

OXIDATIVE STRESS GENERATED BY CARCINOGENS IN *SACCHAROMYCES CEREVISIAE* ALLOWS THEIR DETECTION WITH A SHORT-TERM TEST

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Summary: Furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone are widely used in industry and preparation of human food. Although they are not detected in any short-term genotoxicity assay, their carcinogenic potential has been proved in long-term experiments with rodents. We found that furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone activate the mobility of Ty1 transposon in *Saccharomyces cerevisiae* cells to positive values of the Ty1 transposition assay. This assay is a short-term test for detection of carcinogens based on induction the transposition of an oncogene-like Ty1 genetic element. Upon treatment with furfuryl alcohol, tetrahydrofuran or *tert*-butylhydroquinone the Ty1 transposition rate in tester cells increases in a concentration and time-dependent manner and the increased mobility is due to *de novo* Ty1 transposition. We also found that the studied carcinogens are powerful ROS generators in yeast cells and provide evidence for causality between the increase of ROS level and the activated mobility of Ty1 transposon. The oxidative stress raised by the carcinogens is modified by the intrinsic antioxidant properties we found for furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone using *in vitro* and *in vivo* assays for measuring antioxidant capacity. High *in vitro* and moderate *in vivo* antioxidant activities were determined showing that the studied carcinogens except being chemical reducers are also biological antioxidants able to modulate the redox state of *S. cerevisiae* cells. The consequences of the interplay between oxidative and antioxidant properties of furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone for cellular physiology are discussed.

Keywords: carcinogens, oxidative stress, Ty1 short-term test.

Abbreviations: DPPH – diphenylpicrylhydrazyl; FFA – furfuryl alcohol; NAC – N-acetylcysteine; PCR – polymerase chain reaction; ROS – reactive oxygen species; SD – standard deviation; *t*BHQ – *tert*-Butylhydroquinone; THF – tetrahydrofuran; Ty – transposon yeast; YEPD – yeast extract peptone dextrose.

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INTRODUCTION

A large fraction of genotoxins with carcinogenic potential which have been proved in long-term experiments with rodents did not induce any of the *in vitro* or *in vivo* short-term assays known. These substances have been tested up to the maximum solubility with and without metabolite activation and the reasons for the negative responses are not clear (International Agency for Research on Cancer, 1999; National Toxicology Program 1998, 1999; US Environmental Protection Agency, 2007). Furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone belong to this group of carcinogens.

Furfuryl alcohol (FFA) is widely used in industry for production of polymers and resins. It is also present in numerous foodstuffs of everyday human meal as a product of the thermal dehydration of hexoses (Glatt et al., 2012). A two years study in mice and rats reveals evidence of carcinogenic activity based on increased incidences of neoplasms in renal tubule and nose (NTP, 1999). In contrast, FFA is not genotoxic in short-term tests (Ashby and Tennant, 1991) and the results from cytogenetic studies are controversial: induction or absence of chromosomal aberrations and sister chromatid exchanges were noted in hamster ovary cells and mice bone marrow cells (Monien et al., 2011).

Tetrahydrofuran (THF) is a high production volume chemical with numerous applications as a solvent and as an intermediate in the synthesis of pharmaceuticals, hormones, pesticides, vitamins. Results obtained in long-term carcinogenicity studies evidence increases of renal adenomas and carcinomas in rats

and of hepatocellular tumors in mice (NTP, 1998). THF has been tested in a number of *in vitro* and *in vivo* short-term assays and the results were negative with the exception of a positive response in a mouse erythrocyte micronucleus test (NTP, 1998). THF did not induce sister chromatid exchanges or chromosomal aberrations in ovary and kidney cells of rats and mice (Gamer et al., 2002). However results from a study in human (Funes-Cravioto et al., 1977) showed increased incidences of chromosomal breaks and sister chromatid exchanges in lymphocytes of workers exposed to THF. Data for a ROS generating or antioxidant activities of FFA and THF in yeasts or other cells have not been reported.

tert-Butylhydroquinone (*t*BHQ) is widely used as a food antioxidant to prevent oils and fats from oxidative deterioration and rancidity. The mode of action of its antilipid peroxidation activity has been well documented (reviewed in Ghavari et al., 2007). Upon *t*BHQ exposure the transcription factor Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE) of genes which leads to transcriptional activation of >200 antioxidant enzymes collectively known as the phase II response. The literature data strongly suggest that exposure to higher levels of *t*BHQ induces neoplastic lesions in the forestomach, kidney and urinary bladder of rats, mice and hamsters. The carcinogenicity of *t*BHQ has been attributed to generation of ROS (Ghavari et al., 2007), however, production of ROS upon *t*BHQ exposure has not been found in a number of studies (Hara et al., 2003; Lee et al., 2001; Nakamura et al., 2003). While *in vitro* short-term carcinogenicity and mutagenicity assays give negative

results with *t*BHQ, the data obtained in *in vivo* cytogenetic studies and micronucleus test are quite contradictory, ranging from absence of genotoxicity (Rogers et al., 1993) to positive results for mutagenicity (van Esch, 1986).

