

# Baker's Yeast Deficient in Storage Lipid Synthesis Uses *cis*-Vaccenic Acid to Reduce Unsaturated Fatty Acid Toxicity

Peter Sec<sup>1</sup> · Martina Garaiova<sup>1</sup> · Peter Gajdos<sup>2</sup> · Milan Certik<sup>2</sup> · Peter Griac<sup>1</sup> · Ivan Hapala<sup>1</sup> · Roman Holic<sup>1</sup>

Received: 18 September 2014 / Accepted: 8 April 2015 / Published online: 24 April 2015  
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**Abstract** The role of *cis*-vaccenic acid (18:1n-7) in the reduction of unsaturated fatty acids toxicity was investigated in baker's yeast *Saccharomyces cerevisiae*. The quadruple mutant (QM, *dga1Δ lro1Δ are1Δ are2Δ*) deficient in enzymes responsible for triacylglycerol and steryl ester synthesis has been previously shown to be highly sensitive to exogenous unsaturated fatty acids. We have found that *cis*-vaccenic acid accumulated during cultivation in the QM cells but not in the corresponding wild type strain. This accumulation was accompanied by a reduction in palmitoleic acid (16:1n-7) content in the QM cells that is consistent with the proposed formation of *cis*-vaccenic acid by elongation of palmitoleic acid. Fatty acid analysis of individual lipid classes from the QM strain revealed that *cis*-vaccenic acid was highly enriched in the free fatty acid pool. Furthermore, production of *cis*-vaccenic acid was arrested if the mechanism of fatty acids release to the medium was activated. We also showed that exogenous *cis*-vaccenic acid did not affect viability of the QM strain at concentrations toxic for palmitoleic or oleic acids. Moreover, addition of *cis*-vaccenic acid to the growth medium provided partial protection against the lipotoxic effects of exogenous oleic acid. Transformation of palmitoleic acid to *cis*-vaccenic acid is thus a rescue mechanism enabling *S. cerevisiae* cells to survive in the absence of triacylglycerol synthesis as the major mechanism for unsaturated fatty acid detoxification.

**Keywords** *Saccharomyces cerevisiae* · Triacylglycerols · Steryl esters · Vaccenic acid · Lipotoxicity · Elongase

## Abbreviations

DAG	Diacylglycerol
FA	Fatty acid(s)
GC	Gas chromatography
NP40	Nonidet P-40
PL	Phospholipid(s)
QM	Quadruple mutant <i>dga1Δ lro1Δ are1Δ are2Δ</i>
SE	Steryl ester(s)
TAG	Triacylglycerol(s)
TLC	Thin layer chromatography
VA	<i>cis</i> -Vaccenic acid

## Introduction

Metabolism of neutral lipids is an important part of lipid homeostasis in eukaryotic organisms. Triacylglycerols (TAG) and steryl esters (SE) represent the storage forms of fatty acids (FA) and sterols that accumulate during the periods of surplus and are released by hydrolysis in the periods of increased need. Released FA are activated by acyl-CoA synthetases and utilized as a source of energy in  $\beta$ -oxidation or for synthesis of membrane constituents, phospholipids and sphingolipids. Sterols released from SE can be incorporated into the membrane bilayer and in higher eukaryotes they can serve as precursors for steroid hormone synthesis [1]. In addition, synthesis of TAG and SE serves as a mechanism for elimination or reduction of lipotoxic effects of excessive free FA and sterols in eukaryotic cells [2–5].

The baker's yeast *Saccharomyces cerevisiae* has been extensively used as a model organism to study key

✉ Roman Holic  
roman.holic@savba.sk

<sup>1</sup> Department of Membrane Biochemistry, Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova 61, 900 28 Ivanka pri Dunaji, Slovakia

<sup>2</sup> Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovakia

factors of eukaryotic lipid metabolism. Four acyltransferases encoded by the *DGAI*, *LROI*, *ARE1*, and *ARE2* genes are responsible for storage lipid accumulation in this yeast [6–11]. Dga1p and Lro1p catalyze TAG synthesis whereas Are1p and Are2p are predominantly involved in SE synthesis. Interestingly, genetic ablation of storage lipid synthesis by disruption of all four genes encoding enzymes responsible for synthesis of storage lipids results in no obvious growth defect in *S. cerevisiae*. This is in sharp contrast with the situation in the fission yeast *Schizosaccharomyces pombe* where cells defective in TAG synthesis undergo apoptosis upon entry into the stationary phase [12]. Although the mechanism of apoptosis induction in TAG deficient *S. pombe* cells is not exactly known, it includes accumulation of FA and DAG as well as increased production of reactive oxygen species [12]. Consistent with the proposed role of TAG synthesis in the detoxification of free FA [12–14], *S. cerevisiae* quadruple mutant (QM, *dga1Δ lro1Δ are1Δ are2Δ*) showed increased sensitivity to external unsaturated FA [15–19]. Otherwise, defective storage lipid synthesis in QM cells manifests only by minor effects on lipid homeostasis, particularly by reduced ergosterol content of the plasma membrane [20] and by increased intracellular levels of *cis*-vaccenic acid (VA, 18:1n-7) [9].

In this study we tested the hypothesis that increased production of VA (18:1n-7) in yeast QM cells defective in storage lipid synthesis [9] serves as a detoxification mechanism of the highly lipotoxic palmitoleic acid (16:1n-7). We demonstrated that VA accumulates in the QM cells, in the same growth phase as TAG in wild type cells. This accumulation of VA in QM cells was accompanied by decrease in palmitoleic acid content. We suppose that VA is less metabolically active compared to other unsaturated FA as it accumulated predominantly in the pool of free FA. Activation of FA release mechanism as another strategy for FA detoxification abolished the accumulation of VA in the QM cells. In addition, we showed that when supplied in the growth media, VA was significantly less toxic to the QM cells compared to other major unsaturated FA present in the yeast cells, palmitoleic and oleic acids. Thus, we conclude that transformation of lipotoxic palmitoleic acid to *cis*-vaccenic acid is a rescue mechanism enabling *S. cerevisiae* cells to survive in the absence of TAG synthesis as the major mechanism for unsaturated FA detoxification.

