Phenotypic analysis of mutant and overexpressing strains of lipid metabolism genes in *Saccharomyces cerevisiae*: implication in growth at low temperatures

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Abbreviations: FA: Fatty Acids; MCFA: Medium Chain Fatty Acids; UFA: Unsaturated Fatty Acids; SFA: Saturated Fatty Acids; ChL: Chain Lengths; TG: Triacylglyceride; DG: Diacylglyceride; PL: Phospholipid; PI: Phosphatidylinositol; PS: Phosphatidylserine; PE: Phosphatidylethanolamine; PC: Phosphatidylcholine; PG: Phosphatidylglycerol; CL: Cardiolipin; PA: Phosphatic Acid; NL: Neutral Lipid; SE: Sterol Esters
Abstract

The growing demand for wines with a more pronounced aromatic profile calls for low-temperature alcoholic fermentations (10 – 15 °C). However, there are certain drawbacks to low temperature fermentations such as reduced growth rate, long lag phase and sluggish or stuck fermentations. The lipid metabolism of *Saccharomyces cerevisiae* plays a central role in low temperature adaptation. The aim of this study was to detect lipid metabolism genes involved in cold adaptation. To do so, we analyzed the growth of knockouts in phospholipids, sterols and sphingolipids, from the EUROSCARF collection *Saccharomyces cerevisiae* BY4742 strain at low and optimal temperatures. Growth rate of these knockouts, compared with the control, enabled us to identify the genes involved, which were also deleted or overexpressed in a derivative haploid of a commercial wine strain. We identified genes involved in the phospholipid (*PSD1* and *OPI3*), sterol (*ERG3* and *IDI1*) and sphingolipid (*LCB3*) pathways, whose deletion strongly impaired growth at low temperature and whose overexpression reduced generation or division time by almost half. Our study also reveals many phenotypic differences between the laboratory strain and the commercial wine yeast strain, showing the importance of constructing mutant and overexpressing strains in both genetic backgrounds. The phenotypic differences in the mutant and overexpressing strains were correlated with changes in their lipid composition.

Keywords: lipids, mutant, overexpressing strains, cold, yeast
1. Introduction

Temperature fluctuations are an inevitable part of microbial life in exposed natural environments; however, sub-optimal temperatures are also common in industrial processes. Low temperatures (10-15 ºC) are used in wine fermentations to enhance production and retain flavor volatiles. In this way, white and rosé wines of greater aromatic complexity can be achieved (Beltran et al., 2008; Torija et al., 2003). The optimum fermentation temperature for Saccharomyces is between 25 and 28 ºC. Therefore, among the difficulties inherent to wine fermentation (high concentration of sugars, low pH, presence of ethanol, nutrient deficiency, etc.), we should add a sub-optimal temperature for the primary fermentation agent. Temperature affects both yeast growth and fermentation rate, with lower temperatures giving rise to a very long latency phase of up to one week or longer and sluggish fermentations (Bisson, 1999; Meurgues, 1996), dramatically lengthening alcoholic fermentation with the consequent energy expenditure.

Low temperature has several effects on biochemical and physiological properties in yeast cells: low efficiency of protein translation, low fluidity membrane, change in lipid composition, slow protein folding, stabilization of mRNA secondary structures and decrease in enzymatic activities (Aguilera et al., 2007; Hunter and Rose, 1972; Sahara et al., 2002; Schade et al., 2004). To date, most studies have mainly focused on the genome-wide transcriptional responses to cold-shock (Beltran et al., 2006; Homma et al., 2003; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004). In a decisive study, Tai et al. (2007) compared their transcriptomic results obtained in a steady-state chemostat culture with other previous genome-wide transcriptional studies of batch cultures at low temperature. Interestingly, lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature. This is consistent with the
notion that after a temperature downshift, homeoviscous adaptation of the membrane composition is essential for growth (Beltran et al., 2006; Beltran et al., 2007; Hunter and Rose, 1972; Torija et al., 2003).

Biological membranes are the first barrier between the cell interior and its environment and a primary target for damage during cold stress. Major lipid components of eukaryotic membranes are phospholipids, sterols, sphingolipids and glycerolipids. The main species of fatty acids of S. cerevisiae are C16 and C18, with or without a double bond. The composition of these lipid components is important for the physical properties of the membrane, such as fluidity. Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Russell, 1990). Yeasts are known to have developed several strategies to maintain appropriate membrane fluidity.

The most commonly studied involves the increase in unsaturation (mainly palmitoleic C16:1 and oleic C18:1 acids). Phospholipids with unsaturated fatty acids (UFA) have a lower melting point and greater flexibility than phospholipids with saturated acyl chains. Another way of increasing membrane lipid fluidity is to decrease the chain length (ChL) of these FA by increasing the synthesis of medium chain fatty acids (MCFA; C6 to C14) (Beltran et al., 2008; Torija et al., 2003). Recently, Redón et al. (2011) also reported new common changes in the lipid composition of different industrial species and strains of Saccharomyces after low temperature growth. Despite specific strains/species dependent responses, the results showed that at low temperatures the MCFA and triacylglyceride (TG) content increased, whereas the phosphatidic acid content (PA) and the phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio decreased.