This brief survey illustrates that large groups of people are exposed to FFA, THF or *t*BHQ in their working or occupational environments and during the preparation and consumption of food. Although FFA, THF and *t*BHQ showed negative results in *in vitro* short-term experiments and gave controversial results in *in vivo* cytogenetic studies, they represent a potential risk for the human health since their carcinogenicity in rodents has been firmly documented. It becomes crucial to have a fast and easy method for detection of FFA, THF and *t*BHQ as environmental pollutants or as food additives to allow trustworthy and relevant research on their effect in eukaryotic cells prior to animal studies. None of the existing short-term tests reaches these criteria, even when used in batteries of assays as it was recommended (Eastmond et al., 2009).

In this communication we report that the Ty1 transposition assay can detect the carcinogenic potential of FFA, THF and *t*BHQ. The Ty1 transposition assay is a short-term test based on induction of the mobility of a gene engineered oncogene-like Ty1 transposon in the yeast *Saccharomyces cerevisiae*. Laboratory and field studies evidenced a positive response of Ty1 test to carcinogens and environmental samples containing carcinogenic pollutants (Pesheva et al., 2008). We show that treatment of yeast cells with FFA, THF and *t*BHQ activates the transposition of Ty1 transposon and gives a positive response of the Ty1 short-

term test. We also supply evidence for ROS generating and antioxidant properties of FFA, THF and *t*BHQ in *S. cerevisiae* cells and show that the increased levels of ROS have a primal role for the activation of the Ty1 transposition process.

MATERIALS AND METHODS

Materials and chemicals

All carcinogens used, including hexavalent chromium (as CrO₃), N-acetylcysteine and vitamin C are from Sigma Aldrich (Germany). The S9 metabolite mix is from Microbiological Associates (Rockville, MD, USA). The components for the nutritional media to cultivate yeast cells are from Difco Chem. Co. (USA).

Strains and cultivation procedures

The *S. cerevisiae* 551 strain with genotype *MATa ura3-167 his3Δ200: TymHIS3AI sec53 rho⁺* (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria, Cat№ 8719) was used as a tester strain in Ty1 transposition and Ty1 antiROS assays. This strain is a derivative of *S. cerevisiae* DG1141 (Curcio and Garfinkel, 1991). *S. cerevisiae* DG1141 strain has a Ty1 element marked with the indicator gene *HIS3AI* which allows determination of the Ty1 transposition in the genome as a whole. In *S. cerevisiae* 551 the *SEC53* gene is replaced with a *sec53* mutation which destroys the permeability barrier of cell wall and membrane (Bernstein et al., 1985). The *rho⁻* mutants of *S. cerevisiae* 551 are induced by ethidium bromide treatment (Sherman et al., 2001). The isogenic strains *S. cerevisiae 551sco1Δ* and *S. cerevisiae 551yap1Δ* were obtained

by integrative transformation of 551 cells with cassettes *sco1::URA3* and *yap1::hisG-URA-hisG*, respectively (Akada et al., 2007). URA⁺ integrative transformants were purified and disruption of genes was confirmed by PCR analysis.

Strains were cultivated at 30°C on a rotary water bath shaker in YEPD liquid medium (Sherman et al., 2001) to cell density of 5-7x10⁷ cells/ml and used in the experiments.

The Ty1 transposition test

The Ty1 transposition test was performed as described by Pesheva et al. (2008). Growing cells were treated with carcinogens for 30 min, washed, suspended in fresh YEPD and cultivated at 20°C for 24h to complete the initiated transposition events. Appropriate dilutions of cells were plated to determine survivals (on YEPD) and number of His⁺ transposants (on CM-HIS). The rate of transposition was determined and the mean values ±SD from 5-10 repetitions were calculated. Results are presented as fold increases of Ty1 transposition rate related to control sample taken as a fold increase of 1.0. A fold increase >2.0 is considered as a positive result of the assay. In experiments with scavengers of ROS, N-acetylcysteine (NAC) was added 60 min before the treatment of cells with genotoxins.

