosylated anthocyanins (Table 3).

Phenotypic screening of red wine fractions with a set of *S. cerevisiae* deletion mutants

The existence of a complete set of *S. cerevisiae* deletion mutants allows phenotypic genome-wide screenings to be carried out to identify all the nonessential genes that could

Table 1. Phenolic composition of the fractions obtained from Tempranillo red wine ($\mu\text{g mL}^{-1}$)

	F1	F2	F3	F4	F5	F6	F7	F8	F9
Red pigments*	ND	2.51	6.39	27.5	131	56.50	9.46	1.51	1.12
Flavanols	0.23	0.45	5.70	3.66	2.66	5.59	4.53	0.60	ND
Phenolic acids	0.11	0.20	1.80	3.25	3.11	3.18	1.37	0.18	0.39
Flavonols	ND	1.14	25.60	11.30	75.70	37.50	5.98	ND	ND
Total	0.34	4.30	39.50	45.70	212.47	102.77	21.30	2.29	1.50

*Anthocyanins and anthocyanin-derived pigments.
ND, not detected.

Table 2. Content of red pigments in the fractions obtained from Tempranillo red wine ($\mu\text{g mL}^{-1}$)

	F1	F2	F3	F4	F5	F6	F7	F8	F9
Anthocyanin monoglucosides	ND	0.79	3.87	19.90	70.50	26.30	2.91	1.51	1.12
Acylated anthocyanins	ND	0.97	ND	4.11	30.0	10.80	3.27	ND	ND
Anthocyanin-derived pigments	ND	0.31	2.52	2.13	22.30	13.10	2.69	ND	ND
Total	ND	2.07	6.39	26.14	122.80	50.20	8.87	1.51	1.12

ND, not detected.

Table 3. Content of the main anthocyanin monoglucosides in the fractions obtained from Tempranillo red wine ($\mu\text{g mL}^{-1}$)

	F1	F2	F3	F4	F5	F6	F7	F8	F9
Dp3g	ND	0.20	2.02	1.31	22.10	18.20	0.81	0.85	0.65
Cy3g	ND	0.17	ND	0.35	4.98	2.11	0.54	ND	N.D.
Pt3g	ND	0.20	ND	1.42	28.2	2.59	0.59	ND	ND
Pn3g	ND	0.05	ND	1.04	2.98	0.54	0.19	ND	ND
Mv3g	ND	0.17	1.85	15.60	8.27	1.76	0.79	0.65	0.47
Total	ND	0.79	3.87	19.70	66.53	25.20	2.91	1.51	1.12

ND, not detected; Dp3g, delphinidin-3-glucoside; Cy3g, cyanidin-3-glucoside; Pt3g, petunidin-3-glucoside; Pn3g, peonidin-3-glucoside; Mv3g, malvidin-3-glucoside.

affect the cellular response to a specific treatment (Giaever *et al.*, 2002). In this study, we wished to evaluate the biological effect of red wine polyphenols obtained after the chemical fractionation described above. The limited availability of our polyphenolic fractions made it impossible to carry out a phenotypic screening of the genome-wide set of *S. cerevisiae* knockout mutants, and hence we chose a subset of 44 mutants lacking genes involved in redox metabolism and stress defense to assess the effect of the red wine fractions (Table S2).

Screening of the wine fractions was performed in both the presence and the absence of the pro-oxidants H_2O_2 and *t*-BOOH. Our results revealed marked differences among the phenolic fractions when their effect on the growth rate of the *S. cerevisiae* mutants was analyzed (Fig. 1 and Fig. S1). Fractions F6, F8, and F9 increased the growth rate of about 50% of the mutants in the absence of pro-oxidants. Additionally, fractions F2, F3, and F6 were able to alleviate the cytotoxic effect of H_2O_2 in a high number of mutants. Finally, fraction F5 improved the growth rate of most of the mutants in the presence of *t*-BOOH to a considerable extent (Fig. 1). Thus, anthocyanin-enriched fractions (especially fractions F5 and F6) were able to improve the growth rate of a higher number of mutants than the other fractions.