Reshaping the plasma membrane composition might be a good strategy for adapting yeast cells to low temperatures, reducing the lag-phase and speeding up fermentation.
onset. In this respect, knock-out or overexpression of genes related with lipid metabolism can modify the architecture of this plasma membrane. In a preliminary study (Redón et al., 2012), we tested various phospholipid mutants from the EUROSCARF collection of S. cerevisiae BY4742 to ascertain whether the suppression of some genes could improve the fermentation vitality of the cells at low temperature. The aim of this study was to detect key genes in the lipid metabolism pathways which play an important role in the adaptation of S. cerevisiae to low temperature. To achieve this objective, we analyzed the growth of several knockouts of phospholipid, sterol and sphingolipid pathways at 12 ºC and 28 ºC and compared them to the wild type BY4742. This first screening of the laboratory strain enabled us to select genes for deletion and overexpression in the genetic background of a derivative haploid of the commercial wine strain, QA23. The phenotypic differences in the mutant and overexpressing strains were correlated with the changes in their lipid composition.

2. Material and methods

2.1 Strains and growth media

S. cerevisiae strains used in this study were: a total of 34 phospholipid, sterol and sphingolipid mutants of the laboratory strain BY4742 (MATα, his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0), from the EUROSCARF collection (Frankfurt, Germany) and the derivative haploid of the commercial wine strain QA23 (Lallemand S.A., Canada), hoQA23 (Salvado et al., 2012).

These strains were cultured on SC (6.7 g/L Difco Yeast Nitrogen Base (w/o amino acids), 20 g/L glucose, 0.83 g/L synthetic complete drop-out mix (2 g Histidine, 4 g Leucine, 2 g Lysine 1.2 g Uracil)). They were grown in Erlenmeyer flasks (250 mL) filled with 50 mL of medium, fitted with cotton and shaken at 200 rpm at 30 ºC for 48
hours. The population inoculated in every flask was $2 \times 10^6$ cells/mL from an overnight culture in YPD at 30 °C.

2.2 Construction of mutant and overexpressing strains

Mutated genes which showed growth insufficiency in the background of the laboratory strain BY4742 were deleted on the derivative haploid of a commercial wine strain, hoQA23. Genes were deleted using the short flanking homology (SFH) method based on the KanMX4 deletion cassette (Güldener et al., 1996). The primers used for amplification of the $\text{loxP-KanMX4-loxP}$ cassette from the plasmid pUG6 have 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The PCR fragments were used to transform the haploid hoQA23 strain using the lithium acetate procedure (Gietz et al., 2002). After transformation, strain selection was done using Geneticin (G418) added to YPD solid media at a concentration of 200 mg/L. Total DNA from transformants resistant to G418 Geneticin was analyzed by PCR using primers upstream and downstream of the deleted region combined with primers of the KanMX gene.

The genes, whose deletion significantly impaired growth in the hoQA23, were overexpressed by cloning into the centromeric plasmid pGREG505, as described in Jansen et al. (2005). All genes were amplified from approximately 600 nucleotides upstream of the start codon to 400 nucleotides downstream of the stop codon to ensure that the promoter and terminator regions were included. The PCR protocol involved an initial denaturation at 94 °C (2 min), followed by 30 cycles of 10 s at 94 °C, 30 s at 49-50 °C (depending on the different primers) and 3-4 min at 72 °C (depending on the different PCR product length). The last cycle was followed by a final extension step of
10 min at 72 ºC. PCR fragments were generated with oligonucleotides that contained the short sequences rec5 (forward) and rec2 (reverse), which are homologous to the sequences in the plasmid (about 35 bp). The plasmid was linearized by SalI digestion and digested with AslI to avoid sticky ends and to make the recombination process easier (Jansen et al., 2005). The wine yeast hoQA23 was co-transformed with the digested pGREG505 plasmid together with the PCR amplified target gene, flanked by recombination sequences homologues to the plasmid ends. This co-transformation promotes an in vivo homologous recombination between both fragments. This recombination process also deleted the GAL1 promoter of the plasmid (the genes were cloned with their own promoters). The transformants were selected by Geneticin resistance, which is encoded by the KanMX gene in the plasmid. Correct yeast transformations were verified by plasmid DNA isolation using a modification of the protocol described by Robzyk and Kassir (1992) and subsequently amplification with the Illustra TempliPhi Amplification Kit (GE Healthcare, UK). Then, to verify the correct integration of the gene into the vector, plasmids were checked by PCR using primers specified for sequences rec5 and rec2. All the strains (mutants and overexpressing) constructed in this study are shown in Table 1.

2.3 Generation time (GT)

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 12 ºC and 28 ºC. Measurements were taken, after pre-shaking the microplate for 20 s, every half hour over 3 days. However at 12 ºC the microplate had to be incubated outside the SPECTROstar spectrophotometer and then transferred inside to take measurements every 8 hours during the lag phase and every 3 hours during the exponential phase. The microplate wells were filled with 0.25 mL of
SC medium, reaching an initial OD of approximately 0.2 (inoculum level of 2 x 10^6 CFU/mL). Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All experiments were carried out in triplicate.

Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering et al. (1990):

\[
y = D \cdot \exp\{-\exp\left[\frac{(\mu_{\text{max}} \cdot e)}{D} \cdot (\lambda - t)\right] + 1\}
\]

where \( y = \ln(\text{OD}_t/\text{OD}_0) \), \( \text{OD}_0 \) is the initial OD and \( \text{OD}_t \) is the OD at time \( t \); \( D = \ln(\text{OD}_t/\text{OD}_0) \) is the asymptotic maximum, \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\)), and \( \lambda \) the lag phase period (h) (Salvadó et al., 2011). Generation time (GT) was calculated using the equation \( \text{GT} = \ln2/\mu_{\text{max}} \). We normalized this value by subtracting the GT of \( S. \) cerevisiae BY4742 and hoQA23 (control strains).

2.4 Spot test

To analyze growth phenotypes of mutant strains, cells grown on SC to stationary phase (OD600 ~ 4) were harvested by centrifugation, washed with sterile water, resuspended in sterile water to an OD (600 nm) value of 0.5, followed by serial dilution of 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\) and 10\(^{-4}\). From each dilution, 3.5 \( \mu \)L was spotted onto SC agar plates. The plates were incubated at 28 °C and 12 °C for 2-9 days.

2.5 Lipid extraction

Mutant and overexpressing strains were grown in SC for 48 hours at 28 °C. Geneticin was also added (200 mg/L) to the SC medium of the overexpressing strains to stabilize the plasmid and promote overexpression of the genes. Cells were frozen until the
different lipid analyses. Prior to lipid extraction, a solution of 100 µL of cold methanol and 20 µL of EDTA 0.1 mM was added to the yeast cells (5-10 mg dry mass) with 1 g glass beads (0.5 mm, Biospec Products) in Eppendorf, and then mixed for 5 minutes in a mini-bead-beater-8 (Biospec Products, Qiagen). Lipid extraction was performed in two steps with 1 mL chloroform/methanol (2:1, v/v, for 1 hour), one step with 1 mL chloroform/methanol (1:1, v/v, for 1 hour) and one step with 1 mL chloroform/methanol (1:2, v/v, for 1 hour). All the organic phases were transferred to a 15 mL glass screw tube and cleaned twice by adding KCl 0.88% (1/4 of a total volume of the extract). After vortexing and cooling at 4 ºC for 10 minutes, the samples were centrifuged at 3000 rpm for 5 minutes. The inferior organic phase was collected and finally concentrated to dryness under nitrogen stream. The residue was dissolve in 100 µL of chloroform/methanol (2:1, v/v).

2.6 Separation and quantification of the yeast phospholipids (PLs) by HPTLC

The yeast extract phospholipids were separated by one-dimensional HPTLC on silica gel 60F$_{254}$ plates (10 x 20cm, 200 µm) with chloroform: acetone: methanol: glacial acetic acid: water (65:15:10:10:5, v/v/v/v/v). After charring the plate with 10% CuSO$_4$ in 8% H$_3$PO$_4$ and heating to 160 ºC for 4 min, the PLs were identified by known standards purchased from Sigma: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA). The plates were scanned and each spot of the image was quantified in terms of integrated optical densities (IOD) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by applying standards
to each plate in the range of 1-4 µg/µL to quantify the PLs. These values were related to the dry weight of cells and expressed as a percentage of the total PLs extracted.

2.7 Yeast neutral lipid (NL) composition by thin-layer chromatography (TLC)

NL composition of yeast was separated by one-dimensional TLC on silica gel 60F_{254} (10 x 20 cm, 250 µm) (Merck, Germany). The plate was developed in three steps: the first step with hexane, tert-Butyl methyl ether (MTBE) and glacial acetic acid (50:50:2) to 35mm, the second step with hexane, tert-Butyl methyl ether (MTBE) and glacial acetic acid (80:20:1) to 60mm and the last step with hexane to 85mm. The standard lipids lanosterol, ergosterol, squalene, cholesteryl oleate, ethyl oleate, diolein, triolein were purchased from Sigma and were applied to every plate in the range of 1-4 µg/µL. After TLC, lipids were charred with 10% CuSO_{4} in 8% H_{3}PO_{4} and heated to 160 ºC for 4 min on a TLC Plate Heater (CAMAG). Plates were scanned and each spot of the image was quantified as integrated optical densities (IOS) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by applying standards to each plate in the range of 1-4 µg/µL to quantify the NLs. These values were related to the cell dry weight and expressed as a percentage of total NLs extracted.