## Materials and Methods

### Materials

Media components were obtained from Becton–Dickinson (USA) or BioLife (Italy). Tween 80 and FA (palmitic

acid, palmitoleic acid, heptadecanoic acid, oleic acid and VA) were obtained from Sigma-Aldrich (USA). TLC plates and organic solvents (HPLC grade) were from Merck (Germany). Fine chemicals were mostly from Sigma-Aldrich or MP Biomedicals.

### Strains and Cultivation Conditions

Wild type *S. cerevisiae* strains BY4741 (MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (MAT a *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) were obtained from the Euroscarf collection. Deletion strains in fatty acid elongases in BY4741 genetic background, *elo1Δ* (*YJL196C::kanMX4*), *elo2Δ* (*YCR034W::kanMX4*), and *elo3Δ* (*YLR372W::kanMX4*) were obtained from the Euroscarf collection of deletion strains. Quadruple deletion mutant devoid of lipid particles in BY4742 genetic background (MAT a *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::kanMX are2::kanMX dga1::kanMX lro1::kanMX*) was kindly provided by K. Athenstaedt (Graz University of Technology, Austria). Its *faa1,4Δ* derivative mutant was prepared in our laboratory by disruption of *FAA1* and *FAA4* genes using disruption cassettes (*faa1::HIS4*, *faa4::LYS2*) amplified by PCR on MS51 genomic DNA. Genomic DNA was isolated based on the method described in [21]. Yeast strain MS51 was kindly provided by M. Fulda (Georg-August University Göttingen, Germany). Primers used for amplification of *faa1::HIS3* cassette were as follows: 5'-ATACGGGACAGCAGAGATAGGC-3', 5'-AGCTCC TAATTAGACTGCCATGC-3' and for amplification of *faa4::LYS2* cassette: 5'-TCGGCCTTCGTTTCATCTCG-3', 5'-GGGGCATTTCGATGGTTTACAG-3'. Wild type strain W303-1A (MAT a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and quadruple deletion mutant devoid of lipid particles in W303 genetic background H1246 (MAT a *ADE2-1 can1-100 ura3-1 are1-Δ::HIS3 are2-Δ::LEU2 dga1-Δ::KanMX4 lro1-Δ::TRP1*) were kindly provided by M. H. Gustavsson (Uppsala Genetic Center, Sweden) [9]. For overexpression study, genomic clones containing genes encoding FA elongases were obtained from the ThermoScientific Yeast Genomic Tiling Collection. Yeast transformation was performed by the lithium acetate method [22].

Yeasts were grown on YEPD medium (1 % yeast extract, 2 % peptone, 2 % dextrose) or on standard synthetic minimal medium (0.67 % YNB without amino acids, 2 % glucose) supplemented with essential amino acids and bases. Solid YEPD plates were prepared with 2 % agar. Growth in liquid media was performed at 28 °C with continuous shaking. Cell growth was estimated by counting in hemocytometer or by measuring the OD at 600 nm. The optical density unit is equal to one (OD = 1) corresponded approximately to  $1 \times 10^7$  cells/mL. For FA treatment, free FA were dissolved in ethanol to make 5 % (w/v) stock

solutions and added to the agar plates at the indicated concentrations. The amounts of FA in stock solutions were controlled by GC, allowing for a precise determination of FA concentration. Tween 80 in the final concentration of 0.1 % (w/v) was added to all plates. For FA treatment in the liquid media, each FA was added to the YNB complete medium at the final concentration of 0.05 %. Tween 80 in the final concentration of 0.1 % (w/v) was added to each variant. After 24 h, growth cells were washed three times with 0.5 % NP40 and twice with water prior to the FA analysis described below.

### Cellular Lipid Extraction and Thin-Layer Chromatography Analysis of Neutral Lipids

Total lipids for thin-layer chromatography (TLC) analysis were extracted by a modified procedure of [23]. Briefly, cells were washed twice with water, disrupted by a FastPrep disintegrator (MP Biomedicals) using glass beads (diameter 0.4 mm) at  $3 \times 40$  s, at the highest speed, with 5 min cooling on ice between the cycles. Lipids were incubated in hot methanol (15 min at 65 °C) followed by 2-h extraction by chloroform/methanol/water (1:2:0.8, by vol) with occasional vortexing at room temperature. Phase separation was obtained by adjusting the ratio chloroform/methanol/water to 1:1.3:1.2, by vol followed by centrifugation. The organic phase containing lipids was withdrawn and evaporated under a nitrogen stream. The dried lipid residue was dissolved in chloroform/methanol (2:1, by vol) and aliquots corresponding to  $3 \times 10^8$  cells were applied to silica gel TLC plates by a semiautomatic sample applicator (CAMAG Linomat 5, Switzerland). Neutral lipids were separated by ascending two-step TLC as described in [24] (first step: petroleum ether/diethyl ether/acetic acid, 70:30:2, by vol; second step: petroleum ether/diethyl ether, 49:1, by vol). Individual lipid spots were visualized by iodine vapor or by charring the plates with a solution consisting of 0.63 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 60 mL of water, 60 mL of methanol, and 4 mL of concentrated sulfuric acid. Individual spots were identified by lipid standards applied to the same plate.

