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: Phosphatidic 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 ºC). 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, his3*Δ* 1; leu2*Δ* 0; lys2*Δ* 0; ura3*Δ* 0), from the EUROSCARF collection (Frankfurt, Germany) and the derivative haploid of the commercial wine strain QA23 (Lallemand S.A., Canada), *ho*QA23 (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 x 10^6 cells/mL from an overnight culture in YPD at 30 ºC.

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, *ho*QA23. 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 *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 *ho*QA23 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 *ho*QA23, 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 ºC (2 min), followed by 30 cycles of 10 s at 94 ºC, 30 s at 49-50 ºC (depending on the different primers) and 3-4 min at 72 ºC (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 *Sal*I digestion and digested with *Asl*I to avoid sticky ends and to make the recombination process easier (Jansen *et al.*, 2005). The wine yeast *ho*QA23 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

158 SC medium, reaching an initial OD of approximately 0.2 (inoculum level of 2 x 10^6
159 CFU/mL). Uninoculated wells for each experimental series were also included in the
160 microplate to determine, and consequently subtract, the noise signal. All experiments
161 were carried out in triplicate.
162 Growth parameters were calculated from each treatment by directly fitting OD
163 measurements versus time to the reparameterized Gompertz equation proposed by
164 Zwietering *et al.* (1990):
165 $$y=D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$
166 where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t;
167 $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate
168 (h^{-1}), and λ the lag phase period (h) (Salvadó *et al.*, 2011). Generation time (GT) was
169 calculated using the equation $GT=\ln2/\mu_{max}$. We normalized this value by subtracting the
170 GT of *S. cerevisiae* BY4742 and *ho*QA23 (control strains).
171
172 *2.4 Spot test*
173 To analyze growth phenotypes of mutant strains, cells grown on SC to stationary phase
174 (OD600 ~ 4) were harvested by centrifugation, washed with sterile water, resuspended
175 in sterile water to an OD (600 nm) value of 0.5, followed by serial dilution of 10^{-1}, 10^{-2},
176 10^{-3} and 10^{-4}. From each dilution, 3.5 µL was spotted onto SC agar plates. The plates
177 were incubated at 28 ºC and 12 ºC for 2-9 days.
178
179 *2.5 Lipid extraction*
180 Mutant and overexpressing strains were grown in SC for 48 hours at 28 ºC. Geneticin
181 was also added (200 mg/L) to the SC medium of the overexpressing strains to stabilize
182 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_3PO_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_3PO_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 *Δysr3, 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 ho*QA23

A total of 15 genes, whose deletion showed significant differences in GT in BY4742, were also deleted in the haploid wine strain *ho*QA23. 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 *ho*QA23 (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. *Δlcb3*) in the *ho*QA23. Other remarkable differences between both strains were the phenotypes observed for the mutant strains of genes *CHO2* and *CRD1* in the wine strain *ho*QA23. 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 *ho*QA23. Regarding *CRD1*, we were unable to delete this gene in *ho*QA23 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

306 phospholipid mutants also presented impaired growth at 28 ºC, but, as in the case of
307 BY4742, these increases in GT were much more moderate than at low temperature.
308 The six genes whose deletion produced slowest growth were also overexpressed in the
309 wine strain *ho*QA23. Although *Δlcb4* did not show significant differences in GT, we
310 decided to overexpress this gene because it encodes the enzyme Lcb4, a sphingoid long-
311 chain base kinase, which catalyzes the reversible step of Lcb3, and has been related with
312 heat shock adaptation (Dickson *et al.*, 2006). We also constructed strains
313 overexpressing genes *IDI1* and *OLE1*, whose deletion produced an unviable phenotype
314 (Giaever *et al.*, 2002) and *CHO2*, whose knock-out also yielded an auxotrophic
315 phenotype for choline. *IDI1* is involved in the ergosterol biosynthesis pathway (Fig. 1)
316 and *OLE1* encodes the only desaturase in *S. cerevisiae*, required for monounsaturated
317 fatty acid synthesis (Mitchell and Martin, 1995). The overexpression of the selected
318 genes decreased GT at low temperature, although only the strains pGREG *LCB3*,
319 pGREG *IDI1*, pGREG *ERG3*, pGREG *OPI3* and pGREG *PSD1* showed significant
320 decreases in GT (Fig. 3B).
321
322 *3.2 Lipid composition*
323 The lipid composition (fatty acids, phospholipids, neutral lipids and sterols) of the
324 mutant and overexpressing strains selected in the previous section was compared with
325 the control strains (*ho*QA23 and *ho*QA23 pGREG). It is worth mentioning that TLC
326 enables us to detect only the main metabolites of the phospholipid and ergosterol
327 biosynthesis pathways. Unfortunately we were not able to analyze sphingolipids with
328 the methodology available in our laboratory. The percentages of the different lipids in
329 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 *ho*QA23 (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 *Δerg*6 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 (Rodríguez-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 *ho*QA23, 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 *ho*QA23. *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 *ho*QA23. 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 *Δpsd1* 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 *Δpsd1* and *Δopi3* 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 *Δerg3* mutants. Another overexpressed gene in the ergosterol pathway that significantly reduced its duplication time was *IDI1* (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*)