Quantitative assay for superoxide anions

An assay based on reduction the tetrazolium dye XTT and adapted to yeast cells was used (Stamenova et al., 2008). XTT is taken only by living cells and has a molar extinction coefficient of 2.16 x 10⁴m⁻¹/s⁻¹ at 470nm which allows determination of superoxide anions

concentration per cell. The superoxide anions assay was performed immediately after the treatment with genotoxins and before the cultivation of cells at 20°C in the Ty1 transposition assay. The number of live cells was determined as colony forming units and the results obtained are presented as pM O₂⁻/cell±SD.

Determination of antioxidant activity

The *in vitro* assay based on measurement of the loss of DPPH (diphenylpicrylhydrazyl) color at 515 nm after reaction with test components was used (Bondet et al., 1997). The percentage of remaining DPPH increases proportionally to the antioxidant concentration and in our experiments the antioxidant activity of FFA, THF and tBHQ was determined in 3mM solutions of these genotoxins.

The Ty1 antiROS test is an *in vivo* assay based on the reduction by antioxidants of the Ty1 transposition rate enhanced by ROS in *S. cerevisiae* cells (Dimitrov et al., 2013). Culture aliquots were treated with increasing concentrations of the studied compound; cells were washed and suspended in YEPD medium containing hexavalent chromium as inducer of ROS. Part of the generated ROS were scavenged by the antioxidant properties of the studied substance and the remaining ROS induced Ty1 transposition proportionally to their concentration. Data obtained for Ty1 transposition rates were plotted against the concentrations of the studied antioxidants and IC₅₀ value was calculated. The IC₅₀ is the concentration of the studied substance (µg/ml) inhibiting 50% of the transposition rate in the control sample. The smaller IC₅₀ is for a compound the higher is its antioxidant activity.

Southern blot analysis

Cells were treated for 30 min with FA (6mM), THF (5mM), *t*BHQ (0.05mM) or methylmethane sulfonate (3.5mM) as a positive control. His⁺ transposants were purified and total yeast DNA was isolated from single transposants by the miniprep technique (Sherman et al., 2001). DNA samples were digested with *PvuII*, separated by gel electrophoresis (0.8% agarose gel) and transferred to Hybond filters. DNA immobilized on filters was hybridized with a *HIS3* gene probe purified from plasmid BJC38 (Curcio and Garfinkel, 1991) and digoxigenine labeled by randomly primed DNA synthesis (McCreery and Helentjaris, 1994).

Statistical analysis

Results are presented as means \pm SD from 4 to 10 independent experiments. Comparison between two means was performed using unpaired Student's *t*-test. The 0.05 probability level is chosen as the point of statistical significance throughout.

RESULTS

Ty1 test response to furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone

The treatment with FFA, THF and *t*BHQ for 30 min increased Ty1 transposition in tester cells relative to the untreated controls (Table 1). Values

Table 1. Ty1 test response to furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone.

Carcinogen ^{a)}	Concentration [mM]	S9 mix	Cell survival [%]	Ty1 transposition [fold increase]	Superoxide anions ^{b)} [pM/cell]
Controls (H ₂ O)	–	–	100	1.0	0.04
B(a)P	0.20	–	92	1.5	0.05
	0.20	+	53	18.7	2.08
FFA	3	–	84	3.2	0.24
	6	–	75	4.9	0.48
	6	+	78	5.1	0.52
	20	–	48	15.8	1.41
THF	3	–	90	3.5	0.31
	5	–	68	5.1	0.55
	5	+	62	5.5	0.49
	15	–	51	16.8	1.55
<i>t</i> BHQ	0.03	–	87	4.3	0.53
	0.05	–	63	6.2	0.68
	0.05	+	66	6.8	0.85
	0.15	–	48	18.8	2.01

^{a)} FFA – furfuryl alcohol, THF – tetrahydrofuran, *t*BHQ – *tert*-butylhydroquinone, B(a)P – benzo(a)pyrene used as a positive control.