### Cellular Lipid Extraction and Thin-Layer Chromatography Analysis of Phospholipids

Cellular lipids for phospholipid analysis were extracted by a modified procedure of [25]. Briefly, cells were washed twice with water, disrupted with a FastPrep disintegrator (MP Biomedicals) using glass beads (diameter 0.4 mm) at  $3 \times 40$  s at the highest speed, with 5 min cooling on ice between the cycles. Lipids were incubated in hot methanol (15 min at 65 °C) followed by 2-h extraction by chloroform/methanol/hydrochloric acid (124:65:1, by vol) with

occasional vortexing at room temperature. Phase separation was obtained by adjusting the ratio chloroform/methanol/water to 1:1.1:0.9, by vol followed by centrifugation. The organic phase containing lipids was withdrawn and evaporated under nitrogen stream. The dried lipid residue was dissolved in chloroform/methanol (2:1, by vol) and aliquots corresponding to  $1 \times 10^9$  cells were applied to silica gel TLC plates by a semiautomatic sample applicator (CAMAG Linomat 5, Switzerland). Phospholipids were separated by one-step TLC as described in [26] using the solvent system chloroform/methanol/acetic acid (65:25:8, by vol). Phospholipid spots were visualized by iodine vapor and identified by lipid standards applied to the same plate. Individual phospholipids were extracted from the spots and their FA content estimated by GC as described below.

### Extraction of Lipids from Media

Extraction of lipids/FA from yeast cultivation medium was performed as described in [27]. Briefly, lipids were extracted from the supernatant of harvested cells according to the procedure of [23]. Supernatant (2 mL) was mixed with 2 mL of chloroform and 4 mL of methanol, and the tubes were shaken and vortexed. The chloroform phase was separated by addition of 2 mL of chloroform and 2 mL of water and centrifugation at  $2500 \times g$  for 5 min. The chloroform phase was withdrawn and evaporated under nitrogen stream. For FA composition analysis by GC, the FA in dried samples were directly methylated as described [28] and the FA composition was determined and evaluated as described below.

### Fatty Acid Analysis

Total FA contents of yeast cells and culture medium samples were determined after transmethylation by gas chromatographic (GC) analysis as described [28]. FA analysis was performed by the application of 1- $\mu\text{L}$  aliquots to a GC apparatus (GC2010Plus, Shimadzu, Japan) equipped with BPX70 capillary column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ , SGE Analytical Science, Australia) under temperature programming (160–234 °C at 4.5 °C/min increments). Individual FA methyl esters were identified by comparison with authentic standards of  $\text{C}_4$ – $\text{C}_{24}$  FA methyl esters mixture (Supelco, USA). Quantification of individual FA was performed using the heptadecanoic acid (C17:0) internal standard.

### Fatty Acid Analysis of Individual Lipid Classes

Cellular lipids were extracted from  $2 \times 10^9$  cells and lipid extracts corresponding to  $1.7 \times 10^9$  cells were analyzed by TLC as described above. Individual lipid spots were

visualized with iodine vapor and scraped off for FA analysis. Samples were directly methylated as described [28] and analyzed by GC as described above. Individual FA were quantified using heptadecanoic acid (C17:0) internal standard.

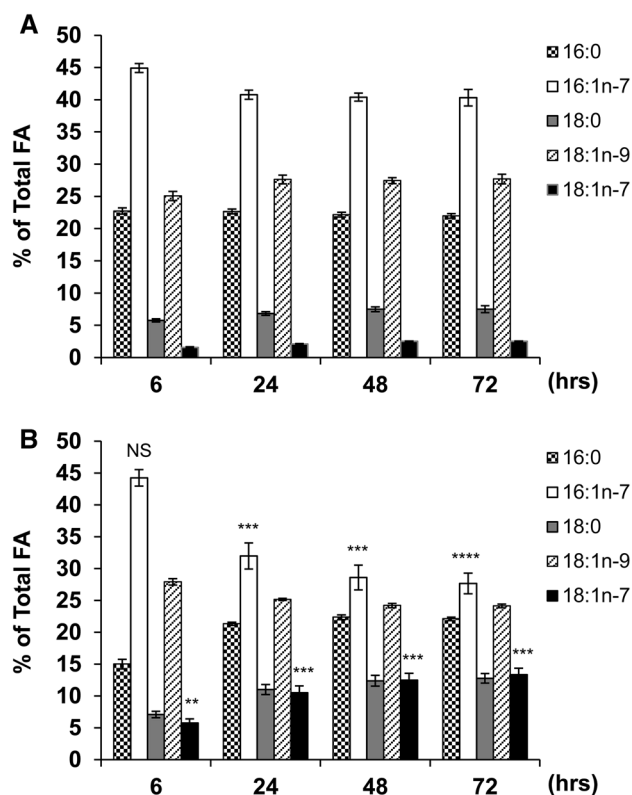
### Statistical Evaluation

Statistical significance of differences in FA composition was evaluated by the Student *t*-test and by ANOVA one-way analysis of variance. In Fig. 1, the Student *t*-test was performed to compare palmitoleic acid and VA levels in BY4742 and QM cells at each time point (6, 24, 48, and 72 h). In Fig. 3, the analysis of variance (ANOVA) was performed for VA of BY4742, QM and QM *faa1,4Δ* cells, followed by the Student *t*-test to test each pair of mean.

## Results

### Defects in Storage Lipid Synthesis are Associated with Accumulation of *cis*-Vaccenic Acid

Increased levels of *cis*-vaccenic acid (VA) in the QM strain (*dga1Δ lro1Δ are1Δ are2Δ*) deficient in storage lipid synthesis were observed already in the first study on the relationship between yeast storage lipid synthesis and cell viability [9]. To determine if this observation was not related to some peculiarities of the W303 genetic background we analyzed the FA profile of the QM mutants in W303 and BY4742 backgrounds and confirmed similar accumulation of VA in both strains (results not shown). To obtain more detailed information on VA accumulation we examined VA content in the BY4742 wild type and corresponding QM cells after various cultivation periods (Fig. 1). We have found that accumulation of VA in the QM cells increased during stationary growth phase (Fig. 1b). The relative content of VA in total FA in the QM cells reached  $12.47 \pm 1.10$  % compared to  $2.51 \pm 0.03$  % in wild type cells after 48 h of cultivation. The accumulation of VA in the QM cells was accompanied by reduction in palmitoleic acid content, which corresponds well with the proposed mechanism of VA formation by elongation of palmitoleic acid [29, 30]. In yeasts, three FA elongases have been described, among them Elo1 as a medium chain FA elongase [31]. To better understand the mechanism of VA formation we analyzed FA in yeast elongase deletion mutants and also in strains overexpressing individual FA elongases from multicopy genomic clones (Table 1). The data show that deletion of the *ELO1* gene decreases the VA content to about half of the wild type strain. In addition, overexpression of the *ELO1* gene approximately doubles the VA content. Deletion or overexpression of two other