Anthocyanin-enriched fractions diminish oxidative-induced toxicity in *S. cerevisiae*

In order to gain further insight into the protective effect of fractions F5 and F6, we analyzed their ability to increase the growth rate of three selected mutant strains that have been proposed to be adequate models for analyses of oxidative

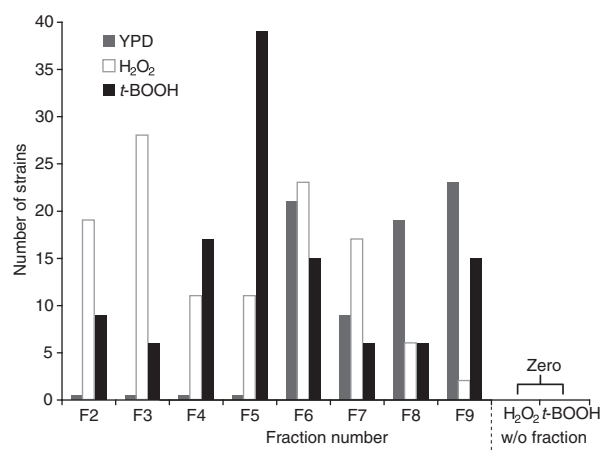


Fig. 1. Phenotypic analysis of the phenolic fractions of red wine. Bar diagram representing the number of *Saccharomyces cerevisiae* mutants whose growth rate was improved after the addition either of fractions of red wine (F2–F9) or pro-oxidants alone (zero strains). YPD-rich media, no pro-oxidant was added; H_2O_2 or *t*-BOOH (1 mM) was added when indicated.

stress, namely, *grx5*, *ahp1*, and *tsa2*. In this sense, the mutant *grx5*, which lacks a mitochondrial glutaredoxin, is hypersensitive to H_2O_2 , while the *ahp1* mutant is unable to grow in the presence of alkyl hydroperoxides such as *t*-BOOH. The mutant *tsa2* lacks a thioredoxin peroxidase with ROS-scavenging activity. The growth kinetics of the liquid cultures was obtained for each yeast strain and the results are shown in Fig. 2. In the absence of pro-oxidants, the four strains analyzed did not show any significant increase in their growth rates after the addition of the F5 and F6 fractions to the cultures (data not shown). However, both

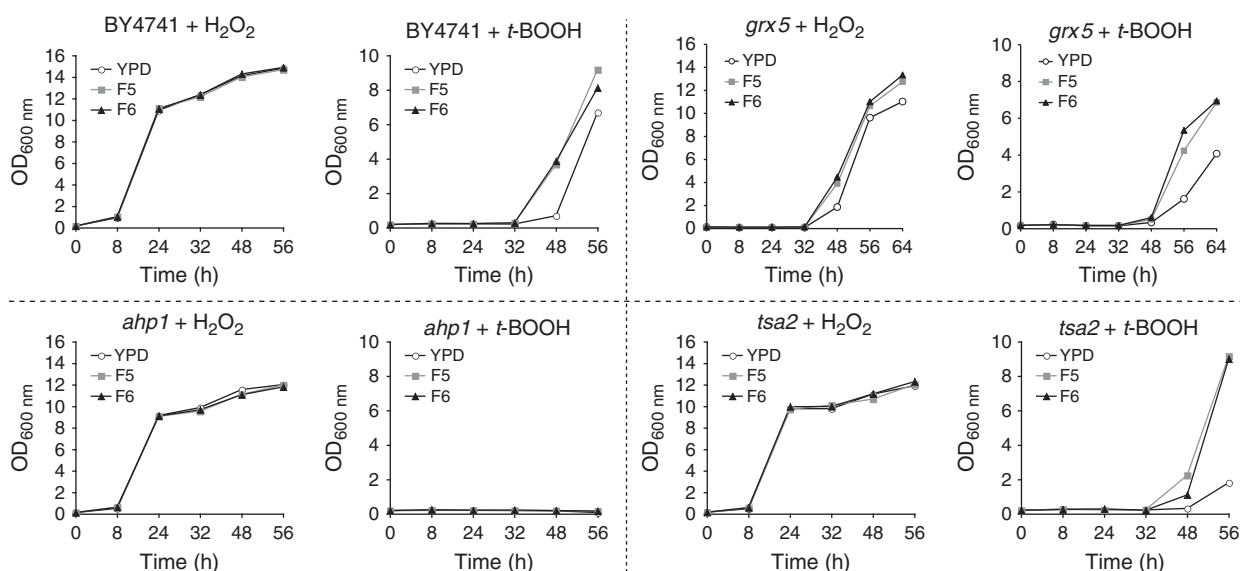


Fig. 2. Growth kinetics of the anthocyanin-enriched fractions. The *Saccharomyces cerevisiae* strains BY4741, *grx5*, *ahp1*, and *tsa2* were cultured in 2 mL of YPD-rich media with either the F5 or the F6 fraction, in the presence or absence of either H₂O₂ (1 mM) or *t*-BOOH (0.5 mM). The cell growth was scored by measuring the OD_{600 nm} of the cultures over a period of 56–64 h. The cultures were carried out in duplicate and in at least three independent experiments.

