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Antioxidant Small Molecules Confer Variable Protection against Oxidative Damage in Yeast Mutants

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To assess the capacity of small molecules to function as antioxidants in pathologic conditions, a set of yeast assays utilizing strains deficient in the antioxidant machinery was applied with measurements of reactive oxygen species (ROS), glutathione (GSH/GSSG), and induction of the stress responsive proteins oye2 and oye3. Yeast strains deficient in superoxide dismutase (Δ*sod1*), catalase A (Δ*cta1*), and double-deficient in Old Yellow enzyme 2 and glutathione reductase 1 (Δ*oye2 glr1*) were supplemented with ascorbic acid, β-carotene, caffeic acid, or quercetin, subjected to pro-oxidant insult, and monitored for growth recovery. Ascorbic acid and caffeic acid protected cells under most circumstances, whereas β-carotene and quercetin protection was highly context dependent, exhibiting protection in some cases and inhibition in others. β-Carotene and quercetin elevated substantially endogenous levels of ROS in some yeast mutants. Quercetin supplementation increased significantly GSH and GSSG levels but could not maintain GSH levels in H_2O_2-exposed cells. Induction of the stress response machinery was manifested by the strong up-regulation of a chromosomally encoded OYE2−GFP fusion. In the case of quercetin, there was simultaneous induction of OYE3−GFP, which was previously shown to sensitize cells to H_2O_2-induced programmed cell death (PCD). Taken together, the results show that mutations in the antioxidant machinery affect significantly the capacity of dietary antioxidants to protect cells.

KEYWORDS: Antioxidants; oxidative damage; glutathione; Old Yellow enzymes; yeast

INTRODUCTION

Aerobic organisms counter oxygen toxicity by operating an antioxidant defense system capable of neutralizing and removing reactive oxygen species (ROS), as well as eliminating damaged cell constituents and repairing the damage. Disturbance of the pro-oxidant antioxidant balance in favor of the former will cause oxidative damage and eventual cell death. Several pathological conditions such as neurodegenerative disorders, cancer, atherosclerosis, and cardiac disease have been linked to impairment of the antioxidant machinery (*1*). Research in the yeast *Saccharomyces cerevisiae* has provided considerable information on oxidative stress and the mechanisms employed by cells in response to increased ROS. An elaborate enzymatic machinery consisting of superoxide dismutases, reductases, catalases, peroxiredoxins, glutaredoxins, and glutathione transferases is utilized to maintain redox balance (*2*). In addition to the enzymes, antioxidant small molecules are produced by the organism or taken up from the environment, which can act to delay or prevent oxidation of intracellular substrates such as lipids, DNA, or proteins. Such compounds have been at the center of an intense focus of research for their association with health-promoting properties (*3*). However, their antioxidant effects have been questioned in several studies, and for some of them pro-oxidant properties have also been attributed (*4, 5*). Four frequently utilized compounds, ascorbic acid, β-carotene, caffeic acid, and quercetin, were selected for study. Ascorbate (vitamin C) is essential in the human diet for the prevention of scurvy, functions as a cofactor for lysyl and prolyl hydroxylases, and is a good reducing agent. It can be oxidized by most of the reactive oxygen species that are thought to contribute to tissue

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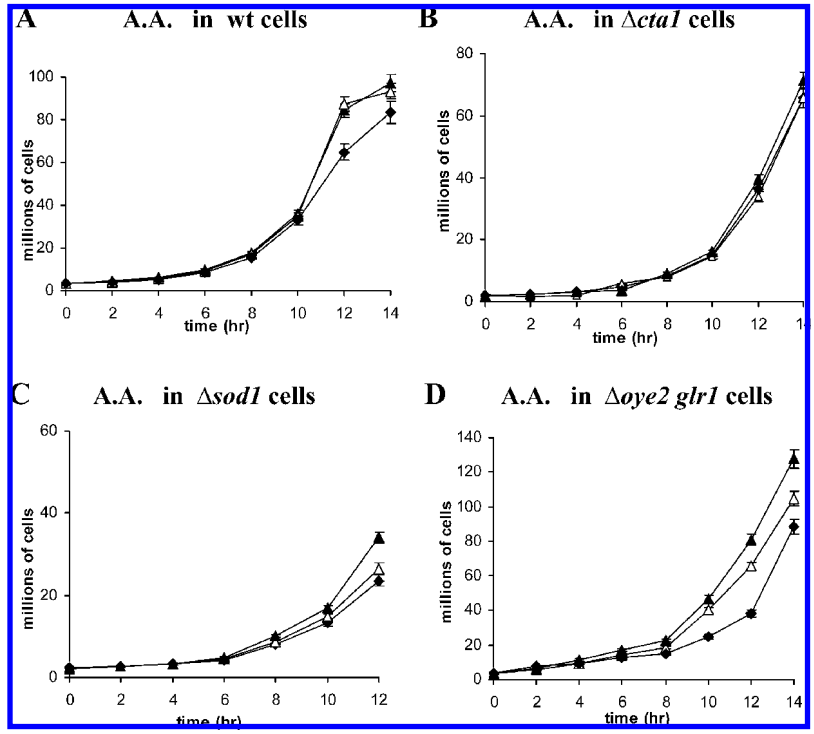

Figure 1. Effect of ascorbic acid on growth in H_2O_2-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 1.25 mM H_2O_2 (◆). Treated cells were additionally supplemented with ascorbic acid (AA) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Changes in growth recovery assays conducted in triplicate are observed at early time points (wt 4 h, 5 μM $P = 0.0065$, 50 μM $P = 0.0001$; Δ*cta1* 6 h, 5 μM NS, 50 μM $P = 0.002$; Δ*sod1* 6 h, 5 μM $P = 0.008$, 50 μM $P = 0.002$; Δ*oye2 glr1* 4 h, 5 μM NS, 50 μM $P = 0.0004$).

injury in several human diseases (*6*). Thus, ascorbate converts ROS into poorly reactive ascorbate-derived products. Yeast does not synthesize ascorbate but a five-carbon analogue, erythroascorbate, that is not thought to play a significant antioxidant role in the cell (*7*). However, a series of data point to a strong protective function of ascorbate in yeast (*8, 9*). β-Carotene, a terpene composed of eight isoprene units, is a member of the carotene family of photosynthetic pigments and is a potent antioxidant. It received high interest for cancer treatment but was eventually found to increase the rates of cancer in smokers (*10*). Caffeic acid and its derivative caffeic acid phenyl ester (CAPE) have been repeatedly linked with anticancer activities, functioning as signaling modulators during angiogenesis and apoptosis, as well as with antifungal activities (*11, 12*). The phenolic compound quercetin has received extensive interest not only for its antioxidant properties but also for its anticarcinogenic effects (*13*). In a recent study in yeast employing brief pulses with quercetin, it was shown that quercetin increased oxidative stress resistance and extended longevity (*14*).