2.8 Determination of total yeast fatty acids

Yeast cells (5-10 mg dry mass) were placed in sealed tubes with a Teflon-lined screw cap and saponified using a 1 mL of 5% NaOH in 50% methanol/water (Rozès et al., 1992). The tubes were placed in a dry bath (100 ºC) for 5 minutes. Samples were vortexed and then the tubes were placed in a dry bath (100 ºC) for another 25 minutes. Then the saponified material was cooled to room temperature and 2 mL HCl 6M was
added. Free fatty acids were extracted by adding 500 µL hexane: tert-Butyl methyl ether (MTBE) (1:1, v/v). Each tube was vortexed twice for 30 seconds. The organic phase was collected after centrifugation at 3000 rpm for 3 minutes. Analytical gas chromatography was performed on a Hewlett-Packard 6850 (Agilent Technologies). 1µL of cellular extract was injected (splitless, 1 minute) into a FFAP-HP column (30m x 0.25mm x 0.25µm from Agilent) with an HP 6850 automatic injector. The initial temperature was set at 140 ºC and increased by 4 ºC/min up to 240 ºC. Injector and detector temperatures were 250 ºC and 280 ºC, respectively. The carrier gas (helium) was at a flow rate 1.4 mL/min. Heptanoic and heptadecanoic acids (10 and 40 mg/mL, respectively) were added as internal standards before cell saponification. Relative amounts of fatty acids were calculated from their respective chromatographic peaks. These values were related to the dry weight of cells and expressed as a percentage of the total fatty acid extracted (Redón et al., 2009).

2.9 Statistical data processing

All experiments were repeated at least three times, and data are reported as the mean value ± SD. Significant differences between the control strain, the mutant and the overexpressing strains were determined by t-tests (SPSS 13 software package). The statistical level of significance was set at $P \leq 0.05$. Principal component analysis (PCA) was done using vegan package (rda function) from the statistical software R v.2.15 (R Development Core Team, 2010).

3. Results

3.1 Generation time (GT)

3.1.1 Determination of generation time and spot test in BY4742 lipid mutants
In order to determine the importance of lipid metabolism genes on growth at low temperature, we determined GT and carried out spot test for the screening of the BY4742 lipid mutants at 12 ºC and at 28 ºC. Most of the deleted genes analyzed (some deletions produce unviable phenotypes) are shown graphically in their respective pathways (Fig. 1). The GT of these mutant strains is also grouped on the basis of the biosynthesis pathway to which they belong (phospholipids, sterols and sphingolipids) (Fig. 2).

The strains with deletions in the genes OPI3, CHO2 and PSD1, encoding enzymes of the phospholipid pathway; ERG24, ERG6 and ERG3, from the sterol biosynthesis pathway; and DPL1, involved in sphingolipid pathway significantly increased their GT at 12 ºC compared to the control strain BY4742. Some of these mutant strains also showed significant differences at 28 ºC, but, most of them, these were not as extreme as at 12 ºC (Fig. 2).

Conversely, some mutant strains improved their relative growth. A remarkable decrease in GT was detected for the deletion of ERG2, involved in the synthesis of a precursor of ergosterol (episterol). Likewise, several mutant strains of the sphingolipid synthesis, such as Δyrs3, Δcsg2, Δipt1, Δsur2, Δydc1, Δlcb4 and Δlcb3, also decreased their GT significantly compared to control strain BY4742.

This data on growth in liquid SC were corroborated by a drop test on a SC agar plate at 12 ºC and 28 ºC. Generally, the same mutant strains also showed an impaired growth at low temperature whereas they were hardly affected at 28 ºC. As an example, the drop test for the ERG genes (ergosterol pathway) is shown in Figure 2D. Only the deletion of cardiolipin synthesis (CRD1) led to worse growth on solid medium than in liquid medium at 12 ºC (data no shown).
3.1.2 Determination of generation time in lipid mutants and overexpressing strains of *hoQA23*

A total of 15 genes, whose deletion showed significant differences in GT in BY4742, were also deleted in the haploid wine strain *hoQA23*. The first remarkable result was the difference in growth behavior observed depending on the genetic background of the strains in which the genes were deleted. In contrast to the laboratory strain, no deletion yielded better growth than the parental wine strain *hoQA23* (Fig. 3A). Most of the deleted genes from the sphingolipid pathway with a lower GT in BY4742 mutants did not show differences or displayed slow growth (i.e. Δlcβ3) in the *hoQA23*. Other remarkable differences between both strains were the phenotypes observed for the mutant strains of genes *CHO2* and *CRD1* in the wine strain *hoQA23*. The Δcho2 strain was unable to grow in SC medium (only grew in YPD) but growth was recovered when SC medium was supplemented with choline. Thus the mutation of this gene caused auxotrophy for choline in the wine strain *hoQA23*. Regarding *CRD1*, we were unable to delete this gene in *hoQA23* because the *CRD1* knock-out made this strain unviable. We confirmed that *CRD1* is required for viability of this wine strain by further deleting one of the copies of the diploid commercial strain QA23. This heterozygous mutant strain (CRD1/Δcrd1) was sporulated but only the spores of the wild copy (non Geneticin resistant) were recovered in the YPD medium. The heterozygous mutant strain (CRD1/Δcrd1) did not show any differences in terms of GT with the parental strain QA23 (data not shown). Thus this mutation produces unviability but not haploinsufficiency.