**Fig. 1** QM strain accumulates *cis*-vaccenic acid in the stationary phase. Cells were grown on YNB complete liquid media and harvested for analysis at the indicated times. Cellular FA profiles were analyzed by GC as described in “Materials and Methods”. **a** FA profile of wild-type BY4742 strain. **b** FA profile of quadruple mutant strain (QM). 16:0 palmitic acid, 16:1n-7 palmitoleic, 18:0 stearic acid, 18:1n-9 oleic acid, 18:1n-7 *cis*-vaccenic acid. Data represent mean  $\pm$  SD from independent experiments. Statistical analysis was performed as described in “Materials and Methods”. Asterisks indicate the significance of difference in palmitoleic acid or VA content in QM cells compared to BY4742 cells (**a**) in specified time point (NS not significant, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ )

FA elongases, Elo2 and Elo3, has no major impact on VA formation. Taken together, these results are in agreement with the production of VA by elongation of palmitoleic acid as already described for adipocytes and soybeans seeds [29, 30]. The percentage of oleic acid in the QM cells was stable during the cultivation time (Fig. 1). Similar time course pattern (accumulation of VA, reduced level of palmitoleic acid and a stable level of oleic acid) was observed also in the W303 genetic background (data not shown).

### Vaccenic Acid Accumulates in the Free Fatty Acid Pool in Yeast Cells Unable to Synthesize Storage Lipids

The general response of lipid metabolism to defective storage lipid synthesis in the QM cells was investigated

**Table 1** Fatty acid profile of *Saccharomyces cerevisiae* strains with deleted or overexpressed genes for fatty acid elongases

Strain	Fatty acid composition (%)						
	14:0	16:0	16:1n-7	18:0	18:1n-9	18:1n-7	22:0
BY4741	1.74 ± 0.07	22.77 ± 0.21	41.12 ± 0.66	4.96 ± 0.24	24.18 ± 0.29	1.74 ± 0.09	3.49 ± 0.10
<i>elo1</i> Δ	4.78 ± 0.03	20.62 ± 0.09	35.54 ± 0.12	7.19 ± 0.06	27.27 ± 0.13	1.09 ± 0.02	3.51 ± 0.00
<i>elo2</i> Δ	1.02 ± 0.34	24.70 ± 1.57	32.59 ± 2.90	10.67 ± 2.52	29.27 ± 1.60	1.57 ± 0.10	0.19 ± 0.16
<i>elo3</i> Δ	2.18 ± 0.03	22.59 ± 0.26	50.30 ± 0.27	4.77 ± 0.05	18.60 ± 0.06	1.57 ± 0.02	ND
pGP564	1.89 ± 0.04	21.09 ± 0.02	41.26 ± 0.26	6.01 ± 0.10	25.22 ± 0.18	1.69 ± 0.01	2.84 ± 0.09
YGPM28m16 ( <i>ELO1</i> )	0.81 ± 0.11	22.20 ± 0.23	40.13 ± 0.75	6.21 ± 0.51	24.46 ± 0.21	3.12 ± 0.18	3.07 ± 0.22
YGPM28o02 ( <i>ELO2</i> )	2.00 ± 0.06	21.54 ± 0.33	40.88 ± 0.28	6.14 ± 0.27	25.08 ± 0.32	1.46 ± 0.09	2.91 ± 0.07
YGPM23113 ( <i>ELO3</i> )	1.82 ± 0.16	20.37 ± 0.16	38.20 ± 0.56	7.12 ± 0.47	26.39 ± 0.25	1.40 ± 0.01	4.70 ± 0.17

BY4741 cells and corresponding deletion strains in fatty acid elongases (*elo1*Δ, *elo2*Δ, and *elo3*Δ) were grown in YNB liquid media for 24 h at 28 °C. BY4742 cells harboring empty multicopy plasmid pGP564 or indicated genomic clones (Yeast Genomic Tiling Collection, ThermoScientific) containing *ELO1*, *ELO2*, and *ELO3* genes were grown in YNB liquid media lacking leucine for 24 h at 28 °C. Fatty acid content in cells was quantified after transesterification by gas chromatography as described in “Materials and Methods”. The data represent mean ± SD of three independent experiments

ND not detected

by TLC of neutral lipids (Fig. 2). An increased content of ergosterol, DAG, lanosterol and free FA was observed in the QM strain compared to the wild type strain (Fig. 2a). A decrease in the squalene content in the QM cells was also detected when compared to the wild type strain. As in previous studies, the QM cells defective in storage lipid synthesis did not synthesize storage lipids, namely TAG and SE [9, 16, 17].