S. cerevisiae strains harboring defects in the antioxidant machinery can be used to emulate the altered intracellular redox environments, frequently encountered in human pathologic conditions, and assess the capacity of antioxidant small molecules to protect against oxidative insults (*8, 9, 14*−*17*). The first strain chosen had a deletion in the gene catalase A (Δ*cta1*). Catalase A is primarily found in the peroxisomal matrix and in mitochondria is responsible for neutralizing H_2O_2 (*18*). Yeasts have two catalase enzymes, ctt1p and cta1p, but only the deletion of *cta1* rendered cells more sensitive to oxidative stress (*19*). The second strain lacks the cytosolic superoxide dismutase 1 (Δ*sod1*), which catalyzes the dismutation of superoxide $O_2^{\bullet-}$ to H_2O_2. Deletion of *sod1* increases endogenous ROS, sensitizes cells to pro-oxidant insult, and causes methionine auxotrophy. Mutations of SOD1 in humans are linked to familial amyotrophic lateral sclerosis (*20*). The two other strains employed show defects in glutathione metabolism. The strain Δ*gsh1* cannot synthesize glutathione and is unable to grow in minimal media without the addition of exogenous GSH. Glutathione is an essential metabolite for resistance to oxidative stress (*21, 22*). Changes in glutathione homeostasis have been associated with aging and Parkinson's disease (*23*). Glutathione reductase is the enzyme responsible for recycling oxidized glutathione back to the reduced form. A *glr1* mutant strain exhibits high sensitivity to oxidants (*24*). The double-knockout strain Δ*oye2 glr1*, which lacks the NADPH-oxidoreductase OYE2 and the glutathione reductase enzymes, exhibits a dramatically altered GSH/GSSG ratio in favor of the latter, higher than *glr1 cells* (*19, 24*). Old Yellow enzymes (OYE) have been implicated in modulating oxidative stress responses program cell death responses by altering actin polymerization (*19, 25, 26*). Deletion of OYE2 renders cells more sensitive to H_2O_2-induced programmed cell death (PCD) (*19*). The oye2 protein was shown to bind to actin at the proximity of two cysteine residues (cys^{374} and cys^{285}), which are susceptible to oxidation or glutathionylation. Changes in their status of oxidation can accelerate cell death in yeast and are countered by oye2 binding (*25, 26*). Modification of these cysteine residues is a universal phenomenon and has been implicated in human pathologies (*27*).

To address the capacity of small dietary antioxidant molecules to promote cell survival from oxidative damage, we tested four frequently utilized compounds, ascorbic acid, β-carotene, caffeic acid, and quercetin. Yeast strains compromised in important components of their antioxidant machinery were used as model systems to emulate common cellular dysfunctions in oxidative stress. Hydrogen peroxide and the organic pro-oxidant cumene hydroperoxide (CHP) were used as pro-oxidant insults in growth recovery assays. The results of the current study show that ascorbic acid and caffeic acid exerted a protective effect under

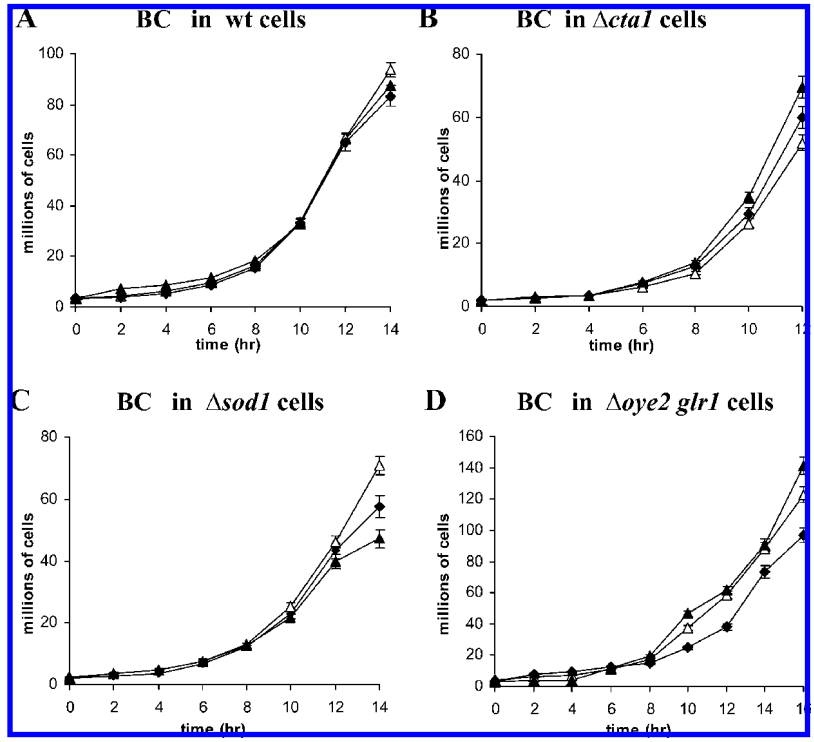

Figure 2. Effect of β-carotene on growth in H_2O_2-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 1.25 mM H_2O_2 (◆). Treated cells were additionally supplemented with β-carotene (BC) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Statistically significant changes are observed at early time points (wt 4 h, 5 μM $P = 0.0019$, 50 μM NS; Δ*cta1* 6 h, 5 μM $P = 0.0004$ inhib, 50 μM $P = 0.01$; Δ*sod1* 6 h, 5 μM NS, 50 μM $P = 0.008$; Δ*oye2 glr1* 4 h, 5 μM NS, 50 μM $P = 0.04$).

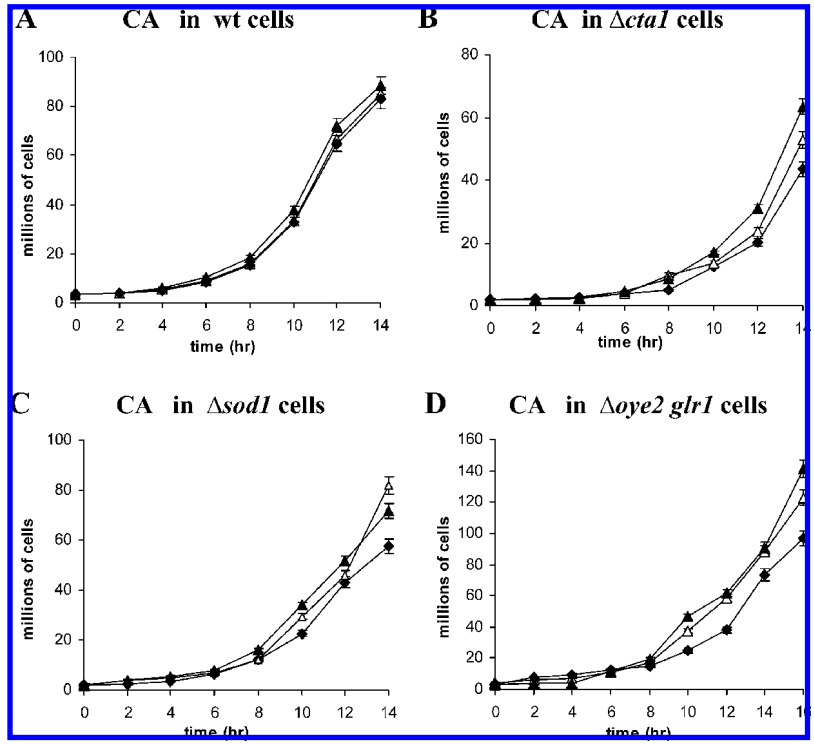

Figure 3. Effect of caffeic acid on growth in H_2O_2-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 1.25 mM H_2O_2 (◆). Treated cells were additionally supplemented with caffeic acid (CA) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Statistically significant changes are observed at early time points (wt 4 h, 5 μM $P = 0.02$, 50 μM $P = 0.01$; Δ*cta1* 6 h, 5 μM NS, 50 μM $P = 0.0002$; Δ*sod1* 6 h, 5 μM NS, 50 μM $P = 0.001$; Δ*oye2 glr1* 4 h, 5 μM NS, 50 μM NS).

most settings, unlike β-carotene and quercetin, which varied dramatically in their effect depending on the cell context and concentration employed. Both compounds increased intracellular ROS. In particular, quercetin at 50 μM induced glutathione synthesis and expression of high oye2 and oye3 protein levels, previously linked to H_2O_2 cell death sensitization (*19*). Thus,