the F5 and the F6 fractions contributed toward reducing the toxicity of H₂O₂ in the *grx5* mutant. Additionally, the *grx5*, *tsa2* mutants, and the BY4741 parental strain displayed reduced sensitivity to *t*-BOOH after the addition of both the F5 and F6 phenolic fractions to the culture medium. The *ahp1* mutant, which lacks an alkyl hydroperoxide reductase, was unable to grow when *t*-BOOH was present in the culture medium. Overall, our results indicate that red wine anthocyanins may elicit a cellular response contributing toward alleviating oxidative stress in *S. cerevisiae*.

Anthocyanin-enriched fractions induce the nuclear accumulation of Yap1 and Msn2

Our results suggested that the addition of anthocyanin-enriched fractions might trigger an antioxidant response in *S. cerevisiae*. Yap1 and Msn2/Msn4 are stress-responsive factors that induce the expression of genes that participate in cellular defense against oxidative stress. Accordingly, we decided to investigate whether our phenolic fractions might affect the localization of Yap1 and Msn2, which determines their activity as transcriptional activators.

We used GFP-tagged Yap1 and Msn2 to analyze their localization after the addition of the F5 and F6 red wine fractions by epifluorescence microscopy. Fraction F5 induced a reversible nuclear translocation of both Yap1-GFP and Msn2-GFP (Fig. 3). The translocation of Msn2-GFP was much faster than that of Yap1-GFP, which occurred approximately 1 h after the addition of the F5 fraction to the culture.

In contrast, the F6 fraction was only able to induce the nuclear translocation of Msn2-GFP, which appeared in the nucleus immediately after the addition of that fraction to the culture (Fig. 3). In this case, Msn2-GFP shifted to the cytosol after 1 h, but was again localized to the nucleus after 2 h. Nuclear localization of both Yap1-GFP and Msn2-GFP was confirmed by DAPI staining of the corresponding samples (Fig. S2).

Dp3g and Pt3g improve the fitness of *S. cerevisiae* in an Msn2-, Msn4-dependent manner

The phenolic fractions F5 and F6 used in our previous analyses indicated a possible mode of action regarding the subcellular localization of both Yap1-GFP and Msn2-GFP. However, to better understand the molecular mechanisms underlying such effects, it is important to determine the specific compounds that are responsible for those biological effects. As evidenced by our HPLC analyses, the F5 fraction was mainly composed of Pt3g and Dp3g, whereas the F6 fraction was essentially constituted by Dp3g. Consequently, we analyzed the effect of these two phenolic standards on the growth rate of *S. cerevisiae* in the presence and absence of *t*-BOOH. We used the parental strain BY4741 and several single, double, and triple mutants lacking genes encoding the stress regulators Msn2 and Msn4 (Fig. 4a)

Dp3g increased the fitness of *S. cerevisiae* in SC medium and it clearly alleviated the toxic effect of *t*-BOOH (Fig. 4b–c). Moreover, the protective effect of Dp3g seems to be