^{b)} Average of 6 experiments; $p < 0.05$.

for Ty1 transposition obtained in the presence or absence of S9 activation mix were similar making a pro-carcinogenic status of the studied genotoxins highly unlikely. The positive answer appeared at low concentrations of carcinogens having negligible effect on cell survival and the fold increase of transposition rates enhanced in a concentration-dependent manner. At concentrations of the genotoxins killing about 50% of cells the fold increases of Ty1 transposition were in the range of 15-18. Such values for the fold increase of Ty1 transposition are very similar to the results obtained previously for different carcinogenic genotoxins (Pesheva et al., 2008) and for the positive control with benzo(a)pyrene, known as a strong human carcinogen. Increased Ty1 transposition rates were also found for FFA, THF and *t*BHQ in kinetic experiments with tester cells treated for 15 to 90 min (data not shown).

In addition to the transposition process the translocation and integration of Ty1 transposon to a new location in the genome can also occur by gene conversion (Roeder and Fink, 1982). There are data in the literature (Hori et al., 2009) indicating activation of the process of homologous recombination by elevated levels of ROS, and increased production of ROS has been found in some experiments with *t*BHQ (Ghavyar et al., 2007). Therefore, we studied the possibility of having increased gene conversion instead of transposition *de novo* in our studies. Southern blot experiments were performed with restricted DNAs isolated from single transposants induced by FFA, THF or *t*BHQ and hybridized to a *HIS3* gene (Figure 1). All DNA samples including the control from spontaneous (not induced by carcinogens) transposants

have a 5kb band representing the parental Ty1 insertion (Staleva and Venkov, 2001). The treatment with carcinogens, such as methyl methanesulfonate (used as a positive control) led to the appearance of additional dispersed bands each one representing *de novo* transposition event of the Ty1 transposon. The same results were obtained after treatment of yeast cells with FFA, THF or *t*BHQ and the analysis of DNAs isolated from the transposants induced by the carcinogens. Figure 1 shows the results from the analysis of single carcinogen-induced transposants and the same picture of the DNA:DNA hybridization was obtained by the study of 10 transposants induced by each one of the studied carcinogens.

Taken together, the results obtained in concentrations and time-dependent experiments evidence an activation of Ty1 transposon mobility following treatment with FFA, THF or *t*BHQ of *S. cerevisiae* cells as it has been found in studies of different carcinogens with the Ty1 test (Pesheva et al., 2008). The positive response of Ty1 short-term test with FFA, THF and *t*BHQ is due to increased Ty1 transposition *de novo* induced by the studied carcinogens.

Furfuryl alcohol, tetrahydrofuran and tert-butylhydroquinone generate oxidative stress in *S. cerevisiae*

Most carcinogens are ROS generators and we studied the production of ROS in yeast cells treated with FFA, THF and *t*BHQ, as well as the dependence between ROS levels and Ty1 transposition rates. The results obtained for superoxide anion (O_2^-) production following a 30 min treatment of cells with different concentrations of FFA, THF or *t*BHQ are presented in Table 1

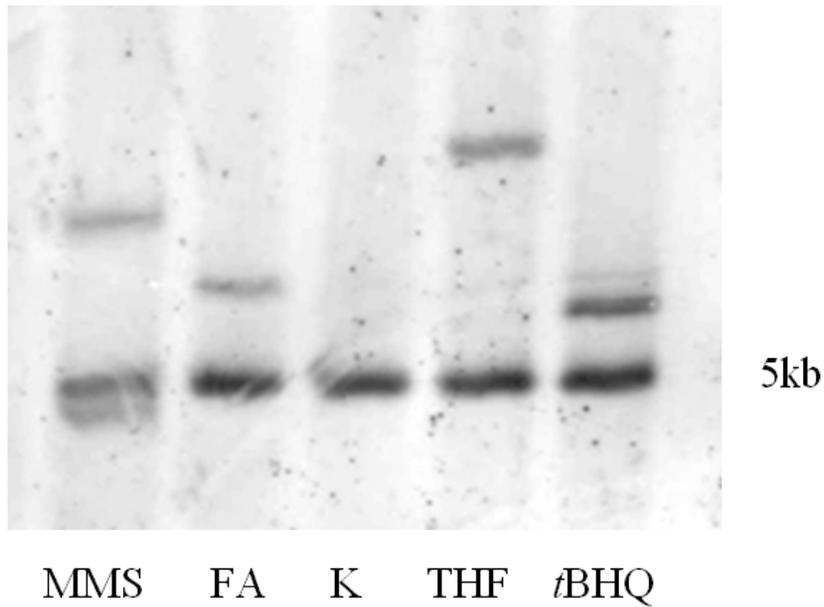


Figure 1. Southern blot analysis of Ty1 transposants. FA, THF and *t*BHQ and MMS – DNA from transposants induced by furfuryl alcohol, tetrahydrofuran, *tert*-butylhydroquinone and methylmethane sulfonate (positive control). K – DNA from spontaneous transposant.