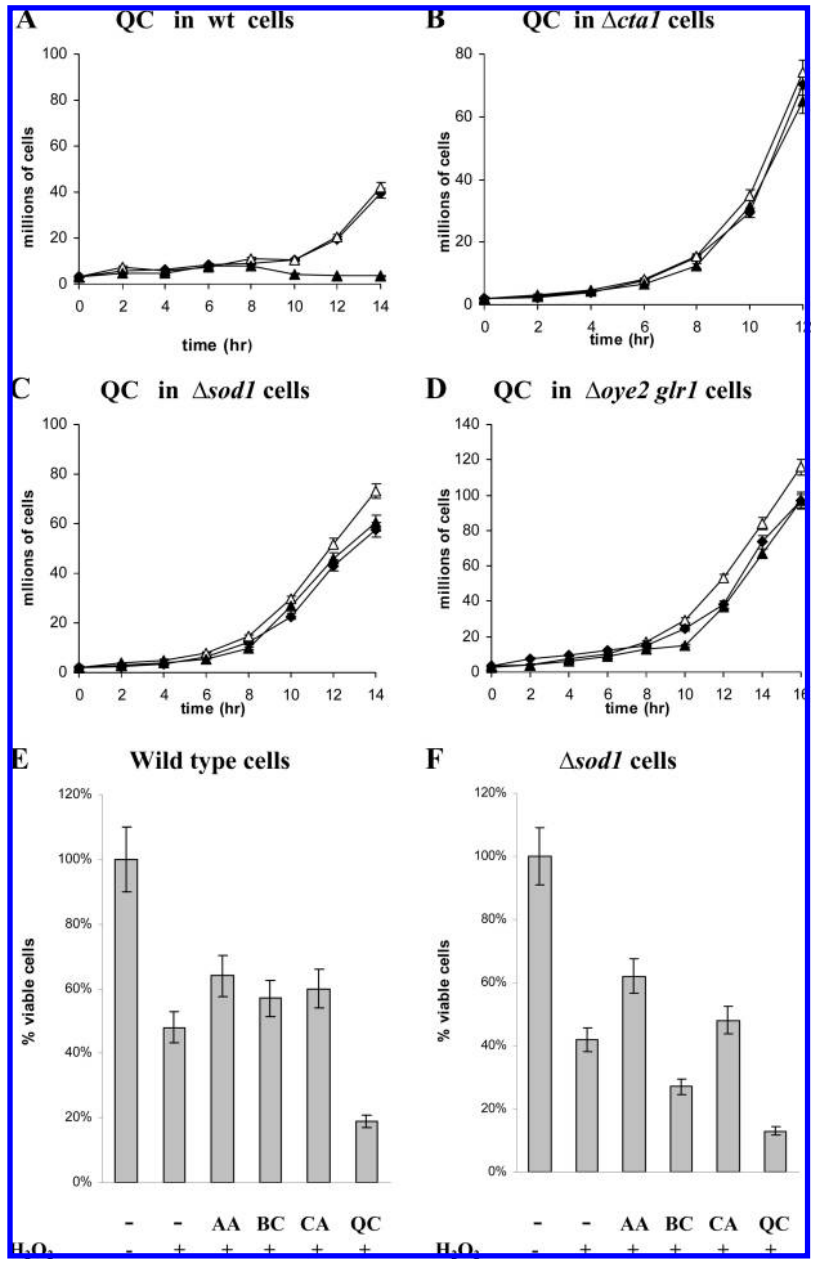

Figure 4. Effect of quercetin on growth in H_2O_2-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 1.25 mM H_2O_2 (◆). Treated cells were additionally supplemented with quercetin (QC) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Statistically significant changes are observed at early time points (wt 4 h, 5 μM NS, 50 μM $P = 0.0045$ inhib; Δ*cta1* 6 h, 5 μM NS, 50 μM NS; Δ*sod1* 6 h, 5 μM NS, 50 μM NS; Δ*oye2 glr1* 4 h, 5 μM NS, 50 μM $P = 0.023$ inhib). Wild type (*E*) and Δ*sod1* (**F**) cells grown at midlog phase were used to inoculate glucose CM media supplemented with 50 μM of each antioxidant small molecule. Cells were immediately insulted with 1.5 mM H_2O_2 and incubated with shaking at 30 °C for 2 h. Viable cells were enumerated by plating dilutions on YPD plates, taken prior or subsequent to treatment. All experiments were performed independently in triplicate. The effects of antioxidant supplementation were statistically significant (AA, $P = 0.0086$ wt, $P = 0.0006$ Δ*sod1*; BC, $P = 0.046$ wt, $P = 0.0007$ Δ*sod1*; CA, $P = 0.036$ wt, $P = 0.032$ Δ*sod1*; QC, $P = 0.001$ wt, $P = 0.0001$ Δ*sod1*).

quercetin triggers the stress responsive machinery as well as signaling components, which may determine the final outcome in cell viability.

MATERIALS AND METHODS

Yeast Strains. The *S. cerevisiae* strain BY4741 and the derived deletion mutants Δ*sod1*, Δ*cta1*, and Δ*gsh1* were obtained from Research Genetics. The double-knockout strain Δ*oye2 glr1* was previously described (*19*). Green Fluorescent Protein fusions to the C terminus of the chromosomal genes OYE2 and OYE3 were constructed as described by Longtine et al. (*28*). The primers OYE2-F2 5′-TTGACTACCCTACGTACGAAGAA-GCTCTAAAACTCGGTTGGGACAAAAATCGGATCCCCGGGTTA-ATTAA-3′ with OYE2-R1 5′-AGTTCTGTTAATATTTCATTAAAG-GCTATTTATCCCAAATATATATATTCGAATTCGAGCTCGTTTA-AAC-3′ and OYE3-F2 5′-CTACCCAACATATGAAGAGGCAGTA-GATTTAGGTTGGAACAAGAACCGGATCCCCGGGTTAATTAA-3′ with OYE3-R1 5′-TTATTGATGTATCAAATATAAATATCTA-TATGTGAGTTTTGTAAGAATTCGAGCTCGTTTAAAC-3′ were used to PCR amplify the homologous recombination module pFA6a (GFP F64A, S65T, R80Q, V163)-HIS3MX6. BY4741 wild-type cells were transformed with the linear amplified products and selected in glucose Complete Minimal (CM) media lacking histidine. To verify proper insertion of the GFP module, genomic DNA was extracted from several growing colonies and used in PCR reactions and the OYE2(1110) 5′-GACAGAGA-

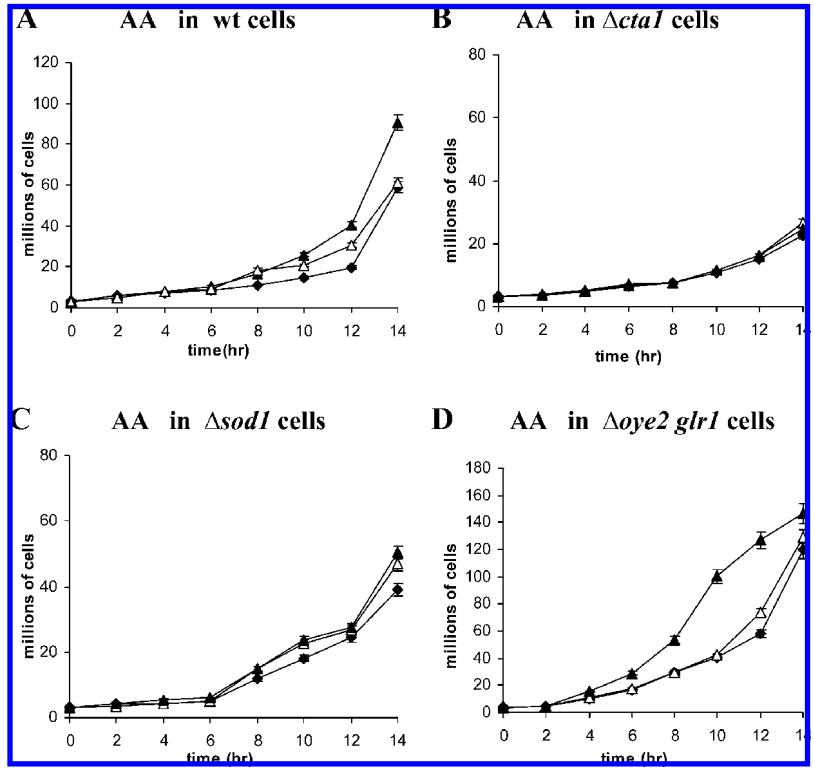

Figure 5. Effect of ascorbic acid on growth in CHP-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 60 μM (Δ*sod1*) or 80 μM CHP (◆). Treated cells were additionally supplemented with ascorbic acid (AA) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Statistically significant changes are observed at early time points (wt 4 h, NS, 50 μM $P = 0.0038$; Δ*cta1* 6 h, 5 μM NS, 50 μM NS; Δ*sod1* 6 h, 5 μM NS, 50 μM $P = 0.001$; Δ*oye2 glr1* 4 h, 5 μM NS, 50 μM $P = 0.0002$).