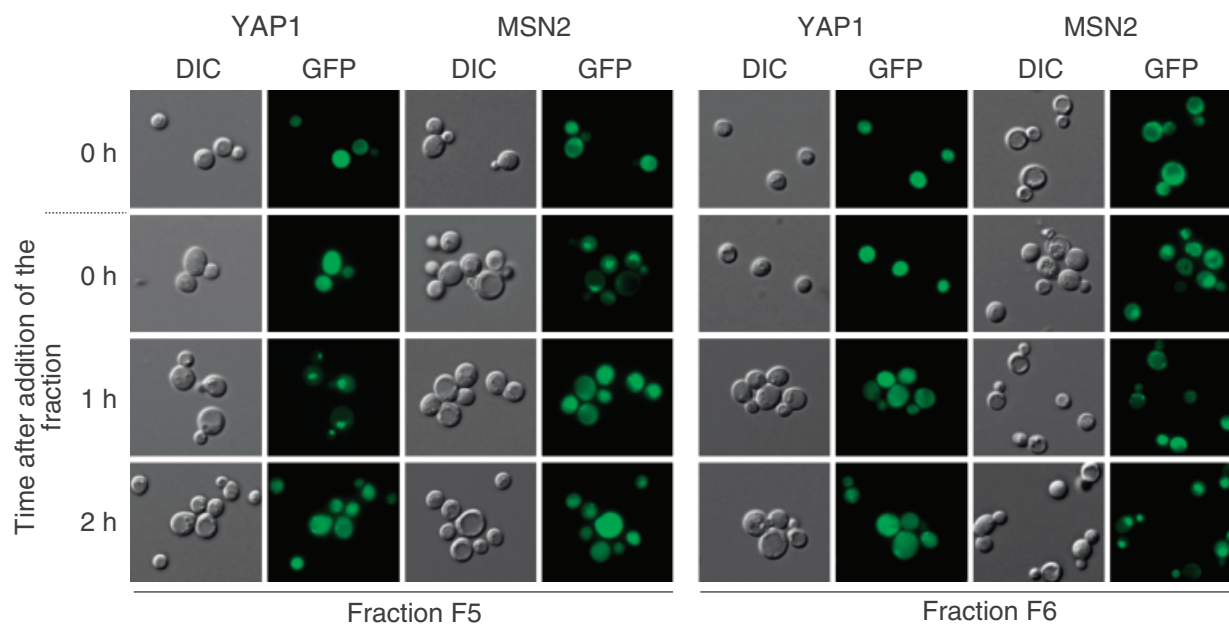


Fig. 3. Anthocyanin-enriched fractions trigger Yap1 and Msn2 nuclear translocation. BY4741 cells transformed with either YAP1-GFP or MSN2-GFP plasmids were incubated with either the F5 or the F6 fraction and the distribution of the GFP fluorescence was visualized at different time-points after the addition of the polyphenols. The concentration of the phenolic fractions was $12.5 \mu\text{g mL}^{-1}$ in all the assays. The corresponding volume of wine-like solution was also added to the negative controls.

dependent on the presence of both Msn2 and Msn4 because neither the double *msn2*, *msn4* nor the triple *msn2*, *msn4*, *pnc1* mutants improved their growth rates after Dp3g treatment (Fig. 4). Indeed, only those mutants lacking both *MSN2* and *MSN4* were not able to achieve a growth rate similar to the BY4741 parental strain when *t*-BOOH was added to the culture. The same results were obtained when Pt3g was analyzed (data not shown), indicating that both anthocyanins may exert identical effects.

Dp3g and Pt3g affect the nucleocytoplasmic trafficking of Msn2

Our previous data indicate that stress-responsive factors participate in the biological activity of the wine anthocyanins Dp3g and Pt3g. Therefore, we examined the effect of pure Dp3g on the nuclear accumulation of both Yap1-GFP and Msn2-GFP by epifluorescence microscopy (Fig. 5). Our results revealed that Dp3g was unable to induce the nuclear translocation of Yap1-GFP. However, Msn2-GFP was localized rapidly to the nucleus after the addition of Dp3g to the culture medium. As occurred when the F6 fraction was used, Msn2-GFP again relocated to the cytosol after 1 h, and it appeared once again in the nucleus after 2 h. These results indicate that the effect of the F6 fraction on the nucleocytoplasmic trafficking of Msn2-GFP could be attributed to the anthocyanin Dp3g.

We also investigated the effect of Pt3g on the subcellular localization of both Yap1-GFP and Msn2-GFP. In this case, we used either pure Pt3g (Fig. 6) or Pt3g combined with Dp3g at the same proportion as observed in the F5 fraction (data not shown), obtaining the same results. Pt3g was also unable to induce the nuclear localization of Yap1-GFP. However, the dynamics of Msn2-GFP resembled those observed when the F5 fraction was used. Thus, Pt3g, either combined with Dp3g or alone, elicited a rapid translocation of Msn2-GFP from the cytosol to the nucleus, but the fluorescence was seen in the cytosol after only 1 h. In contrast to what was observed with Dp3g, Pt3g did not induce a second nuclear relocalization of Msn2-GFP.

Transcriptional activation of Msn2 targets induced by Dp3g

The nuclear localization of Msn2-GFP upon either Dp3g or Pt3g treatments suggests that the putative target genes of the transcription factor Msn2 should be activated. Therefore, we analyzed the transcription levels of different Msn2 target genes (*PNC1*, *AHP1*, *TRX2*, *CTT1*) after Dp3g treatment. *TRX2* and *CTT1*, and to a lesser extent *AHP1*, showed a significant increase in their transcription after the addition of Dp3g to the cultures (Fig. 7). However, we did not detect a significant variation in the transcription of *PNC1*. The mRNA levels of the antioxidant genes *AHP1*, *TRX2*, and