The mutant strains with significant differences in GT are shown in Figure 3A. Δerg3, Δpsd1 and Δopi3 showed the most important increases in GT. These two latter
phospholipid mutants also presented impaired growth at 28 °C, but, as in the case of
BY4742, these increases in GT were much more moderate than at low temperature.
The six genes whose deletion produced slowest growth were also overexpressed in the
wine strain hoQA23. Although Δlcb4 did not show significant differences in GT, we
decided to overexpress this gene because it encodes the enzyme Lcb4, a sphingoid long-
chain base kinase, which catalyzes the reversible step of Lcb3, and has been related with
heat shock adaptation (Dickson *et al.*, 2006). We also constructed strains
overexpressing genes IDI1 and OLE1, whose deletion produced an unviable phenotype
(Giaever *et al.*, 2002) and CHO2, whose knock-out also yielded an auxotrophic
phenotype for choline. IDI1 is involved in the ergosterol biosynthesis pathway (Fig. 1)
and OLE1 encodes the only desaturase in *S. cerevisiae*, required for monounsaturated
fatty acid synthesis (Mitchell and Martin, 1995). The overexpression of the selected
genes decreased GT at low temperature, although only the strains pGREG LCB3,
pGREG IDI1, pGREG ERG3, pGREG OPI3 and pGREG PSD1 showed significant
decreases in GT (Fig. 3B).

3.2 Lipid composition

The lipid composition (fatty acids, phospholipids, neutral lipids and sterols) of the
mutant and overexpressing strains selected in the previous section was compared with
the control strains (*hoQA23* and *hoQA23 pGREG*). It is worth mentioning that TLC
enables us to detect only the main metabolites of the phospholipid and ergosterol
biosynthesis pathways. Unfortunately we were not able to analyze sphingolipids with
the methodology available in our laboratory. **The percentages of the different lipids in
the constructed strains are shown in Table S1.** The impact of deleting or overexpressing
a gene on the compounds of its respective pathway is graphically shown in Figures 4 and 5.

As expected, the most important modification in phospholipid composition was observed in the mutant strains of phospholipid pathway Δpsd1 and Δopi3, which showed a significant increase in PI and important reduction of PS, PC and PE (Fig. 4 A.1). In fact, PE and PC were not detected in Δpsd1 and Δopi3 respectively. Moreover, the blockage in PC synthesis in Δopi3 yielded a new band on HPLTC plates, which may suggest the detection of PMPE and PMMPE intermediates (Fig. 1A). For Δpsd1, the strong PE reduction seemed to be compensated by a significant increase in CL (Fig. 4 A.1). It should be kept in mind that we cannot analyze PL composition of Δcho2 because this mutant was unable to grow in SC medium.

It should be highlighted that the parental hoQA23 (control strain of the mutants; panel 1) and the same strain transformed with the empty vector pGREG (control strain of the overexpressing strains; panel 2) differed in the composition of some PLs. These differences may be explained by the presence of Geneticin in the growth medium of the overexpressing strains and resistance to this antibiotic encoded in the plasmid. For all the phospholipid overexpressing strains, the most important changes were observed in pGREG CHO2 (Fig. 4 A.2). Contrary to the expected result, overexpression of CHO2 induced a significant increase in PE and CL percentages, but a decrease in PC. In fact, most of the overexpressing strains showing significant differences seemed to follow the same trend: to decrease their PC content and increase in PE and CL percentage, except pGREG OLE1 and pGREG DPL1 which had less PE (Table S1).

In contrast to PL composition, the mutant and overexpressing strains involved in sterol synthesis did not show important changes in sterol composition (Fig. 4 B.1 and 4 B.2).
The most remarkable trend is that the overexpressing strains increased the sterol esters and decreased squalene.

As expected, pGREG OLE1 significantly increased UFA (mainly in palmitoleic acid C16:1) and decreased saturated fatty acids (SFA) (mainly in palmitic acid C16) (Fig. 5). However most of the overexpressing strains which showed significant differences in their GT (pGREG OPI3, pGREG IDI1 and pGREG LCB3) also significantly increased the UFA/SFA ratio (Table S1). In the case of the mutant strains, the general trend was an increase in C16 and C16:1 and a decrease in C18 and C18:1. This increase in shorter-chain fatty acids resulted in a decrease in the average fatty acid chain length of most of the mutant strains. As a paradigm of this trend, we were able to detect the myristoleic acid (C14:1) in Δerg6 whereas C18 was undetectable in Δlcb3 (Table S1).

3.3 Principal component analysis (PCA)

In order to explore the effect of the deletion and overexpression of the target genes in lipid composition, a PCA was performed on the 19 strains using the untransformed relative concentration of the 18 compounds measured in all strains (Fig. 6). The two first components were retained explaining 80.8% of the total variance. The first component explained 66.2% of the variation and was marked by high positive component loadings for sterol esters (+0.605) and PC (+0.430) and high negative loadings for PI (-0.429) and FA (-0.406). The second component explained 14.6% of the variation and was marked by high positive component loadings for sterol esters (+0.620) and PI (+0.511) and high negative loadings for PE (-0.395) and TG (-0.320).

The general pattern provided by the PCA is the formation of two groups: deletion and overexpressing strains associated with low and high amounts of sterol esters,
respectively. Moreover deletion strains were grouped by PI content and overexpressing strains by PE content.