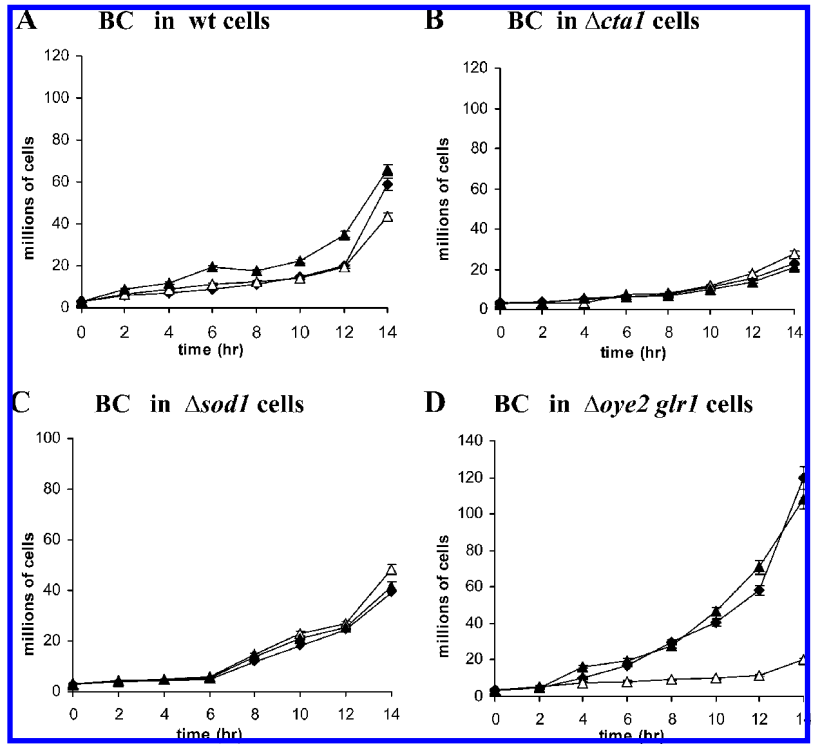

Figure 6. Effect of β-carotene on growth in CHP-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 60 μM (Δ*sod1*) or 80 μM CHP (◆). Treated cells were additionally supplemented with β-carotene (BC) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Statistically significant changes are observed at early time points (wt 4 h, 5 μM NS, 50 μM $P = 0.0033$; Δ*cta1* 6 h, 5 μM $P = 0.02$, 50 μM NS; Δ*sod1* 6 h, 5 μM 0.02, 50 μM NS; Δ*oye2 glr1* 4 h, 5 μM NS, 50 μM NS).

CACTTTCTACAAAATG-3′ with pFA6a conf 5′-GCAACCTGACCTACAGGAAAGAGTT-3′ primers or the OYE3(1110) 5′-GACAGAAGTACCTTCTACACCATGTCC-3′ with pFA6a conf primers.

Growth Recovery Curves. Growth recovery curves were performed as previously described (*19*). To examine the effect of small molecule antioxidants, ascorbic acid (100 mM stock in H_2O), β-carotene (10 mM

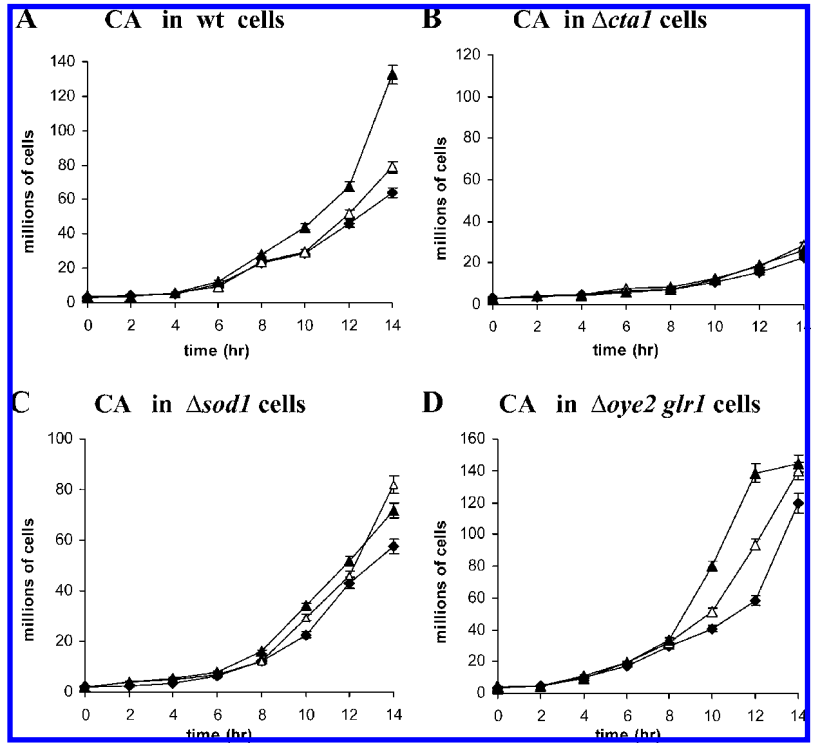

Figure 7. Effect of caffeic acid on growth in CHP-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 60 μM (Δ*sod1*) or 80 μM CHP (◆). Treated cells were additionally supplemented with caffeic acid (CA) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Statistically significant changes are observed at early time points (wt 4 h, 5 μM $P = 0.0004$, 50 μM $P = 0.0001$; Δ*cta1* 6 h, 5 μM NS, 50 μM NS; Δ*sod1* 6 h, 5 μM $P < 0.0001$, 50 μM $P < 0.0001$; Δ*oye2 glr1* 4 h, $P = 0.0007$, 50 μM $P = 0.002$).

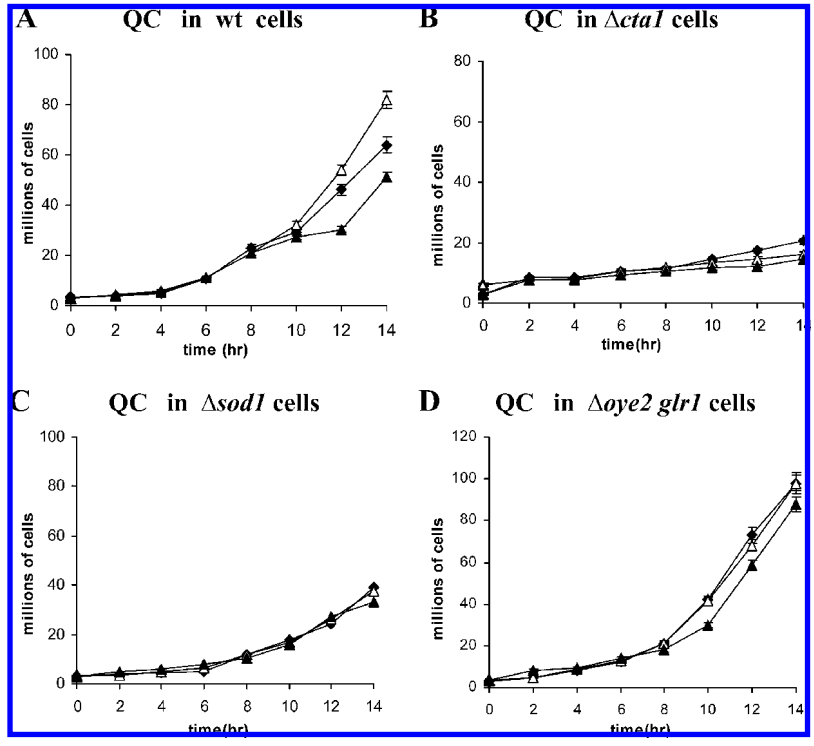

Figure 8. Effect of quercetin on growth in CHP-treated cells. BY4741 wild-type cells (**A**), Δ*cta1* cells (**B**), Δ*sod1* cells (**C**), and Δ*oye2 glr1* cells (**D**) were treated with 60 μM (Δ*sod1*) or 80 μM CHP (◆). Treated cells were additionally supplemented with quercetin (QC) at 5 μM (△) and 50 μM (▲). Growth was monitored by measuring OD_{600} every 2 h. Statistically significant changes are observed at early time points (wt 4 h, 5 μM $P = 0.01$, 50 μM NS; Δ*cta1* 6 h, 5 μM NS, 50 μM NS; Δ*sod1* 6 h, 5 μM NS, 50 μM NS; Δ*oye2 glr1* 4 h, 5 μM NS, 50 μM NS).