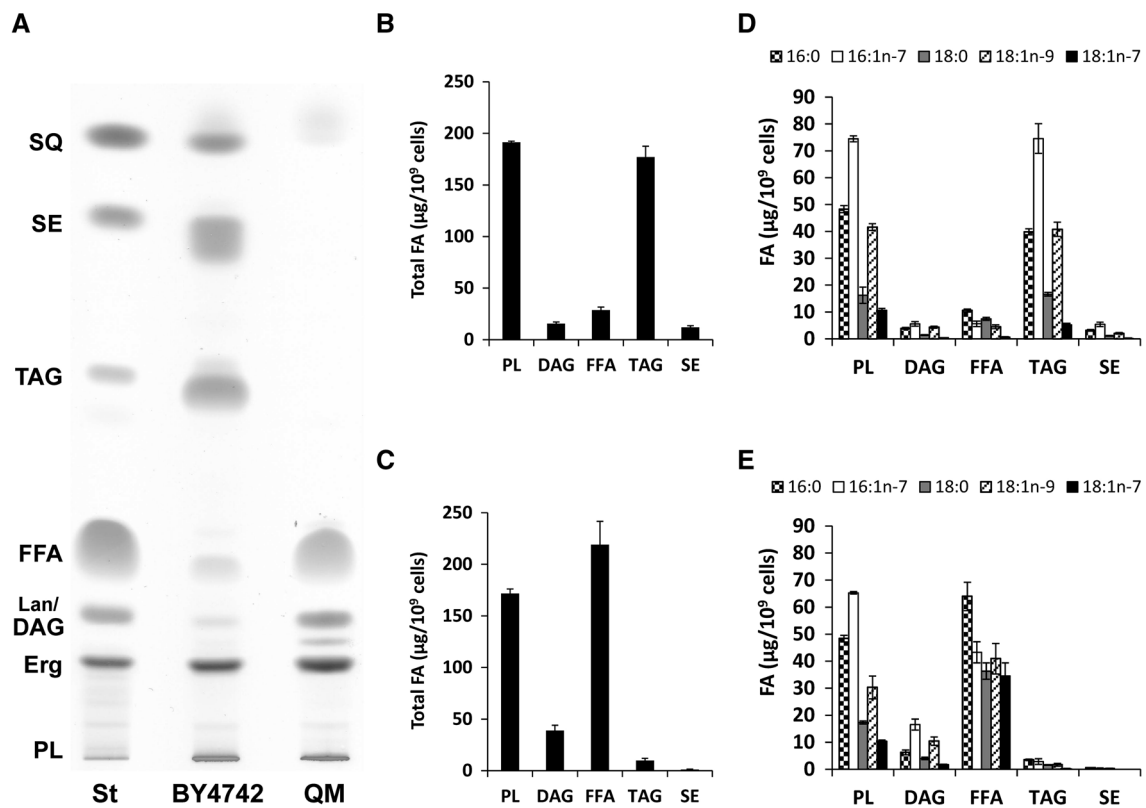
To analyze the accumulation of VA in more detail, the FA content of individual lipid classes/spots after TLC separation was determined by GC. In the wild type strain the majority of FA was detected in PL and TAG (Fig. 2b). In the QM strain the majority of FA was found in the free FA pool and PL (Fig. 2c), with both strains having similar amounts of FA in PL (Fig. 2b, c). Compared to the wild type strain, deficiency in storage lipid biosynthesis in the QM cells led to an increase in DAG and free FA (2.5- and 7.6-fold, respectively, see Fig. 2b, c). A small amount of TAG and trace amount of SE were found in the QM strain. The small amount of TAG might be explained by non-enzymatic process described previously [9].

FA composition analysis of individual lipids revealed differences between the wild type and the QM cells. The levels of palmitoleic acid, stearic acid (18:0) and VA were elevated in DAG, whereas the levels of palmitic (16:0) and oleic acids were reduced in the QM strain compared to the wild type (Fig. 2d, e). The content of VA in the PL fraction of the QM cells was similar in both strains (6.1 ± 0.1 % in the QM cells compared to 5.6 ± 0.6 % in the wild type strain). VA was abundant in the free FA pool of QM cells (Fig. 2c), comprising 15.7 ± 0.8 % of

free FA compared to 2.2 ± 0.3 % in the wild type. This result implies that VA may represent a metabolically less active form of unsaturated free FA. Based on these data, we hypothesize that detoxification of intracellular palmitoleic acid by elongation to VA in the QM cells serves to reduce lipotoxicity of unsaturated FA in cells defective in storage lipid synthesis.

#### Ability to Release Fatty Acids Prevents *cis*-Vaccenic Acid Production in Quadruple Mutant Cells

It is known that disruption of the *FAA1* and *FAA4* genes encoding two acyl-CoA synthetases enables the yeast cells to release free FA into their environment [32]. By deleting two acyl-CoA synthetase genes in the QM strain we generated strain QM *faa1,4*Δ without TAG and SE synthesis and with the capability to release free FA. We hypothesized that cells with the ability to release free FA will be able to eliminate toxic free FA avoiding thus the need for VA biosynthesis. As shown in Fig. 3a, activation of FA release mechanism in the QM strain strongly reduced the cellular content of VA (0.29 ± 0.04 μg/OD for the wild type, 0.88 ± 0.08 μg/OD for the QM and 0.08 ± 0.01 μg/OD for the QM *faa1,4*Δ strain). To test the possibility that VA produced in QM *faa1,4*Δ cells was released into the environment, the growth medium was analyzed for FA content. As shown in Fig. 3b, the extracellular level of VA in the growth medium of the QM *faa1,4*Δ strain was low (0.2 ± 0.04 μg/OD). Taken together, the wild type strain and the QM with the capability to release FA into the growth medium produced comparable amount of VA, which was 3-times less than the amount produced by the QM strain. This