4. Discussion

Yeast adaptation at low temperature is an interesting feature from an industrial viewpoint, especially in the wine industry, where low temperatures are used to enhance production and retain flavor volatiles. Lipid composition of the cellular membranes has been directly related with yeast adaptive response at different environmental temperatures in many studies (Beltran et al., 2008; Henschke and Rose, 1991; Redón et al., 2011; Torija et al., 2003). A possible adaptation might be the reshaping of the plasma membrane composition, which would reduce the lag phase, increase growth and speedup fermentation onset. In a previous study, we modified lipid composition by incubating yeast cells in the presence of different lipid compounds, improving growth and fermentation activity at low temperature (Redón et al., 2009). Another strategy to redesign the cellular lipid composition is to alter transcriptional activity by deleting or overexpressing key genes of lipid metabolism. This latter strategy has previously been and successfully assayed, though not as comprehensively as in this study. Some authors have overexpressed the gene encoding the S. cerevisiae desaturase OLE1 (Kajiwara et al., 2000) or other heterologous desaturases (Rodríquez-Vargas et al., 2006) in order to increase the degree of unsaturation and membrane fluidity, while improving the cold resistance of these engineered strains. In a recent work (Redón et al., 2012), we have also detected an improved or impaired fermentation vitality in some mutants of the phospholipid biosynthesis. In the present study, we have screened most of the mutants of the laboratory strain BY4742 encoding enzymes of the phospholipid, sterol and sphingolipid pathways in terms of their growth capacity at low temperature. The GT of
these mutant strains was used to select genes which were further deleted in a derivative industrial strain. Again, the deleted genes showing impaired growth at low temperature were overexpressed in the genetic background of this industrial strain. The main objective of this study was to identify lipid-metabolism genes that play a key role in the adaptive response of wine yeast to low temperature and to verify the correlation between growth at low temperature and lipid composition.

Phenotypic differences between strains mutated in the same gene constructed in the laboratory and wine yeast showed the importance of the genetic background (Pizarro et al., 2008; Redón et al., 2011). In the wine strain hoQA23, the deletion of CRD1 led to unviability whereas BY4742 was hardly affected by the deletion of this gene. CRD1 encodes cardiolipin synthase which catalyses the last step in CL synthesis, but it is not essential for growth (Breslow et al., 2008). Thus, other complementary mutations confer synthetic lethality in the haploid wine strain hoQA23. ERG24 provides another example of the genetic background effect. This gene encodes a C-14 sterol reductase and the mutants accumulate the abnormal sterol ignosterol (ergosta-8,14 dienol), and are viable under anaerobic growth conditions but unviable on rich medium under aerobic conditions (Marcireau et al., 1992). We detected important impaired growth in the BY4742 Δerg24, although this mutation hardly affected growth fitness in hoQA23. However, in spite of these differences, we detected gene deletions which significantly affected growth fitness at low temperature in both studied strains.

The mutants in the PL synthesis pathway Δpsd1 and Δopi3 (and Δcho2 in the BY4742) showed the greatest increases in terms of GT in comparison with the parental strains. These genes encode the enzymes involved in synthesis of the most important plasma membrane phospholipids, PE and PC, by the de novo pathway (Daum et al., 1998). As expected, these mutant strains were characterized by a strong reduction in the
proportion of PE and PC. *S. cerevisiae* had two PS-decarboxylases, one located in the mitochondrial inner membrane (encoded by *PSD1*) and another located in the Golgi and vacuolar membranes (encoded by *PSD2*). Daum *et al.* (1998) reported *PSD1* had no effect on cell viability because Δ*psd1* had residual PSD activity attributed to Psd2p. However the presence of the isoenzyme Psd2p was not enough to counterbalance the lack of Psd1p growing at low temperature. The decrease in PE and PC in the Δ*psd1* and Δ*opi3* strains was counterbalanced by the increase in PI and CL. All these PLs have the same precursor CDP-DAG and, the blockage in the PE and PC biosynthetic branch increased the flux in the other two branches, leading to PI and CL increases (Fig. 1A).

Contrary to PL mutant strains, the overexpression of genes *OPI3* and *PSD1* produced a significant reduction in GT in the wine strain at low temperatures. Enhanced growth in the PL overexpressing strains could be correlated with changes in lipid composition; however, these overexpressing strains did not significantly increase PE and PC. Even the overexpression of *OPI3*, which catalyzes the last two steps in PC biosynthesis, decreased the proportion of this PL. This metabolic route must be fine-tuned to avoid imbalances in PL proportion as a consequence of increasing the gene-dosage of some enzymes in the pathway.

The deletion and overexpression of *ERG3* also produced a phenotype with worse and better growth, respectively, in comparison with the parental strain. This gene encodes a sterol desaturase, which catalyzes the insertion of a double bond into episterol, a precursor in ergosterol biosynthesis. The deletion of this gene has previously been related with cold sensitivity (Hemmi *et al.*, 1995). These authors correlated the growth defect at low temperature with a defect in tryptophan uptake in the Δ*erg3* mutants. Another overexpressed gene in the ergosterol pathway that significantly reduced its duplication time was *ID11* (mutant strain is unviable). We selected this gene because
Beltran et al. (2006) previously reported a strong up-regulation of this gene at low temperature fermentation in a global transcriptomic analysis of the same industrial wine yeast. IDI1 encodes the isopentenyl-diphosphate delta-isomerase which catalyzes the isomerization between isopentenyl pyrophosphate and dimethylallyl pyrophosphate (Fig. 1B). In terms of sterol composition, the overexpression of this gene did not change the proportion of the main sterols substantially. As in the PL overexpressing strains, it is difficult to correlate improved growth with changes in the composition of the main metabolites of the pathways involving these genes. It must be borne in mind that our methodology was unable to detect ergosterol precursors. Thus, the possibility that deletion or overexpression may produce changes in the concentration of these precursors cannot be ruled out.