stock in acetone), caffeic acid (10 mM stock in methanol), and quercetin (10 mM stock in DMSO) were added to glucose CM media at concentrations of 5 and 50 μM. H_2O_2 at 1.25 mM final concentration and cumene hydroperoxide (CHP) at 60 μM (for Δ*sod1*) or 80 μM final concentration were added at the end of the lag period, and cells were further incubated by shaking at 30 °C. The ability of the cell population to recover from pro-oxidant insult was assessed by measuring growth at OD_{600nm} at regular intervals. All growth recovery assays were

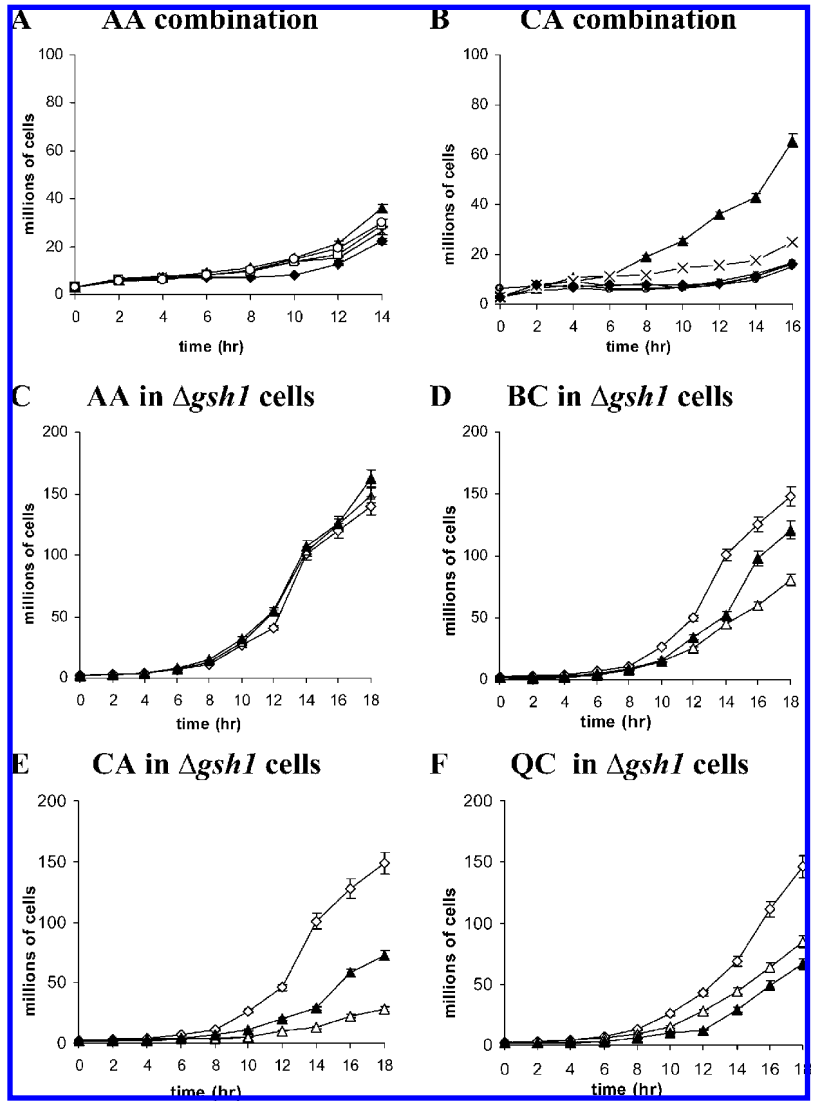

Figure 9. Effect on growth of binary combination of ascorbic acid (**A**) and caffeic acid (**B**) at high concentrations with all other antioxidant molecules at low concentrations (50 μM + 5 μM = 55 μM total antioxidants); (▲) ascorbic acid only (**A**) and caffeic acid only (**B**), (×) + β-carotene, (□) + caffeic acid (**A**) or ascorbic acid (**B**), (○) + quercetin. Δ*gsh1* cells growing in CM media with 100 nM GSH (◇) were supplemented with 5 μM (△) and 50 μM (▲) of ascorbic acid (**C**) β-carotene (**D**), caffeic acid (**E**), and quercetin (**F**).

performed independently in triplicate. For Δ*gsh1* cells growth assays were performed as follows: freshly grown Δ*gsh1* cells in YPD media were washed with H_2O and resuspended in glucose CM media with the addition of specific concentrations of reduced glutathione (GSH). Cells were monitored for growth as above. Measurement values from independent experiments of the earliest time points at which the effects were observable, that is 4 h for wild-type and Δ*oye2 glr1* cells and 6 h for the slower growing Δ*sod1* and Δ*cta1*, were used for analysis by the GraphPad statistical software.

Viability Colony Assays. Wild-type and Δ*sod1* cells were freshly grown overnight in glucose CM media (*29*). The next day, the cells were resuspended in new Glu/CM media at OD_{600} = 0.1 and were incubated with shaking at 30 °C until they reached OD_{600} = 0.5. A small aliquot was used to inoculate fresh media that were supplemented with 50 μM of the antioxidant small molecules. Time point 0 samples were quickly taken, and dilutions were plated on YPD plates. H_2O_2 was immediately added to a final concentration 1.5 mM, and cells were incubated for 2 h at 30 °C. At the end of incubation, dilutions were plated on YPD plates and cells were allowed to grow for 72 h. All experiments were performed independently in triplicate. The results were statistically analyzed as above.

Microscopy and Fluorescence Measurements. Yeast cells growing in glucose CM at midlogarithmic phase OD_{600} = 0.5 were split into 1 mL aliquots in Eppendorf tubes. Cells were washed with PBS and resuspended in an equal volume of PBS. Antioxidants were added at final concentrations of 5 and 50 μM or left untreated, and cells were incubated with shaking for 30 min at 30 °C. At the end of the incubation period cells were washed again with PBS and stained with 0.5 μM Mitotracker Red CMXRos (M7512, Molecular Probes) in PBS for 15 min in the dark with occasional shaking. At the end of the incubation period cells were washed with PBS and resuspended in 2 mL of PBS. Fluorescence was measured using a Perkin-Elmer LS55 instrument at 540 nm excitation and 560−750 nm emission wavelengths.

Cells at mid logarithmic phase harboring endogenous OYE2−GFP or OYE3−GFP fusions were treated with the antioxidant small molecules in PBS for 2 h and were observed for induction of GFP fluorescence under ×1000 magnification in a fluorescent microscope.

Determination of Total Glutathione and Glutathione Disulfide. BY4741 wild-type cells were grown overnight in 20 mL of glucose/CM. The next day cells were resuspended in 400 mL of medium at OD_{600} = 0.1 and grown with shaking at 30 °C until OD_{600} = 0.5. The culture was subsequently split into four equal volumes. Flask A was left untreated, flask B was supplemented with 50 μM quercetin, and flask C was supplemented with 50 μM quercetin and 1 mM H_2O_2. The cultures were further incubated for 5 h with shaking at 30 °C. At the end of the incubation period cells were repeatedly washed with dH_2O. Total glutathione and glutathione disulfide were measured according to a method described by Griffith (*30*) and modified by Kampranis et al. (*31*).