**Fig. 2** *cis*-Vaccenic acid highly accumulates in the pool of free fatty acids. Lipid extracts were prepared from 48-h yeast cultures grown in YNB complete liquid media. Lipid composition was analyzed by TLC and FA profiles of individual lipids were analyzed by GC as described in “Materials and Methods”. FA content for PL was calculated as the sum of values estimated for individual PL spots after acidic extraction and one-dimensional TLC. **a** TLC of neutral lipids isolated from wild type strain BY4742 and its corresponding quadruple mutant strain (QM). **b** Total FA content in individual lipid classes

of wild type strain BY4742. **c** Total FA content in individual lipid classes of QM strain. **d** FA composition of individual lipid classes of wild type strain BY4742. **e** FA composition of individual lipid classes of QM strain. *St* standard, *PL* phospholipids, *Erg* ergosterol, *DAG* diacylglycerol, *Lan* lanosterol, *FFA* free fatty acids, *TAG* triacylglycerols, *SE* steryl esters, *SQ* squalene, 16:0 palmitic acid, 16:1n-7 palmitoleic acid, 18:0 stearic acid, 18:1n-9 oleic acid, 18:1n-7 *cis*-vaccenic acid. Data represent mean  $\pm$  range from two independent experiments

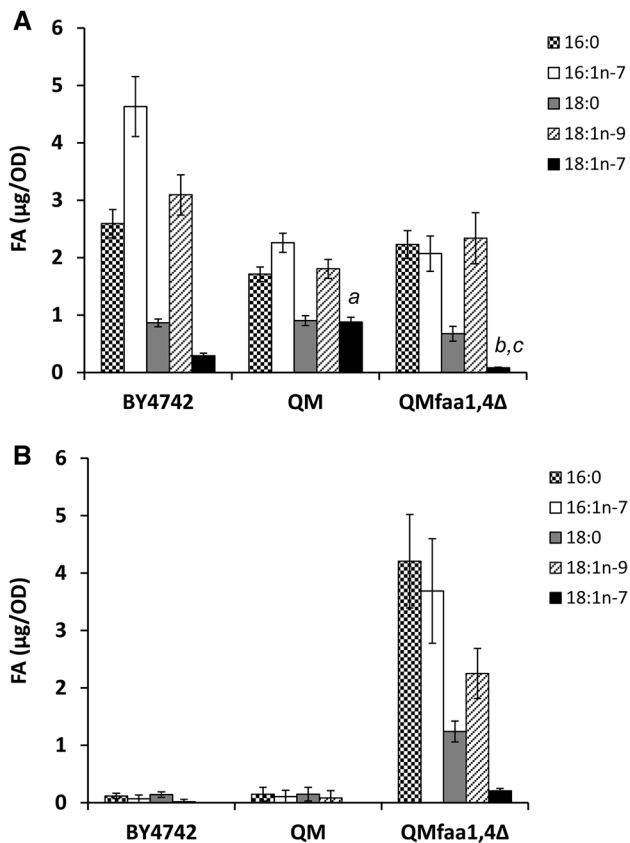
observation confirmed that the ability to remove toxic unsaturated FA by release mechanism will obviate the requirement for VA biosynthesis in the QM cells.

### The Quadruple Mutant Is Tolerant to Exogenous *cis*-Vaccenic Acid

Previous reports on FA lipotoxicity in *S. cerevisiae* have shown that the QM cells defective in storage lipid synthesis are hypersensitive exogenous unsaturated FA [15–19]. We therefore examined the growth of the wild type and the QM strains on agar plates supplemented with various FA: palmitic acid (16:0), palmitoleic acid (16:1n-7), oleic acid (18:1n-9) and VA (18:1n-7). As shown in Fig. 4, the QM cells were tolerant to VA and highly sensitive to the other two unsaturated FA, palmitoleic and oleic acids. Consistent with previous studies, the QM cells were not sensitive to exogenous saturated FA.

### Exogenous *cis*-Vaccenic Acid Partially Restores Growth of Quadruple Mutant Cells on Oleic Acid-Containing Media

Low toxicity of external VA might be explained also by inefficient import of VA compared to other two unsaturated FA used for lipotoxicity test—palmitoleic acid and oleic acid. It had been shown previously that palmitic acid supplementation partially restores the growth of the QM strain unable to synthesize storage lipids in the presence of oleic acid [17]. In this study, the same approach was used to test whether VA is efficiently transported into cells. Cells were spotted on plates supplemented with equal amounts of oleic acid and VA or equal amounts of palmitoleic acid and VA, respectively. As shown in Fig. 5, VA had no effect on the growth of the QM in the presence of palmitoleic acid. However, VA supplementation partially restored the viability of the QM in the presence of oleic acid. This indicated that exogenous VA was efficiently internalized by QM cells.



**Fig. 3** Ability to release FA prevents production of *cis*-vaccenic acid in QM cells. Cells were grown 48 h in YNB complete liquid media and FA content in cells and cultivation media was analyzed as described in “Materials and Methods”. **a** Amount of individual FA in cells. **b** Amount of individual FA released to the medium. BY4742 wild type, QM quadruple mutant, QM *faa1,4Δ* quadruple mutant with *faa1Δ* and *faa4Δ*, 16:0 palmitic acid, 16:1n-7 palmitoleic acid, 18:0 stearic acid, 18:1n-9 oleic acid, 18:1n-7 *cis*-vaccenic acid. Data represent mean  $\pm$  SD from five independent experiments. Statistical analysis was performed as described in “Materials and Methods”. *a* VA levels in QM cells compared to BY4742 cells,  $P \leq 0.0001$ ; *b* VA levels in QM *faa1,4Δ* cells compared to QM cells,  $P \leq 0.0001$ ; *c* VA levels in QM *faa1,4Δ* cells compared to BY4742 cells,  $P \leq 0.0001$

Efficient import of VA from the medium was confirmed also by direct analysis of cellular FA content in QM cells growing in the presence of VA, oleic acid and combination of both FA (Fig. 6). Interestingly, the cellular amounts of

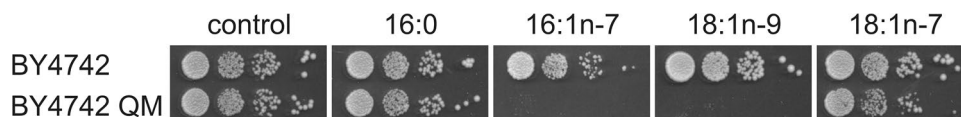
VA and oleic acid in QM cells cultivated on media containing the combination of both FA were similar to their content in cells grown on single FA. This shows that the protective effect of exogenous VA on oleic acid toxicity in QM cells is not mediated by competition in the uptake process but on some yet unknown intracellular mechanism.