(last column). These results demonstrate that the three studied carcinogens are ROS generators in *S. cerevisiae* cells. The data for O_2^- level and the values for the fold increase of Ty1 transposition rate (Table 1) are shown in Figure 2. The increase of superoxide anion is coupled with a linear increase of Ty1 transposition rate, e.g. the dependence between enhanced O_2^- and Ty1 transposition is proportional. The studied carcinogens are not equally powerful ROS generators. For instance, a fivefold increase of Ty1 transposition rate was achieved at O_2^- levels of 0.5-0.6pM/cell generated by 6mM FFA, 5mM THF or 0.05mM *t*BHQ. The finding that Ty1 transposition rate is proportional to the level of O_2^- generated in *S. cerevisiae* strongly suggested causality between the increase in ROS levels and the mobility of Ty1 transposon.

The *S. cerevisiae* *rho*⁻ strains represent

large deletions of mitochondrial DNA that span over different genes, including the genes for the oxidative phosphorylation complexes. *Rho*⁻ cells can not produce ROS of mitochondrial origin and are characterized by very low level of intracellular reactive oxidative species (Stamenova et al., 2008). The study of several *rho*⁻ mutants as tester strains in the Ty1 test (551 *rho*⁻ strains) showed absence of both, generation of O_2^- and activation the Ty1 transposition after treatment with FFA, THF or *t*BHQ (not shown). These preliminary results suggest that the induction of Ty1 mobility depends on mitochondrial integrity and function. The nuclear gene *SCO1* of *S. cerevisiae* codes for a protein that is transported to mitochondria and participates in the formation and functioning of oxidative phosphorylation complexes (Glerum et al., 1996). Mutations in or disruption

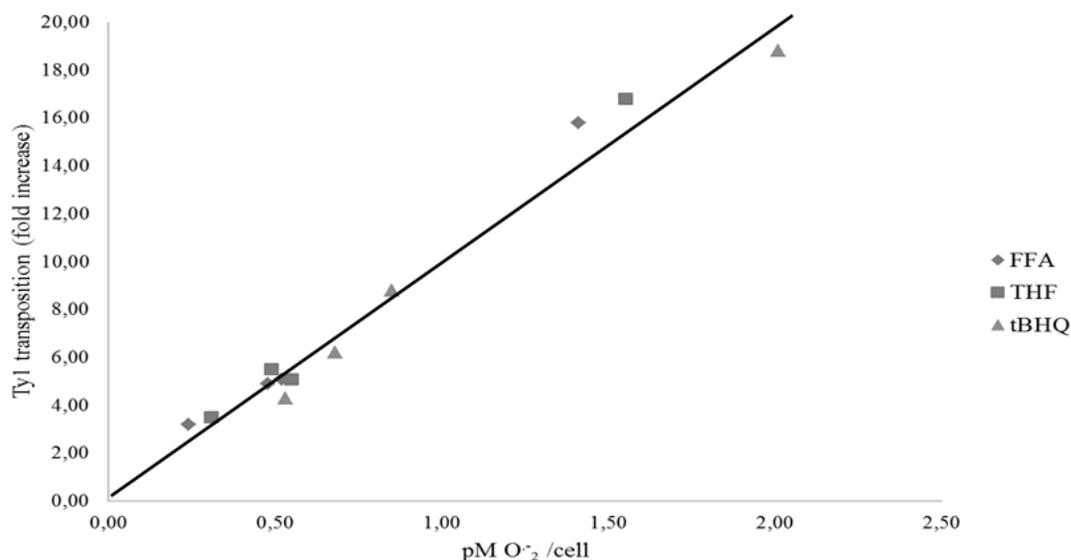


Figure 2. Proportional dependence between Ty1 transposition rate and superoxide anion level. FFA – furfuryl alcohol, THF – tetrahydrofuran, *t*BHQ – *tert*-butylhydroquinone.