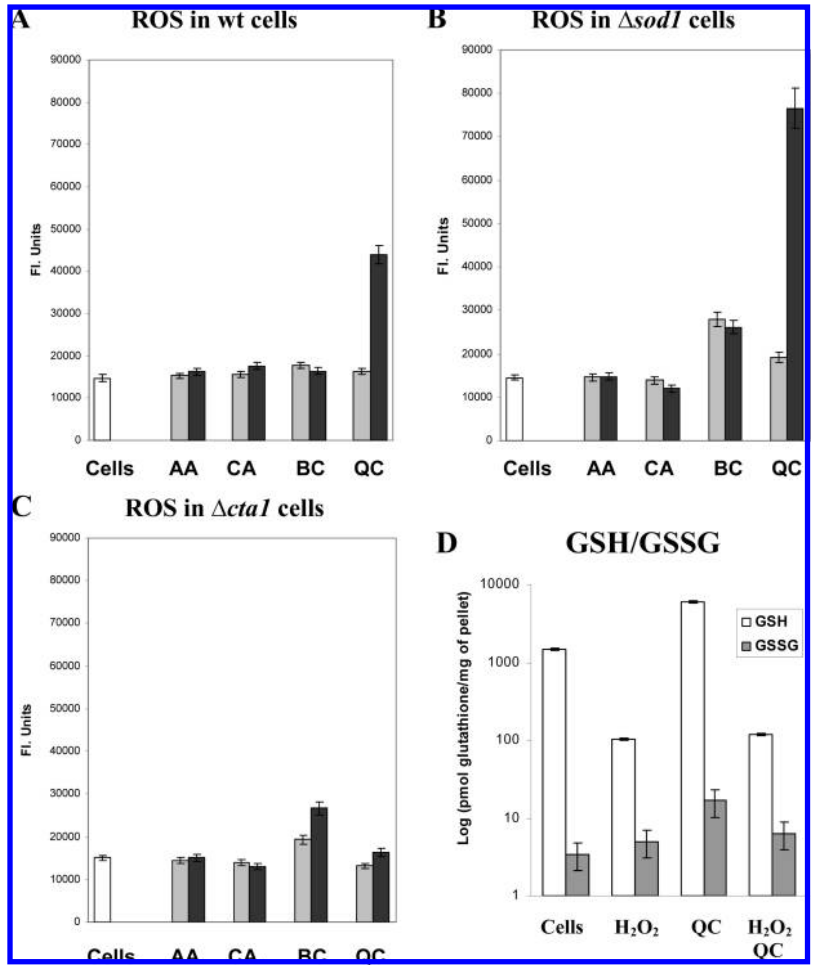

Figure 10. Intracellular ROS levels and glutathione levels of cells treated with antioxidants. Wild-type BY4741 yeast cells (**A**), Δ*sod1* (**B**), and Δ*cta1* (**C**) cells at midlogarithmic phase resuspended in PBS were supplemented with 5 μM (gray bars) or 50 μM (black bars) or left untreated (white bars) and incubated for 30 min at 30 °C. ROS levels were assayed in washed cells by staining with Mitotracker Red CMXRos and measuring fluorescent emission in a fluorometer. Wild-type BY4741 yeast cells at midlog phase were treated with H_2O_2 or 50 μM quercetin, H_2O_2 with 50 μM quercetin or were left untreated for an additional 5 h. Levels of reduced glutathione GSH (white bars) and glutathione disulfide GSSG (gray bars) are shown (**D**).

RESULTS

Effect of Antioxidant Supplementation in H_2O_2-Induced Yeast Programmed Cell Death (PCD). To evaluate the capacity of the four small antioxidant molecules, ascorbic acid, β-carotene, caffeic acid, and quercetin, to protect yeast cells compromised in their enzymatic antioxidant machinery, we used three deletion strains and their isogenic wild-type counterpart in growth recovery assays. Treatment of yeast cells with H_2O_2 is known to cause cell death with the characteristic hallmarks of apoptotic death (*32, 33*). Cells deleted in superoxide dismutase (Δ*sod1*), catalase A (Δ*cta1*), and double knockout in Old Yellow enzyme 2 and glutathione reductase 1 (Δ*oye2 glr1*) containing high levels of oxidized glutathione (GSSG) were subjected to 1.25 mM H_2O_2 pro-oxidant insult, which induces programmed cell death (PCD) in a percentage of the population. The recovery of the surviving cells in complete minimal media supplemented with low (5 μM) or high (50 μM) antioxidants was monitored for a period of 12−14 h. All growth recovery assays were performed independently in triplicate. Detectable statistically significant differences could be seen starting at 4 h for wild-type and Δ*oye2 glr1* cells and at 6 h for the slower growing Δ*sod1* and Δ*cta1* cells. Overall, these differences are also reflected at the end of the assay period. The effects of H_2O_2 in the cultures, as judged by the lag in cell division time compared to untreated cells, appear to last for up to 10 h. Supplementation of strains with ascorbic acid had beneficial effects in every compromised state tested, at both low and high concentrations. The control wild-type cells showed 12% at 5 μM AA and 15% at 50 μM AA improved recovery at the end of recovery period; Δ*cta1* cells showed no improvement at 5 μM AA and 9% minor statistically significant improvement at 50 μM AA; Δ*sod1* cells showed 10 and 47% improvement at 5 and 50 μM AA respectively; and Δ*oye2 glr1* cells showed 18% improvement at 5 μM AA that increased to 47% at 50 μM AA (**Figure 1**). Supplementation of cells with higher concentrations of ascorbic acid (100 μM) was inhibitory to cell recovery (data not shown). β-Carotene supplementation of cells exhibited variable effects ranging from reduction of fitness to significant protection. Wild-type cells showed a 12% minor improvement at 5 μM BC and a 4% not significant change at 50 μM BC; in Δ*cta1* cells BC reduced recovery by 14% at 5 μM and improved recovery by 8% at 50 μM; Δ*sod1* exhibited marked improvement by 30% at 5 μM BC and a reduction by 18% at 50 μM BC; and in Δ*oye2 glr1* cells there was increased protection at both concentrations, 27% for 5 μM BC and 46% for 50 μM BC (**Figure 2**). Supplementation of cells with caffeic acid exerted beneficial effects in most cases and no fitness reduction under any type of treatment. Whereas the protective effect was minimal for wild-type cells at the end of incubation period (0% for 5 μM CA and 4% NS for 50 μM CA), CA was highly protective in all compromised deletion strains. In Δ*cta1* cells there was a 23% improvement with 5 μM CA and a 46%

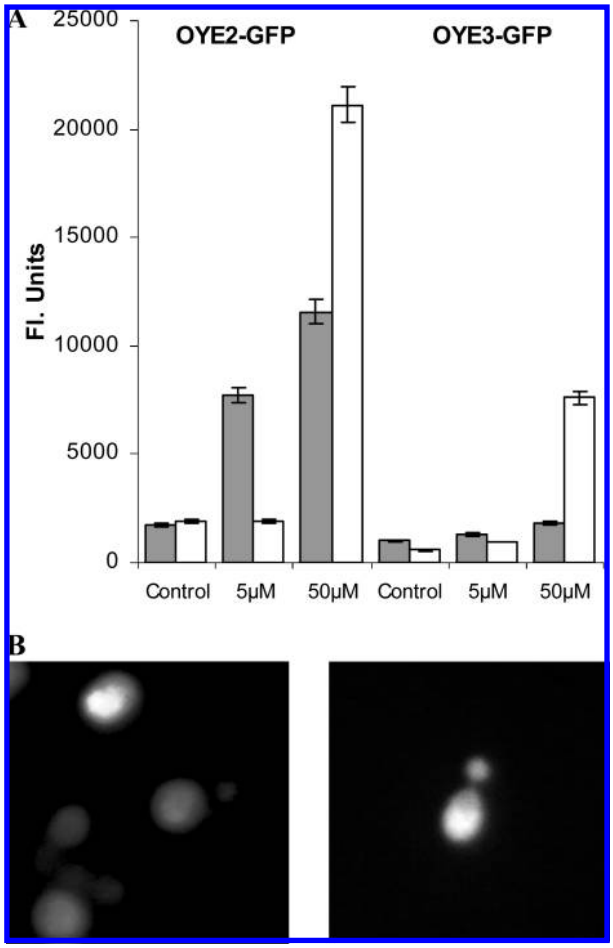

Figure 11. Response of the yeast antioxidant machinery in cells treated with antioxidant small molecules. (**A**) Yeast cells engineered to express a chromosomally encoded C-terminal fusion of GFP to the OYE2 or OYE3 gene from their native promoters were supplemented with β-carotene (gray bars) or quercetin (white bars) at 5 and 50 μM concentrations and incubated for 2 h. GFP fluorescence revealing the extent of OYE protein induction was monitored by fluorometry. (**B**) OYE2−GFP cells treated with β-carotene (left) and quercetin (right) were visualized for GFP localization microscopically.