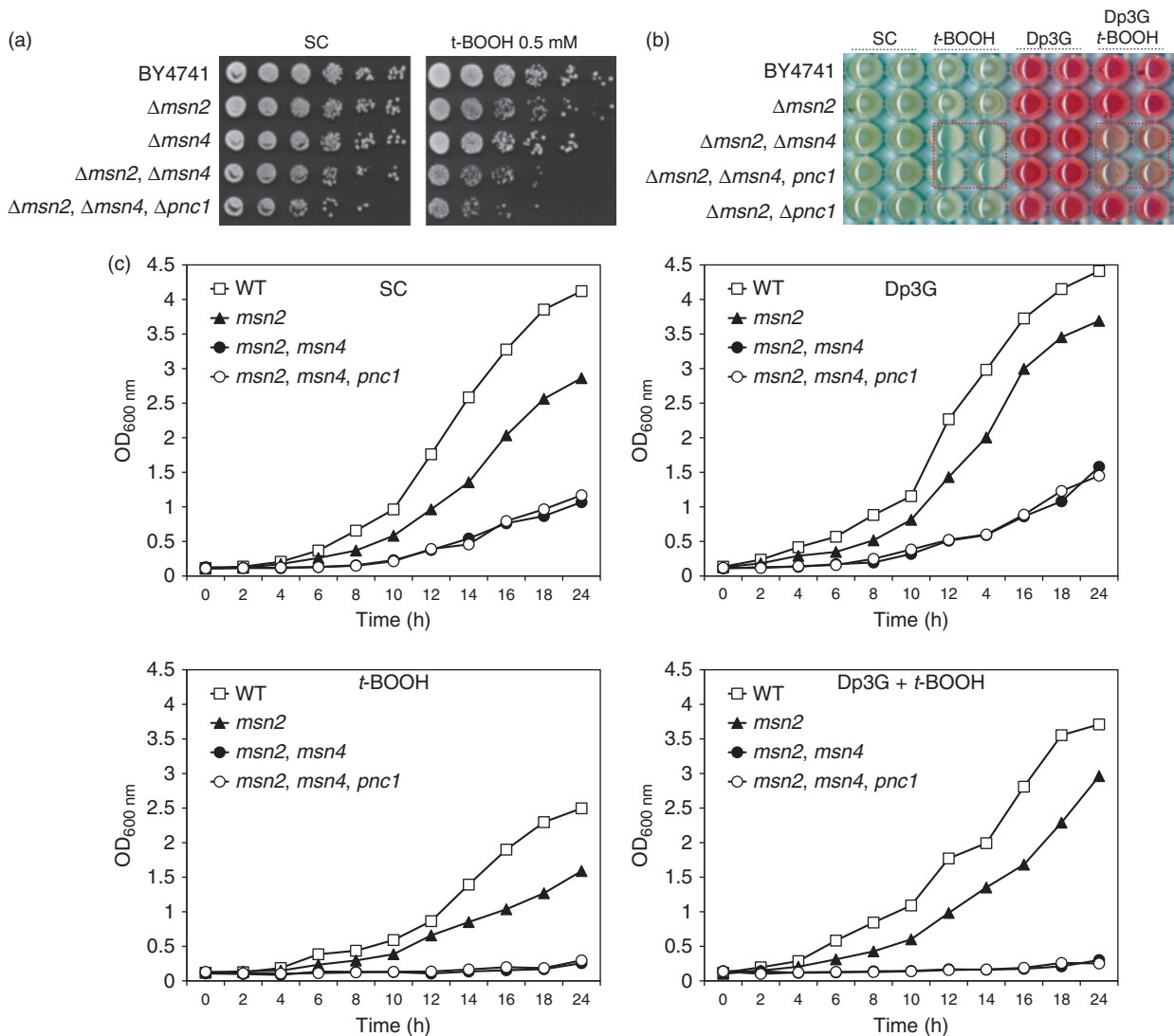


Fig. 4. Dp3g protects *Saccharomyces cerevisiae* against oxidative stress in an MSN2- and MSN4-dependent manner. (a) Plate assay of BY4741 and $\Delta msn2$, $\Delta msn4$, $\Delta pnc1$ mutants in the presence of 0.5 mM *t*-BOOH. (b) Microtiter plate of duplicate liquid cultures of BY4741 and $\Delta msn2$, $\Delta msn4$, $\Delta pnc1$ mutants after 24 h. Dp3g (25 $\mu\text{g mL}^{-1}$) and/or *t*-BOOH (0.5 mM) was added when indicated. Wells corresponding to the double or the triple $\Delta msn2$, $\Delta msn4$ or $\Delta msn2$, $\Delta msn4$, $\Delta pnc1$ without cellular growth are marked with a red square. (c) Growth kinetics of BY4741 and $\Delta msn2$, $\Delta msn4$, $\Delta pnc1$ mutants in either SC medium or SC media containing Dp3g (25 $\mu\text{g mL}^{-1}$) and/or *t*-BOOH (0.5 mM). In all assays, the corresponding volume of wine-like solution was also added to the negative controls.