of *SCO1* gene are associated with a respiratory deficient phenotype, however in contrast to *rho*⁻ strains, *scol* mutant cells lack only the mitochondrial oxidative phosphorylation in otherwise intact and functioning mitochondria. We studied the effect of FFA, THF and *t*BHQ in the Ty1 test using 551 *scolΔ* derivatives as tester strains (Table 2) and found that the levels of O₂⁻ and the Ty1 transposition rates did not increase following treatment with these carcinogens. Almost equal values were obtained for control cells or *scolΔ* and *rho*⁻ cells treated with the studied carcinogens. The dependence of activation the Ty1 mobility on accumulation of O₂⁻ was further evidenced by results obtained in experiments with NAC, a scavenger of O₂⁻. Previously, it has been found that treatment with NAC in concentrations of 60 mM scavenges the existing O₂⁻ in *S. cerevisiae* cells and did not allow an increase of ROS following treatment with laboratory ROS generators (Cojocel et al., 2006). The results we obtained

showed that in cells pretreated with NAC the addition of FFA, THF or *t*BHQ was without effect on Ty1 transposition (Table 2). Quantitative measurements evidenced the absence of both ROS production and activation of Ty1 mobility in such cells.

Based on these results we conclude that the activation of Ty1 transposition by FFA, THF or *t*BHQ depends proportionally on the generation of ROS by the oxidative phosphorylation process and not on another mitochondrial function.

Increased levels of H₂O₂ participate in activation the Ty1 transposition by furfuryl alcohol, tetrahydrofuran or *tert*-butylhydroquinone

S. cerevisiae has been shown to have distinct stress responses to superoxides and peroxides (Jamieson et al., 1992). The *YAP1* gene encodes a transcription factor which binds to AP-1 sites in promoters of target genes involved in the major defense response against H₂O₂ (Nguyen et al., 2001). Mutants deleted for *YAP1*

Table 2. ROS levels participate in activation the mobility of Ty1 transposon.

Strain	Carcinogen ^{a)}	Concentration [mM]	NAC [60mM]	Survival [%]	Superoxide anions ^{b)} [pM/cell]	Ty1 transposition [fold increase]
Controls						
551	–	–	–	100	0.04±0.01	1.0
551 <i>sco1Δ</i>	–	–	–	100	0.03±0.01	1.0
551 <i>yap1Δ</i>	–	–	–	100	0.07±0.02	1.0
551 <i>sco1Δ</i>	FFA	20	–	49	0.05±0.02	1.2
	THF	15	–	66	0.03±0.01	1.0
	<i>t</i> BHQ	0.15	–	61	0.04±0.02	1.4
551	FFA	20	–	53	1.55±0.21	16.3
	FFA	20	+	69	0.03±0.01	1.2
	THF	15	–	54	1.64±0.22	15.1
	THF	15	+	74	0.04±0.01	0.9
	<i>t</i> BHQ	0.15	–	48	2.05±0.30	19.3
	<i>t</i> BHQ	0.15	+	60	0.04±0.01	1.2
551 <i>yap1Δ</i>	FFA	6	–	41	0.97±0.10	9.3
	THF	5	–	43	0.81±0.09	8.6
	<i>t</i> BHQ	0.05	–	38	1.29±0.12	10.6

^{a)} FFA – furfuryl alcohol, THF – tetrahydrofuran, *t*BHQ – *tert*-butylhydroquinone.

^{b)} Average of 5 experiments; $p < 0.05$.

accumulate H_2O_2 and are hypersensitive to peroxides. We took advantage of this observation and studied the involvement of intracellularly accumulated H_2O_2 in the activation of Ty1 transposition. The *YAP1* gene in *S. cerevisiae* 551 strain was disrupted and the obtained 551*yap1Δ* mutants used as tester strains in the Ty1 assay. 551*yap1Δ* cells not treated with carcinogen showed a moderately increased background level of O_2^- (Table 2) due to the accumulated H_2O_2 , known as a generator of ROS (Stephen et al., 1995). Given the higher sensitivity of *yap1Δ* cells to H_2O_2 , lower concentrations of FFA, THF and *t*BHQ were used in experiments

to avoid cell-killing. Results obtained in Ty1 test after treatment of *yap1Δ* cells with FFA, THF or *t*BHQ showed that the production of ROS and the induction of Ty1 transposition were increased compared to cells with a functioning *YAP1* gene. For instance, the treatment with FFA at a concentration of 6mM induced in 551*yap1Δ* cells an O_2^- level of 0.97pM/cell and Ty1 fold increase of 9.3 (Table 2), compared to 0.48pM H_2O_2 /cell and Ty1 fold increase of 4.9 in 551 cells with intact *YAP1* gene (Table 1). Similar higher O_2^- levels and Ty1 transposition rates in 551*yap1Δ* were also obtained after treatment with THF or *t*BHQ.