improvement with 50 μM CA; in Δ*sod1* cells, 43 and 25% improvements were observed for 5 and 50 μM CA, respectively; and in Δ*oye2 glr1* cells 22 and 27% improvements were found for the low and high regimens (**Figure 3**). Finally, quercetin supplementation exhibited the most dramatic variability among the tested antioxidant small molecules. In wild-type cells at 5 μM QC, no protection was seen, whereas 50 μM QC totally inhibited recovery; in Δ*cta1* there was minimal protection of 5% at 5 μM QC and inhibition by 8% at 50 μM QC; in Δ*sod1* a significant improvement by 28% at 5 μM QC was observed, but almost no protection at 50 μM QC; and in Δ*oye2 glr1* cells a 20% improvement at 5 μM QC and none at 50 μM QC (**Figure 4**) were found.

To assess the comparability of the growth recovery assay data with the frequently employed viability colony assay, wild-type and Δ*sod1* cells were grown to midlog phase at $OD_{600} = 0.5$ in glucose CM media. Aliquots of cells were used to inoculate fresh media containing 50 μM concentrations of the four antioxidant small molecules. H_2O_2 was subsequently added at a concentration of 1.5 mM, and cells were incubated at 30 °C for 2 h. The numbers of viable cells were enumerated prior and subsequent to treatment. As seen in **Figure 4E**,**F** the percentage of surviving colonies after H_2O_2 treatment was reduced by more than half—to an average of 48% for wild-type cells and 42% for Δ*sod1*. Supplementation with ascorbic and caffeic acid was beneficial to cell survival for both strains, 64 and 62% for AA and 59 and 48% for CA in wild-type cells and Δ*sod1*, respectively. BC gave mixed results as in the growth recovery assays, 56% viability in wild type and 27% survival in Δ*sod1*, which is a significant reduction in viability compared to unsupplemented H_2O_2-treated cells. Quercetin supplementation was inhibitory for strains, 18 and 13% viability for wild type and Δ*sod1*, respectively. The results of colony viability assays are highly comparable to the corresponding growth recovery assays, and the effects of supplementation were statistically significant in all cases (**Figure 4**).

Effect of Antioxidant Supplementation in Cumene Hydroperoxide-Induced Cell Death. It is known that distinct components of the stress responsive machinery respond to different types of oxidative stress (*34*). The capacity of the four small molecule antioxidants to protect against organic prooxidant insult was also examined, by treating cells with CHP at 60 μM for Δ*sod1* cells and at 80 μM for all other strains. As with H_2O_2 treatment, ascorbic acid supplementation offered significant protection under most treatments. In wild-type cells AA caused a minor improvement at 5 μM and a significant 54% improvement at 50 μM, in Δ*cta1* cells no change was seen at either concentration, in Δ*sod1* protection was significant for both concentrations (22 and 28% for low and high regimens, respectively), and in Δ*oye2 glr1* there was 9% improvement at 5 μM and 24% improvement at 50 μM (**Figure 5**). β-Carotene supplementation of CHP-treated cells exhibited protection only in Δ*sod1* cells and was inhibitory under several treatments. In wild-type cells there was a reduction of recovery by 28% at 5 μM BC and a minor improvement by 8% at 50 μM BC; in Δ*cta1* cells no significant change was seen under either regimen; in Δ*sod1* cells a 22% improvement at 5 μM BC and nonsignificant change for 50 μM BC were observed; and in Δ*oye2 glr1* cells total inhibition at 5 μM BC and an 8% reduction of recovery at 50 μM BC were found (**Figure 6**).

In the same manner as with H_2O_2-induced PCD, caffeic acid supplementation upon CHP treatment was highly beneficial in most cases. In wild-type cells there was a 23% improvement at 5 μM CA that reached 102% at 50 μM CA; no improvement was seen in Δ*cta1* cells; in Δ*sod1* cells a 22% improvement at 5 μM CA and a 38% improvement at 50 μM CA were seen; and in Δ*oye2 glr1* there were 12 and 21% improvements for low and high doses of CA, respectively (**Figure 7**). Finally, quercetin supplementation had beneficial effects only in wild-type cells at low doses, showing a 28% improvement at 5 μM QC. In all other cases there no change or a mild inhibition (**Figure 8**). It is noteworthy, though, that no drastic inhibition was observed as was the case for H_2O_2-treated QC supplemented cells (**Figure 4A**).

In addition to single small antioxidant supplementation, we also tested binary combinations of the most potent compounds, ascorbic acid and caffeic acid, at high concentrations of 50 μM, with the other compounds at low concentrations, 5 μM (total antioxidants = 55 μM) in Δ*sod1* treated with H_2O_2 (**Figure 9A**,**B**). In all cases tested no combination of antioxidants fared better than AA or CA at 50 μM. In contrast, combination of vitamin E and ascorbic acid showed significant improvement over supplementation with only AA (data not shown).

Effects of Small Molecule Antioxidant Supplementation in Cells Deficient in Glutathione. Yeast cells harboring a deletion in the gene GSH1, which encodes a γ-glutamylcysteine synthetase catalyzing the first step in glutathione biosynthesis,

are unable to grow in CM media unless exogenously supplemented with GSH. To emulate conditions of limiting concentrations of intracellular GSH, the medium was supplemented with 100 nM, which enabled cells to grow at a lower rate and a 70% reduction in population density at 18 h (data not shown). The capacity of the small molecule antioxidants to improve the growth properties of Δ*gsh1* cells was subsequently assayed. Supplementation with AA is the only case when improvement in growth properties is observed, ranging from 5% at 5 μM to 16% at 50 μM. All other compounds caused significant inhibition in growth recovery (**Figure 9C−F**).

Reactive Oxygen Species and Glutathione Levels in Cells Treated with Antioxidant Small Molecules. To assess the effect of the four small molecules in intracellular ROS levels, cells at midlog phase resuspended in PBS were supplemented with 5 and 50 μM concentrations of each antioxidant for 30 min. Washed cells were stained with Mitotracker CMXROS, and fluorescent emission was monitored by fluorometry. Ascorbic acid supplementation did not alter significantly ROS levels, whereas caffeic acid contributed to a small reduction in ROS in Δ*sod1* and Δ*cta1* cells but not in wild-type cells. On the other hand, β-carotene supplementation induced a significant ROS increase in Δ*sod1* and Δ*cta1* cells but not in wild-type cells. Quercetin supplementation elicited the most dramatic increase at the 50 μM QC concentration in wild-type and Δ*sod1* cells. At 5 μM QC change in ROS was evident only in Δ*sod1* cells (**Figure 10**). Subsequently, we proceeded to assay the effects of quercetin on endogenous glutathione levels. Wild-type yeast cells growing in glucose CM to midlog phase OD_{600} = 0.5 were split in four aliquots. The first aliquot was left untreated; the second was treated with 1.25 mM H_2O_2, the third with 50 μM quercetin, and the fourth with H_2O_2 and quercetin. The cells were further incubated for 5 h, and total glutathione and glutathione disulfide levels were measured. Continuous exposure for extended periods to H_2O_2 caused a drastic reduction of total glutathione levels and an increase of GSSG levels (**Figure 10D**). Quercetin-supplemented cells exhibited a significant increase in GSH and GSSG levels compared to wild-type cells. However, upon addition of H_2O_2, quercetin failed to protect cells from loss of GSH.