CTT1 increased immediately after the addition of the anthocyanins and decreased rapidly after 30–60 min (Fig. 7).

Discussion

Understanding the molecular mechanisms underlying the 'French paradox' has contributed to the growing interest in investigating the biological activity of the polyphenols of red wine. Thus, recent studies have shown that many of the constituents of red wine, such as resveratrol and its derivatives, may serve as nutraceuticals (Baur *et al.*, 2006; Feige

et al., 2008; Pearson *et al.*, 2008). In addition, the idea that stress-derived plant molecules may act as chemical vaccines against stress, activating certain signalling protective pathways, in heterotrophs organisms (i.e. xenohormesis) is being widely accepted (Lamming *et al.*, 2004; Howitz & Sinclair, 2008).

Relatively little is known about the nature of absorption and the *in vivo* metabolism of polyphenols in humans. Indeed, the apparent bioavailability of polyphenols has been shown to be very low. However, recent studies have reported that, after oral intake, a high proportion of anthocyanins

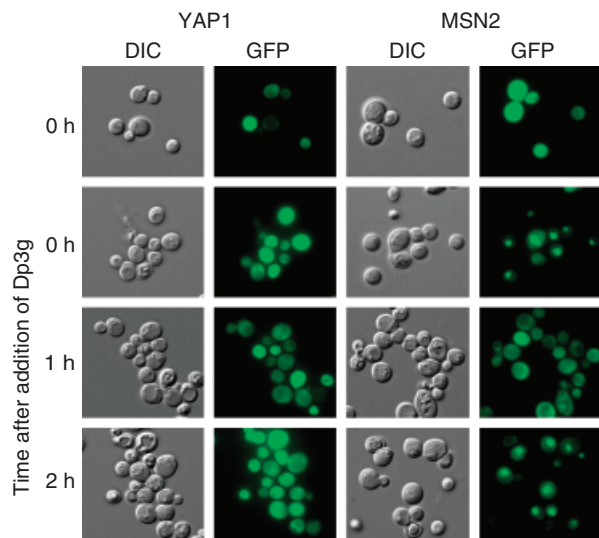


Fig. 5. Effect of Dp3g on the intracellular localization of Yap1 and Msn2. BY4741 cells transformed with either YAP1-GFP or MSN2-GFP plasmids were treated with Dp3g ($12.5 \mu\text{g mL}^{-1}$) and the distribution of the GFP fluorescence was visualized at different time-points.

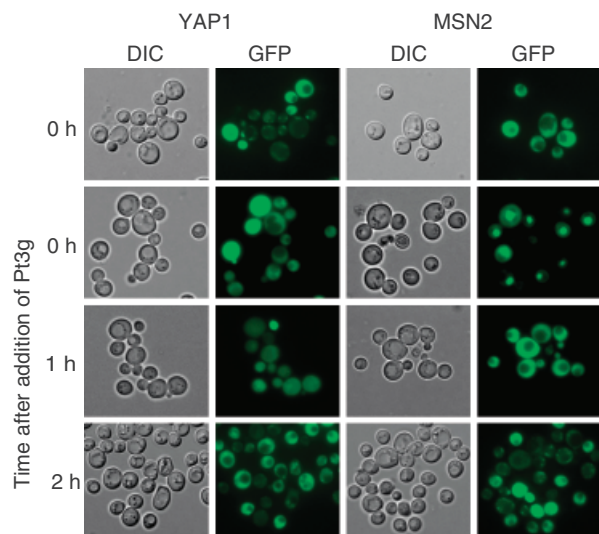


Fig. 6. Effect of Pt3g on the intracellular localization of Yap1 and Msn2. BY4741 cells transformed with either YAP1-GFP or MSN2-GFP plasmids were treated with Pt3g ($20 \mu\text{g mL}^{-1}$) and the distribution of the GFP fluorescence was visualized at different time-points.

could pass the small intestine unmetabolized and reach the colon, where the anthocyanins are absorbed (Kahle *et al.*, 2006; Neto, 2007; Knaup *et al.*, 2009). Furthermore, it has been described that some flavonoids including delphinidin exert some of their activities through their binding to receptors at the plasma membrane and activating important signalling pathways without entering the cell (Fridrich *et al.*, 2008; Kwon *et al.*, 2009; Teller *et al.*, 2009).