Finally, only the mutation and overexpression of the sphingolipid gene LCB3 yielded a significant increase and decrease, respectively, in GT. This gene encodes a phosphatase with specificity for dihydrosphingosine-1-phosphate, regulating ceramide and long-chain base phosphate levels and involves in incorporation of exogenous long-chain bases in sphingolipids (Mao et al., 1997; Mandala et al., 1998; Qie et al., 1997). Intermediates in sphingolipid biosynthesis, such as sphingolipid long-chain bases (LCBs), dihydrosphingosine (DHS) and phytosphingosine (PHS) (Figure 1C), have been identified as secondary messengers in signaling pathways that regulate the heat stress response (Ferguson-Yankey et al., 2002; Jenkins et al., 1997). Thus, it cannot be ruled out that these sphingolipid intermediates may also contribute to the cold stress response. Unfortunately we were not able to determine how the deletion or overexpression of this gene affected the content of these intermediates.

In an attempt to correlate growth of the different constructed strains at low temperature and modification in their lipid composition, we performed a PCA. This is a useful tool
for identifying similarity and difference patterns among strains for which many data are analyzed. The PCA data clearly separated the mutant strains (left panel) from the overexpressing strains (right panel). Although genes involved in different lipid pathways were deleted or overexpressed, a general modification of the lipid profile can be ascribed to both groups of strains. The mutant strains tended to increase PI and FA, whereas the overexpressing strains increased sterol esters and the phospholipids PC and PE. Both lipid compounds have been linked to low temperature growth or fermentation activity in previous works by our research team. Redon et al. (2011) compared the lipid composition of strains, belonging to different Saccharomyces species and isolated from different fermentative processes (wine, beer, bread), after growing at optimum (25 ºC) and low temperatures (13 ºC). A common change in all the strains under study was the increase in PE and reduction in the PC/PE ratio. In a similar study, Tronchoni et al. (2012) also compared the lipid composition of different S. kudriavzevii strains (a more psychrophilic species than S. cerevisiae) and hybrid strains between S. cerevisiae and S. kudriavzevii. In these strains, in terms of neutral lipids, a common response to low temperature was an increase in TG and SE, the main storage lipids. These storage lipids are mainly synthesized during the stationary phase, when the growth is arrested, and there is an excess of intermediates of the biosynthetic pathways (Czabany et al., 2007), in a similar manner to the accumulation of carbohydrates such as glycogen and trehalose. Thus, the excess of intermediates of the sterol pathway in the overexpressing strains can produce an increase in the synthesis of sterol esters.

In conclusion, here we report a study aiming to detect the role of key lipid metabolism genes in promoting better growth at low temperature and which can be important in the adaptation to industrial processes. The study has identified genes involved in the phospholipid (PSD1 and OPI3), sterol (ERG3 and IDI1) and sphingolipid (LCB3)
pathways whose deletion strongly impaired growth at low temperature, whereas their overexpression reduced generation or division time by almost half. The study also reveals the importance of constructing mutant and overexpressing strains in the genetic background of commercial wine yeast, given the many phenotypic differences observed between these and the laboratory strain. As the impact of all these genes can be modulated by the genetic background, new strains should be tested in future studies to ensure the universality of these mechanisms of adaptation at low temperature.

Moreover, further research will test these strains with improved growth during grape must fermentations and analyze their growth behavior and fermentation performance. This information may help to improve the future performance of wine yeast at low temperature, either by genetic modification or by the selection of strains with a better genetic makeup in terms of low temperature adaptation.

Acknowledgments

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References


for understanding the global responses of low-temperature winemaking fermentations.


Mutant analysis reveals complex regulation of sphingolipid long chain base phosphates and long chain bases during heat stress in yeast. Yeast 19, 573-86.


Table 1. Strains constructed in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>hoQA23</td>
<td>MATα; YDL227C::kanMX4</td>
<td>Derivative wine haploid strain</td>
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<tr>
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<td>OPI3 mutant strain</td>
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<td>Haploid strain with empty plasmid</td>
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<td>pGREG DPL1</td>
<td>hoQA23-pGREG DPL1</td>
<td>DPL1 overexpressing strain</td>
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**Table S1.** Percentage of phospholipids (PI, PS, PC, PE, CL, PA and MM-PE), neutral lipids (DG and TG), sterols (squalene, lanosterol, ergosterol and sterol esters) and fatty acids (C14:1, C16, C16:1, C18 and C18:1) expressed as the mean ± SEM (standard error of the mean) of total cellular concentration of these compounds. *Significant differences compared with their respective control strains (hoQA23 and hoQA23 pGREG).