Induction of the Stress Responsive Machinery by β-Carotene and Quercetin. Old Yellow enzymes 2 and 3 (OYE2 and OYE3) were recently identified for their role in oxidative stress and modulation of PCD processes (*19*). They are two highly homologous proteins that can form homo- or heterodimers. They are transcriptionally differentially regulated and normally coincide at the beginning of stationary phase when cells tend to undergo senescent PCD prior to adaptive regrowth (*35, 36*). Heterodimer formation sensitized cells to undergo apoptosis (*19*). Yeast cells at midlogarithmic phase, harboring an endogenous carboxy-terminal fusion of the green fluorescent protein (GFP) to the OYE2 or OYE3 gene, were treated with low and high doses of antioxidants for 2 h, and the GFP fluorescence was quantified by fluorometry. β-Carotene caused induction of OYE2−GFP at both 5 and 50 μM BC, but no change in OYE3−GFP fluorescence. Quercetin supplementation at 5 μM QC had no effect, whereas at 50 μM QC OYE2−GFP was superinduced and there was concomitant induction of OYE3−GFP (**Figure 11**).

No changes were observed upon ascorbic or caffeic acid supplementation. The formation of oye2p−oye3p heterodimer has previously been associated with sensitization of cells to H_2O_2-induced PCD. This may explain the extreme inhibition of QC supplementation seen in **Figure 4A**.

DISCUSSION

The growth recovery assay upon oxidative insult is a comparable assay to colony viability measurements as seen in **Figure 4** and in previously published data (*19*). It was chosen as the assay of choice as populations of cells maintained in chemically defined glucose minimal media are supplemented with the antioxidant small molecules for the whole assay period, which resembles more closely a real life setting. This enables testing concentrations of antioxidants used at physiologically relevant levels that organisms encounter (5 μM low dose and 50 μM high dose), especially when we also take into consideration the yeast efficient efflux system. Two types of pro-oxidant insults were used to induce oxidative damage, H_2O_2 and the organic pro-oxidant CHP. H_2O_2 is an endogenously produced ROS from the dismutation of superoxide with additional attributes as signaling molecule inducing apoptosis in yeast cells and altering the stress adaptive response (*33*). In the growth recovery assays, upon H_2O_2 insult, as in the colony viability assays, a significant proportion of cells lose viability, although they maintain membrane integrity, and exhibit hallmarks of PCD such as annexin externalization and DNA fragmentation (*19, 33*). Thus, cells that escape from PCD eventually recover and undergo cell divisions. Organic hydroperoxides cause a different type of damage and adaptive response. These affect metabolic functions, detoxification pathways, generation of NADPH, and down-regulation of the rate of protein synthesis but not oxidative stress defense enzymes and general cellular defense systems (*34*). The effects of H_2O_2 insult, as judged by the time period for a cell division compared to untreated cells, last up to approximately 10 h (8 h after the addition of the pro-oxidant), whereas for CHP they last approximately 12 h. The cells were monitored for a period of 14 h. Changes in growth that are statistically significant can be observed from the 4 h time point. Our results clearly emphasize the importance of the antioxidant small molecule concentration supplementing the medium in the growth recovery assays. Ascorbic acid exerts its maximal protection at the high concentration under both H_2O_2 and CHP insult; caffeic acid also functions well at high concentration, although in several circumstances the low concentration protects equally well; β-carotene yielded mixed results, performing better in Δ*sod1* cells at low concentration under both H_2O_2 and CHP and at 50 μM only in H_2O_2-treated Δ*cta1* and Δ*oye2 glr1* cells; quercetin at 5 μM performed better as in all cases it exerted a protective effect.

The protective action of ascorbic acid in yeast cells is expected to be solely based on its antioxidant role, although we should note that ascorbic acid is the only compound that can augment the retarded growth of Δ*gsh1* cells supplemented with minimal glutathione, which could indicate a potential involvement in the GSH metabolic pathway. Recent evidence also shows that ascorbate can be utilized by yeast Prx1 1-Cys peroxiredoxins, thus functioning as ascorbate peroxidase (*37*). Populations that consume diets rich in fruits, vegetables, and grains are expected to have ascorbate levels of >35−60 μM, concentrations that are equivalent to the 50 μM high regimen applied in yeast cells (*38*). Supplementation of yeast with a higher concentration of ascorbic acid (100 μM) was inhibitory to growth recovery (data not shown). The effects of β-carotene on yeast growth recovery are erratic and highly concentration dependent. This is also reflected in the induction of high ROS levels in Δ*sod1* cells and to a lesser extent in Δ*cta1* cells but not in wild-type cells. It was tested as an anticancer agent in clinical trials with adverse effects in lung cancer (*10*). Among

the two plant phenolic compounds tested, caffeic acid was highly effective in protecting cells from oxidative damage, although higher concentrations usually improved potency. The measured plasma concentration of caffeic acid after the consumption of coffee (200 mL) is in the range of 500 nmol/L; however, the concentrations found in the gastrointestinal tract are very high, ranging from 6 to 670 μmol/L. At such concentrations caffeic acid could be expected to function as a potent antioxidant and may additionally affect the composition of microbial flora. In *Aspergillus flavus* cells, treatment with caffeic acid (12 mM) reduced by >95% aflatoxin production, presumably due to its strong antioxidant capacity (*39*). In the current study continuous exposure to quercetin in pro-oxidant-treated cells showed protection only at 5 μM and growth inhibition in several circumstances at 50 μM concentration. The extreme growth inhibition seen was accompanied by high levels of intracellular ROS in wild-type and Δ*sod1* cells. The stimulation of GSH synthesis and increased GSSG levels seen in quercetin-treated cells indicate that the compound is functioning as a xenobiotic and stimulates the stress responsive machinery, which could explain the increased resistance and longevity caused by pulses of quercetin (*14*). Upon prolonged exposure to H_2O_2 not only do yeast cells accumulate oxidized glutathione but the total amounts of reduced and oxidized glutathione are substantially reduced. This indicates inhibition of glutathione biosynthesis that quercetin supplementation cannot alleviate.

Published microarray data from a number of groups show hyperinduction of OYE2 and OYE3 upon arsenic treatments, diamide, menadione, the initial stages of H_2O_2 treatment, and transition from hypoxia to reoxygenation. OYE3 also responds to the DNA-damaging agent MMS (*36, 40, 41*). Although the two genes respond similarly under conditions of oxidative insult, computational studies have shown that the genes are not coregulated, belonging to different regulatory modules (*42*). OYE2 expression is substantially reduced when cells enter stationary phase, whereas no such effect is seen in OYE3. The exquisite transcriptional sensitivity to conditions of oxidative insult renders OYE2 and OYE3 very useful stress biosensors.

Previous data from our laboratory linked the oye2−oye3 heterodimer in sensitization of cells undergoing H_2O_2-induced PCD, which can alter drastically the final outcome of exposure to xenobiotics (*19*). The simultaneous induction of the two stress responsive proteins oye2p and oye3p further supports this assumption. In humans, flavonoids are not readily absorbed in the gastrointestinal tract, unlike vitamin C. It appears that nature has not evolved systems for efficient uptake of these compounds (*38*). The highest plasma concentration that has been measured after the consumption of onions was 7.6 μmol/L (*43*). The extreme sensitivity of yeast cells to the combination of high quercetin levels and H_2O_2 indicates that quercetin may be tapping on yeast intracellular signaling pathways as well.

Taken together, defects of yeast cells in the intracellular antioxidant machinery affect significantly the capacity of small molecules to protect cell viability from oxidative damage. The combination of pro-oxidant recovery assays, ROS, and GSH/GSSG measurements and stress response indicators can provide rapid informative screens for bioactive natural product compounds.

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