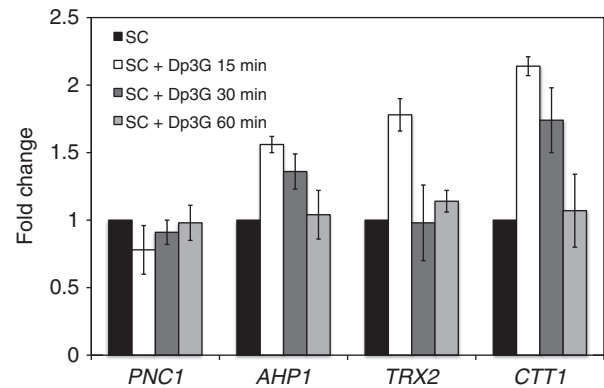


Fig. 7. Relative transcription levels of *PNC1*, *TRX2*, *AHP1*, and *CTT1* genes after various Dp3g treatments. Total mRNA was obtained from BY4741 cells at different time points (0, 15, 30, and 60 min) after the addition of Dp3g ($25 \mu\text{g mL}^{-1}$). Transcription levels are normalized using the *ACT1* gene as a reference. Quantitative analyses were performed using the LIGHTCYCLER 480 software. The results are the average of two independent experiments and are expressed as a ratio of the cDNA abundance of the target genes in the cultures with Dp3g with respect to that without Dp3g. Error bars indicate the SD.

To gain further insight into the cellular effects of such phenolic compounds at the molecular level, here, we wished to investigate whether the colored phenolic fractions of red wine might trigger a xenohormetic response in eukaryotic cells. We therefore decided to perform a phenotypic screening with HPLC-fractionated polyphenols in a set of *S. cerevisiae* deletion mutants lacking the genes involved in redox metabolism.

A Tempranillo red wine was fractionated after postfermentative maceration, and anthocyanin-derived pigments, which are formed during the later steps of the maturation process, were found in a very low proportion. In contrast, monoglucosylated anthocyanins were the red pigments with the highest concentrations in the samples obtained.

The effect of the phenolic fractions on the growth of *S. cerevisiae* mutants was assessed under both nonoxidative and oxidative conditions (using H_2O_2 or *t*-BOOH). Fractions F6, F2, F3, and to a lesser extent F7, were the most active fractions as regards the increase in the growth rate of the mutants when H_2O_2 was included in the analysis; however, among them, only F6 was also able to reduce the cytotoxicity of *t*-BOOH in a significant number of strains. Surprisingly, fraction F5 abolished the toxicity of *t*-BOOH in 80% of the mutants analyzed, and was only seen to be inactive in hypersensitive strains such as *ahp1*. An additional analysis of the F5 and F6 fractions, which were enriched in red pigments, was carried out on the wild-type BY4741 and the *ahp1*, *grx5*, and *tsa2* mutants of *S. cerevisiae*, which have been shown to be hypersensitive to oxidants and thus have a constitutive level of oxidative stress. Our results showed that both fractions F5 and F6 conferred protection

against the oxidative stress in the mutant strains and did not reveal significant differences between the treatments. Thus, it could be speculated that the molecular mechanism underlying the effect of the red pigments in fractions F5 and F6 may be comparable. The mutant *ahp1* is extremely sensitive to hydroperoxides and thus unable to grow in the presence of *t*-BOOH.