These results indicate that the increased levels of H₂O₂ also participate in modulation the Ty1 transposition process in the response of *S. cerevisiae* to treatment with FFA, THF or *t*BHQ.

Antioxidant activity of furfuryl alcohol, tetrahydrofuran and tert-butylhydroquinone

The study of FFA, THF and *t*BHQ in the *in vitro* DPPH and the *in vivo* Ty1 antiROS tests (Table 3) showed that the three carcinogens possess antioxidant properties. Although results obtained in different antioxidant assays are not comparable in between (Prior et al., 2005), the usage of equimolar (for DPPH) or equal weight (for Ty1 antiROS) concentrations allows direct comparison of values obtained in each one assay. The results showed that the antioxidant capacities of the studied carcinogens was *t*BHQ>FFA>THF and this rank order was the same in both DPPH and Ty1 antiROS tests. The high antioxidant activity of *t*BHQ in DPPH assay is in agreement with its wide usage as an effective *in vitro* antioxidant (Ghavari et al., 2007). Considering the biological effect of FFA, THF and *t*BHQ however, the values obtained in the Ty1 antiROS

in vivo assay showed significantly lower antioxidant activities than the strong referent antioxidant vitamin C. The IC₅₀ values were comparable to the IC₅₀ value found for honeybees (250 µg/ml) in the same assay (Dimitrov et al., 2013), suggesting a moderate *in vivo* antioxidant activities of FFA, THF and *t*BHQ. The detection of antioxidant activities for the studied carcinogens in the two assays indicated some characteristics of their mode of action. *In vitro* assays, such as DPPH, are based on chemical reactions, however the tests are conducted under nonphysiological conditions and the obtained results can not be extrapolated to the *in vivo* situation (Prior et al., 2005). The cell-based Ty1 antiROS test as an *in vivo* assay measures the penetration of studied antioxidant into cells, and its ability to scavenge oxidative radicals inside living cells. There is a general agreement that *in vivo* antioxidant methods are a relevant way to study the biological effect of antioxidants. Therefore, the observation that FFA, THF and *t*BHQ demonstrated antioxidant properties *in vitro* and *in vivo* means that these carcinogens are not only chemical reductors but also biological antioxidants able to modulate the redox state of live cells. Previously, chemical

Table 3. Antioxidant activity of furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone.

	DPPH [% activity for 3mM]	Ty1antiROS [IC ₅₀ in µg/ml]
FFA	66±5	585±42
THF	25±2	701±49
<i>t</i> BHQ	>90	55±4
Vitamin C	>90	0.7±0.1

Average of 8 experiments, p<0.05.

reductor properties were suggested for FFA (Xia et al., 2006; Pickering and Wiesner, 2005) and it was shown that THF was able to react with free radicals *in vitro* (Baron et al., 2006), however, data for measurements of antioxidant activity *in vitro* or *in vivo* have not been presented for these carcinogens. The results obtained in our study evidence that in addition of their *in vitro* chemical antioxidant activity, the carcinogenic FFA, THF and *t*BHQ penetrate into cells and can influence the balance between oxidants and antioxidants in live cells.

DISCUSSION

ROS such as O_2^- , H_2O_2 and $OH\cdot$ are produced in all aerobic organisms and normally exist in the cell in balance with antioxidant molecules. Oxidative stress occurs when this critical balance is disturbed due to depletion of antioxidants or excess accumulation of ROS. The formation of ROS can be accelerated as a consequence of various environmental stress conditions or treatment of cells with genotoxins. Many carcinogens are powerful ROS inducers and here we provide evidence for ROS generating activity of FFA, THF and *t*BHQ in *S. cerevisiae*. Considering the biological effect of increased ROS, the first major problem that has to be taken into account is that the free radicals are extremely reactive and consequently short-lived. Any oxidative radical produced in the cell reacts at or close to its source of formation (Dimitrov et al., 2013) making the role of available defense mechanisms in the vicinity extremely important. Since synthesis, activation and bioavailability of the antioxidant systems require some