## Discussion

Lipotoxicity as the detrimental effect of accumulated lipids (sterols, TAG or FA) represents an attractive topic in current biomedical research due to its involvement in the pathology of serious diseases such as atherosclerosis, diabetes, and obesity [33]. The phenomenon of lipotoxicity has been successfully studied also in the yeast *S. cerevisiae* particularly with respect to the toxic effects of over-accumulated unsaturated [15–19] and saturated [34] FA. Several mechanisms for detoxification of harmful lipids are available in this yeast including metabolic transformation, sequestration in specific storage organelles and excretion of toxic lipids from the cell into the environment.

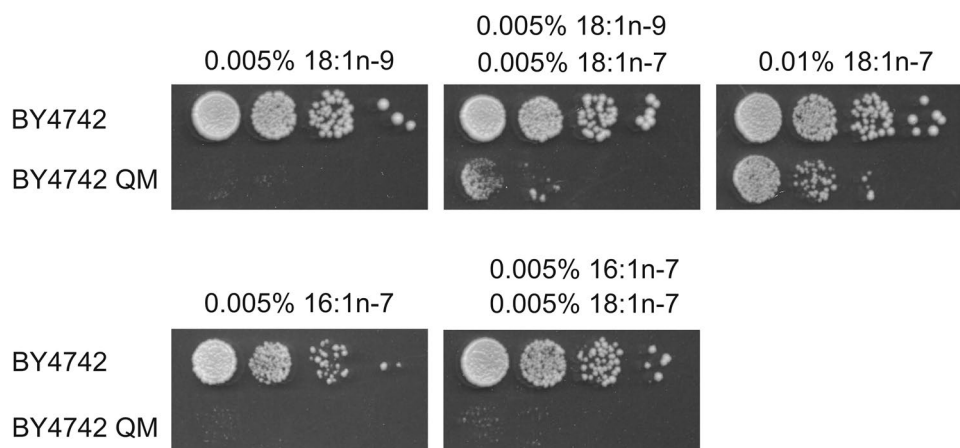
Conversion of free FA to TAG as a metabolically inert form and sequestration of TAG in lipid droplets is the major defense mechanism of yeast cells against the harmful effect of unsaturated FA overload. This mechanism is particularly important upon the entry into the stationary phase of growth where significant lipid accumulation is taking place. As previously published [16], the ability to synthesize storage lipids (including TAG) is important for maintaining the viability of *S. cerevisiae* cells in the stationary phase.

In this study we show that the yeast QM (quadruple mutant, *dgal1Δ lro1Δ are1Δ are2Δ*) with disrupted biosynthesis of storage lipids uses alternative protective mechanism(s) to reduce the lipotoxic effects of free unsaturated FA. Elevated intracellular levels of VA in the QM cells [9] together with the supposed formation of this FA from palmitoleic acid [29, 30] indicated that VA might be involved in alternative protection mechanisms to reduce unsaturated FA toxicity. In our study we have shown that the VA content substantially increases during stationary phase in the QM cells, and this accumulation occurs at the



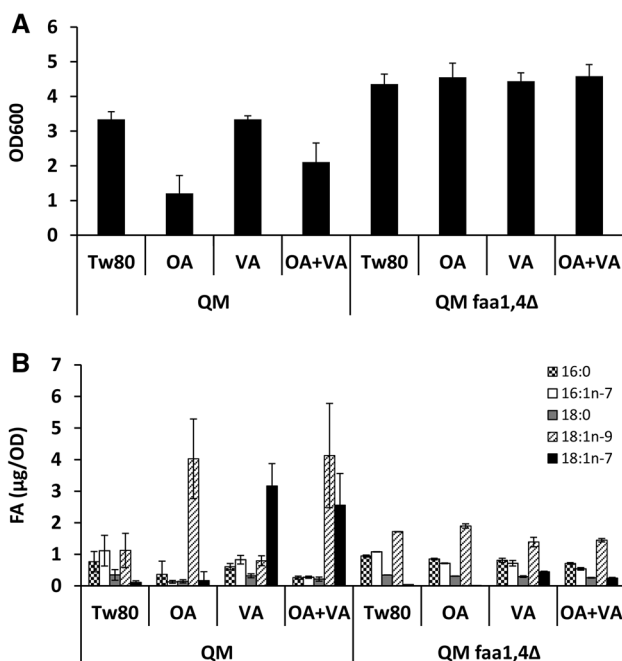
**Fig. 4** Effect of exogenous *cis*-vaccenic acid on growth of QM cells. Sensitivity of the BY4742 strain and from it derived quadruple mutant (QM) strain to external FA was established by drop tests on plates in serial dilutions. 5- $\mu$ L aliquots of diluted cell suspen-

sions containing  $10^4$ – $10^3$ – $10^2$ – $10^1$  cells were spotted on YEPD agar plates containing 0.005 % of external FA and incubated at 28 °C for 2 days. 16:0 palmitic acid, 16:1n-7 palmitoleic acid, 18:0 stearic acid, 18:1n-9 oleic acid, 18:1n-7 *cis*-vaccenic acid



**Fig. 5** Supplementation of *cis*-vaccenic acid partially restores the growth of QM cells on oleic acid-containing media. Sensitivity of the BY4742 strain and from it derived quadruple mutant (QM) to external FA was established by drop tests on plates in serial dilutions. 5- $\mu$ L aliquots of diluted cell suspensions containing  $10^4$ – $10^3$ – $10^2$ – $10^1$

cells were spotted on YEPD agar plates with indicated concentrations of external FA and incubated at 28 °C for 2 days. 16:0 palmitic acid, 16:1n-7 palmitoleic acid, 18:0 stearic acid, 18:1n-9 oleic acid, 18:1n-7 *cis*-vaccenic acid