Cells adapt to environmental changes by modifying their gene expression patterns, mainly through the activation of transcription factors. Several flavonoids such as resveratrol or curcumin have been proposed to regulate the signalling pathways involved in cellular energy supply or stress response in mammals (Howitz & Sinclair, 2008). Previous studies have shown that green tea polyphenols are able to activate oxidative-stress-responsive transcription factors in yeast (Takatsume *et al.*, 2005; Maeta *et al.*, 2007). Yap1 and Msn2/Msn4 are stress-responsive transcription factors that are localized to the nucleus during oxidative stress or under other starving conditions (Temple *et al.*, 2005). We hypothesized that red wine pigments might also promote the nuclear accumulation of those transcription factors, which in turn might trigger the induction of stress-responsive genes and consequently alleviate the effect of oxidative stress. Accordingly, GFP-fusion proteins were used to analyze the subcellular localization of Yap1 and Msn2 upon treatment with fractions F5 and F6. It is remarkable that significant differences were observed between the treatments with fractions F5 and F6; these could be attributed to the different compositions of the phenolic fractions. The effect of the F5 and F6 fractions on Msn2 can certainly be attributed to the monoglucosylated anthocyanins Pt3g and Dp3g. These anthocyanins represent > 75% of the total monoglucosylated anthocyanins in fractions F5 and F6. Indeed, our analyses using pure Pt3g or Dp3g demonstrated that both compounds were able to induce the nuclear import of Msn2-GFP. This effect was completely reversible with Pt3g, as also reported for catechins and green tea extracts (Maeta *et al.*, 2007); however, when Dp3g was used, the nuclear localization of Msn2-GFP was recurrent after 2 h. The structural difference among the anthocyanins tested is the substitution in the B ring. Dp3g contains a gallo catechol structure (3', 4', and 5'-hydroxyl groups), whereas Pt3g contains two hydroxyl groups and one methoxyl group. This variation might be relevant for explaining the behavior of the two anthocyanins examined here. In fact, in a previous study, the authors proposed that it is mainly the presence of the gallo catechol group that determines the prominent *in vitro* antioxidant potential of Dp3g (Garcia-Alonso *et al.*, 2004) and it might also be responsible for the antiangiogenic properties of delphinidin (Lamy *et al.*, 2006). Therefore, the structure of Dp3g provides a chemical reducing potential, which might be responsible for the periodicity in the nucleocyto-

plasmic trafficking of Msn2-GFP when either F6 or Dp3g was used.

Both Dp3g and Pt3g improved the fitness of *S. cerevisiae* and induced a significant resistance to oxidative stress. Moreover, this effect is completely abolished in mutant strains lacking both *MSN2* and *MSN4* genes, indicating that both transcription factors are involved in the biological activity of these wine anthocyanins. This suggests that both Dp3g and Pt3g might induce a xenohormetic response in *S. cerevisiae* that is related to the environmental stress response (Gasch *et al.*, 2000; Causton *et al.*, 2001). In this sense, it has been described that the nuclear accumulation of Msn2 induces the expression of *PNC1*, a nicotinamidase gene that regulates sirtuin activity and extends life span (Medvedik *et al.*, 2007). However, we found that Dp3g did not cause a significant change in the transcription level of *PNC1* after 1 h of treatment, although Msn2-GFP is effectively localized to the nucleus. Again, this suggests that Dp3g triggers a specific xenohormetic response through Msn2/Msn4, which was mainly related to the antioxidant defense as evidenced by the increased transcription of the antioxidant genes *TRX2*, *AHP1*, and *CTT1*, which are also Msn2 targets (Schmitt & McEntee, 1996). Indeed, the induction of *AHP1* expression after Dp3g treatment is consistent with the hypersensitivity to hydroperoxide of the Δ *ahp1* strain and the ineffectiveness of the polyphenols to rescue the growth capacity of this mutant. The activation gene expression kinetics resembles that of the environmental stress response and it might be speculated that these plant polyphenols could activate an adaptive response in *S. cerevisiae* as described for the acquired stress resistance (Berry & Gasch, 2008).

The effect of the F5 fraction on Yap1 cannot be attributed either to Pt3g or to Dp3g. In this case, other constituents of the F5 fraction such as flavonols, which represent 35% of the total phenolic composition, could be responsible for the relocalization of Yap1. In this sense, previous studies have reported a pro-oxidant activity of catechins and other flavanols present in green tea, which may trigger the intramolecular disulfide bonding of Yap1 and hence the nuclear localization of Yap1 (Maeta *et al.*, 2007; Wang *et al.*, 2009). Nonetheless, synergistic interactions between different components of the fraction F5 might also be responsible for the Yap1 relocalization.

The chemical features of Pt3g and Dp3g as natural colorants together with their bioactivity seem to offer a good possibility of using these compounds as nutraceutical food additives. Anthocyanins have been proposed to possess beneficial effects against different human diseases (Hou, 2003; Leifert & Abeywardena, 2008), but further studies using animal models would be required to determine whether monoglucosylated anthocyanins are indeed beneficial to human health.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phenotypic screening of red wine fractions in a subset of yeast mutant strains.

Fig. S2. Yap1-GFP and Msn2-GFP accumulate in the nuclei of *Saccharomyces cerevisiae* cells after the addition of phenolic fractions.

Table S1. Primers used in this study.

Table S2. *Saccharomyces cerevisiae* strains used in this study.

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