<table>
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<tr>
<th>Strains</th>
<th>PI</th>
<th>PS</th>
<th>PC</th>
<th>PE</th>
<th>CL</th>
<th>PA</th>
<th>MM-PE</th>
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<td>20.46 ± 2.43</td>
<td>79.54 ± 2.43</td>
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<td>13.54 ± 4.34*</td>
<td>-</td>
<td>39.05 ± 9.25*</td>
<td>7.81 ± 1.74*</td>
<td>-</td>
<td>39.59 ± 1.19*</td>
<td>60.41 ± 1.19*</td>
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<td>2.02 ± 0.43*</td>
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<td>nq</td>
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<td>C16:1</td>
<td>C18</td>
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<td>14.44 ± 2.03</td>
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<td>18.35 ± 0.99*</td>
<td>35.51 ± 0.67</td>
<td>6.05 ± 1.01</td>
<td>34.02 ± 2.83*</td>
</tr>
<tr>
<td>pGREG IDI1</td>
<td>3.97 ± 0.32*</td>
<td>-</td>
<td>11.26 ± 2.83</td>
<td>84.77 ± 2.88*</td>
<td>-</td>
<td>20.29 ± 0.99*</td>
<td>36.47 ± 0.67*</td>
<td>3.31 ± 3.03</td>
<td>39.93 ± 2.83</td>
</tr>
<tr>
<td>pGREG OLE1</td>
<td>4.61 ± 1.10</td>
<td>2.28 ± 0.52</td>
<td>13.14 ± 1.39</td>
<td>79.98 ± 3.02</td>
<td>-</td>
<td>12.25 ± 0.82*</td>
<td>41.93 ± 0.39*</td>
<td>3.95 ± 0.30</td>
<td>41.88 ± 1.36*</td>
</tr>
<tr>
<td>pGREG DPL1</td>
<td>3.18 ± 0.60*</td>
<td>0.89 ± 0.12*</td>
<td>10.05 ± 0.46</td>
<td>85.87 ± 0.30*</td>
<td>-</td>
<td>16.80 ± 0.34*</td>
<td>35.77 ± 1.74</td>
<td>3.96 ± 0.35</td>
<td>43.20 ± 1.39*</td>
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<tr>
<td>pGREG LCB3</td>
<td>8.01 ± 2.52</td>
<td>9.34 ± 4.55</td>
<td>12.57 ± 1.70</td>
<td>70.08 ± 8.70</td>
<td>-</td>
<td>19.91 ± 0.34</td>
<td>36.37 ± 1.74</td>
<td>3.97 ± 0.35</td>
<td>39.75 ± 1.39*</td>
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<tr>
<td>pGREG LCB4</td>
<td>1.90 ± 0.36*</td>
<td>2.23 ± 0.95</td>
<td>11.45 ± 1.89</td>
<td>84.42 ± 2.05*</td>
<td>-</td>
<td>21.35 ± 0.44*</td>
<td>33.58 ± 2.41</td>
<td>5.06 ± 1.40</td>
<td>38.90 ± 1.80*</td>
</tr>
</tbody>
</table>

(-) not detected, (nq) not quantified
Figure legends

**Figure 1.** Diagrams of the major pathways for lipid biosynthesis in *S. cerevisiae*: A) Phospholipid pathway B) Sterol pathway C) Sphingolipid pathway. Genes in bold indicate viable mutants.

**Figure 2.** Growth of lipid mutant strains compared with control strain BY4742. Generation time of A) phospholipid, B) sterol, C) sphingolipid mutants grown at 12 ºC (black bars) and at 28 ºC (grey bars). The GT of the mutant strains were compared to GT of control strain BY4742 (normalized as value 1). The duplication time for this control strain was 20.24 h at 12 ºC and 3.09 h at 28 ºC. D) Results of sterol mutants spot test at 12º C and 28 ºC. *Significant differences compared with the wild type at the same temperature.

**Figure 3.** Growth of lipid mutant and overexpressing strains compared with their control strains. Generation time of A) mutant and B) overexpressing strains grown at 12 ºC (black bars) and at 28 ºC (grey bars). The GT of the mutant and overexpressing strains compared to GT of their control strains *hoQA23* and *hoQA23 pGREG* (normalized as value 1). The GT for control strains was the following: 15.94 h and 2.58 h for *hoQA23* and 12.18 h and 3.13 h for *hoQA23 pGREG* at 12 ºC and 28 ºC, respectively. *Significant differences compared with the control strains at the same temperature.
**Figure 4.** Percentages of phospholipids (A), neutral lipids and sterols (B) for the mutant (1) and overexpressing (2) strains of these biosynthetic pathways. *Significant differences compared with their respective control strains.

**Figure 5.** Percentages of fatty acids of pGREG *OLE1* strain and their control strain, *hoQA23* pGREG. *Significant differences compared with their respective control strains.

**Figure 6.** Biplot of the first two components of the PCA according to the lipid composition. Variables are represented by grey underlining and samples are represented by black underlining: deletion strains (lower-case letters) and overexpressing strains (capital letters).
Figure 2

A)

B)

C)

D)
Figure 3

A)  

B)
Figure 5

Relative amount of FA (%)

- C16
- C16:1
- C18
- C18:1

Bars represent the relative amount of fatty acids (FA) for pGREG and pGREG OLE1. Significant differences are indicated by asterisks.