time for full expression (Temple et al., 2005), the maintenance of the steady-state ROS level within some subcellular compartments becomes a problem. In the case of FFA, THF and *t*BHQ this problem seems modified because the substances possess intrinsic antioxidant activity which neutralizes part of ROS on the spot and shortly after their production. The *in vivo* antioxidant activities we found for FFA, THF and *t*BHQ were not high and the values obtained were lower compared to those of known strong antioxidants (Table 3). On the contrary, the ROS generating activity of FFA, THF and *t*BHQ seems high and comparable to that of benzo(a)pyrene which is a powerful inducer of ROS production (Table 1). Therefore, in *S. cerevisiae* cells treated with FFA, THF and *t*BHQ the balance between ROS and antioxidants is disrupted in favor of free radicals with all consequences of the raised oxidative stress. The interplay between oxidants and antioxidants is known to depend on multiple cellular factors that are expressed in varying degrees in the different cells (Scandalios, 2005) suggesting that treatment with FFA, THF or *t*BHQ might have all sorts and even opposite effects on intracellular redox state depending on the cell model used in the experiment. Thus, FFA, THF and *t*BHQ provide a paradox in that they possess two antagonistic biological effects and the balance between them depends on cell species. This might explain to some extent the controversial results obtained previously in studies with these carcinogens.

Recently, it has been recognized that the activity of free radicals in living cells goes beyond the generation of oxidative stress and has effects on the physiology of

cells. A large body of experimental data indicates that H_2O_2 is produced transiently and plays a key role as an intracellular messenger or as a mediator in cell-to-cell communications (D'Autreaux and Toledano, 2007). Localized production and accumulation of ROS are critical for signaling function in transduction cascades to modulate their function through reversible oxidation (Janssen-Heininger et al., 2008). We report here that one of the cellular processes that is activated by ROS generated in *S. cerevisiae* cells treated with FFA, THF and *t*BHQ is the transposition of Ty1 transposon. The proportional dependence found between O_2^- levels and Ty1 transposition rates, the absence of Ty1 activation following treatment with FFA, THF and *t*BHQ of cells with compromised mitochondrial ROS production (*rho*⁻, *sco1Δ* mutants), or in cells pretreated with ROS scavengers are data evidencing a key role of the free radicals in initiation the Ty1 transposition process. The Ty1 short-term test is based on activation of the mobility of Ty1 transposon and the positive response of the test appeared at low doses of FFA, THF and *t*BHQ (Table 1) evidencing a sensitive and specific answer of the assay. The increase of Ty1 mobility was due to *de novo* transposition indicating activation of the Ty1 transposition process itself and not another way of translocation that might give false positive results in the assay. These data show that the Ty1 short-term test can be used for detection of FFA, THF and *t*BHQ that are undetectable in other short-term assays. The wider range of Ty1 assay in detection of carcinogens can be explained with the increased permeability of tester cells (Bernstein et al., 1985), structural and

functional similarity of Ty1 transposon to oncoviruses (Garfinkel, 1992) and mainly with the molecular mechanism of the positive Ty1 test response. Most of the short-term tests were constructed to detect a *genetic end-point* raised by one, or several (but not all) carcinogens (Eastmond et al., 2009). Detecting the end-point of a genotoxin has the advantage to unravel its mode of action, however it limits the range of detected substances. Typical example is the recent important finding that FFA becomes detectable in *Salmonella typhimurium* TA100 test only if tester cells are engineered for expression of sulfotransferase because FFA is converted by sulfoconjugation to a mutagen (Glatt et al., 2012). Contrary to such strategy, the positive response of Ty1 test is based on activation the *start of a cellular process*, the Ty1 transposition. This activation is due to increased ROS generated by the carcinogens, which seems to be a feature of all carcinogens tested till now in *S. cerevisiae* (Brennan and Schiestle, 1998; Pesheva et al., 2008; Scandalios, 2005). The need of having a short-term test for detection of FFA, THF and *t*BHQ is strengthened after the critical analysis of data obtained in long-term tests with rodents showing that renal tumor formation in rats and liver tumor appearance in mouse are not relevant to carcinogenicity in humans and should not be considered in further risk assessment efforts (Fenner-Crips et al., 2011a, 2011b).

It might be concluded that the usage of carcinogen-induced generation of ROS in *S. cerevisiae* cells expands the limits in detection of carcinogens with Ty1 test. The detection of FFA, THF and *t*BHQ by their ROS generating capacity is not an indirect or nonspecific approach for evidencing

their carcinogenic potential. Induction of ROS production has been found in different cells treated with carcinogens that have quite different modes of action (reviewed in Scandalios, 2005), and it is now generally accepted that humans are exposed to many carcinogens, but the most significant and common may be the reactive species derived from the metabolism of oxygen and nitrogen (Halliwell, 1999).

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