**Fig. 6** Externally added *cis*-vaccenic acid is efficiently imported into the cells and does not compete with oleic acid uptake. Cells were grown 24 h in YNB complete liquid media and FA content in cells was analyzed as described in “Materials and Methods”. **a** Cell growth. **b** Cellular content of individual FA. QM quadruple mutant, QM *faa1,4*Δ quadruple mutant with *faa1*Δ and *faa4*Δ, 16:0 palmitic acid, 16:1n-7 palmitoleic acid, 18:0 stearic acid, 18:1n-9 oleic acid (OA), 18:1n-7 *cis*-vaccenic acid (VA). Data represent means  $\pm$  SD from three independent experiments

in yeast strains with deletion and overexpression of genes encoding FA elongases we showed that for the formation of VA in yeast cells Elo1, a medium FA chain elongase, is mainly responsible. Our data indicate that QM cells might use the production of VA as an alternative tool to reduce the lipotoxicity of free palmitoleic acid.

The mechanism of sequestration of excess FA in a biologically inert form as TAG and SE is utilized also in cells supplemented with exogenous FA in the growth medium. Recent studies from different laboratories have demonstrated hypersensitivity of the QM cells to exogenous palmitoleic and oleic acids [15–19]. Lipotoxicity of exogenous saturated FA (palmitic or stearic acids) was not noticed [17]. Observed resistance of the QM cells to external VA and the ability of VA to partially suppress oleic acid toxicity are thus rather surprising. Our data suggest that VA is not as harmful as other naturally occurring unsaturated FA in these yeast cells.

One of the most important findings of this study is the fact that the QM cells accumulate VA mainly in the form of free FA (Fig. 2e). It indicates that low toxicity of VA compared to palmitoleic acid or oleic acid may be related to different fate of VA in lipid metabolism. Because of the limited amount of VA in PL and DAG of the QM cells, we hypothesized that yeast acyl-CoA synthetases are able to distinguish between palmitoleic/oleic acid and VA, or alternatively, lipid molecules undergo remodeling and during this process VA is released from PL and DAG into the FFA pool. A recent study in bovine adipocytes supplemented with radioactive palmitoleic acid showed that cells produce VA by elongation of palmitoleic acid [29, 30]. Moreover, the study showed that supplementation of adipocytes with palmitoleic acid or VA reduces lipogenesis [29]. Since the

expense of palmitoleic acid levels. Formation of VA by elongation of palmitoleic acid has already been described in adipocytes and soybean seeds [29, 30]. By analyzing FA



*S. cerevisiae* QM cells accumulate high levels of VA, they might represent a useful model to test the relationship of VA and lipogenesis.

The proposed protective role of VA production in the *S. cerevisiae* mutant defective in TAG synthesis is supported by the situation in another yeast species—*Schizosaccharomyces pombe*. If the TAG synthesis is disabled by disruption of two genes encoding diacylglycerol acyltransferases (*DGAI* and *PLH1*) in this organism, mutant cells undergo apoptosis upon entry into the stationary phase. It is known that there is a significant difference in FA composition of *S. cerevisiae* and *S. pombe* cells. Under standard growth conditions, oleic acid represents the major FA in *S. pombe* cells (75 % of total FA) while only negligible amounts of palmitoleic acid (2.6 % of total FA) are found in this yeast [35]. On the contrary, palmitoleic acid is the most abundant FA in *S. cerevisiae* cells (about 40 % compared to 27 % for oleic acid, see Fig. 1a). Elongation of palmitoleic acid to VA represents thus a mechanism of buffering the free unsaturated FA toxicity in *S. cerevisiae* cells defective in TAG synthesis which is not available in *S. pombe* cells with high levels of oleic acid and very low levels of palmitoleic acid. The absence of this “rescue” mechanism may explain the inability of *S. pombe* cells defective in TAG synthesis to survive the entry into the stationary phase.

In conclusion, our data indicate that the yeast *S. cerevisiae* is equipped with several levels of protection from the cytotoxic effects of unsaturated FA. The first and the most efficient level is the sequestration of unsaturated FA into TAG that are secluded in lipid droplets as specific lipid storage organelles. If TAG synthesis is disabled by simultaneous deletion of four genes encoding yeast acyltransferases (*DGAI*, *LRO1*, *ARE1* and *ARE2*), yeast cells become dependent on other mechanisms of unsaturated FA detoxification. Conversion of palmitoleic acid (16:1n-7) to VA (18:1n-7) seems to be the first-line defense in the QM cells (*dgai1Δ lro1Δ are1Δ are2Δ*) defective in TAG synthesis. Since VA accumulates in the free FA pool in the QM cells, the protective effect may be related to metabolic inertness of this unsaturated FA. Low lipotoxicity of VA compared to other unsaturated FA (palmitoleic and oleic acids) was confirmed also in growth experiments using supplementation of the QM cells with exogenous unsaturated FA. The complex interplay between various protective mechanisms against unsaturated FA toxicity in *S. cerevisiae* is indicated also by the fact that production of VA in the QM cells was arrested by activation of free FA release mechanism. Detailed knowledge about the protective effects of VA in yeast may also contribute to deciphering the roles of this FA in various human pathologies.

**Acknowledgments** We thank K. Athenstaedt (Technical University, Graz, Austria), M. H. Gustavsson (Uppsala Genetic Center, Sweden)

and M. Fulda (University of Göttingen, Germany) for kindly providing yeast strains used in this study. We thank R. Weselake and Ch. Kazala (University of Alberta, Canada) for critical reading of the manuscript. This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0785-11 and APVV-0662-11 and VEGA agency grant No. 2/0180/12.

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