503 pathways whose deletion strongly impaired growth at low temperature, whereas their
504 overexpression reduced generation or division time by almost half. The study also
505 reveals the importance of constructing mutant and overexpressing strains in the genetic
506 background of commercial wine yeast, given the many phenotypic differences observed
507 between these and the laboratory strain. As the impact of all these genes can be
508 modulated by the genetic background, new strains should be tested in future studies to
509 ensure the universality of these mechanisms of adaptation at low temperature.
510 Moreover, further research will test these strains with improved growth during grape
511 must fermentations and analyze their growth behavior and fermentation performance.
512 This information may help to improve the future performance of wine yeast at low
513 temperature, either by genetic modification or by the selection of strains with a better
514 genetic makeup in terms of low temperature adaptation.

515
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643

Figure

Table 1. Strains constructed in this study

Strain	**Genotype**	**Definition**
*ho*QA23	MATα; YDL227C::kanMX4	Derivative wine haploid strain
Δpsd1	*ho*QA23; YNL169c::kanMX4	*PSD1* mutant strain
Δopi3	*ho*QA23; YJR073C::kanMX4	*OPI3* mutant strain
Δerg3	*ho*QA23; YLR056W::kanMX4	*ERG3* mutant strain
Δerg6	*ho*QA23; YML008C::kanMX4	*ERG6* mutant strain
Δlcb3	*ho*QA23; YJL134W::kanMX4	*LCB3* mutant strain
Δlcb4	*ho*QA23; YOR171C::kanMX4	*LCB4* mutant strain
Δdpl1	*ho*QA23; YDR294C::kanMX4	*DPL1* mutant strain
*ho*QA23 pGREG	*ho*QA23-pGREG505	Haploid strain with empty plasmid
pGREG *PSD1*	*ho*QA23-pGREG *PSD1*	*PSD1* overexpressing strain
pGREG *CHO2*	*ho*QA23-pGREG *CHO2*	*CHO2* overexpressing strain
pGREG *OPI3*	*ho*QA23-pGREG *OPI3*	*OPI3* overexpressing strain
pGREG *ERG3*	*ho*QA23-pGREG *ERG3*	*ERG3* overexpressing strain
pGREG *ERG6*	*ho*QA23-pGREG *ERG6*	*ERG6* overexpressing strain
pGREG *IDI1*	*ho*QA23-pGREG *IDI1*	*IDI1* overexpressing strain
pGREG *OLE1*	*ho*QA23-pGREG *OLE1*	*OLE1* overexpressing strain
pGREG *LCB3*	*ho*QA23-pGREG *LCB3*	*LCB3* overexpressing strain
pGREG *LCB4*	*ho*QA23-pGREG *LCB4*	*LCB4* overexpressing strain
pGREG *DPL1*	*ho*QA23-pGREG *DPL1*	*DPL1* overexpressing strain

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 (*ho*QA23 and *ho*QA23 pGREG).

	PHOSPHOLIPIDS							NEUTRAL LIPIDS	
Strains	**PI**	**PS**	**PC**	**PE**	**CL**	**PA**	**MM-PE**	**DG**	**TG**
*ho*QA23	17.89 ± 1.16	6.11 ± 0.86	33.70 ± 2.72	25.25 ± 1.47	12.82 ± 2.83	4.23 ± 0.42	-	20.46 ± 2.43	79.54 ± 2.43
Δ*psd1*	38.10 ± 2.56*	1.50 ± 0.61*	13.54 ± 4.34*	-	39.05 ± 9.25*	7.81 ± 1.74*	-	39.59 ± 1.19*	60.41 ± 1.19*
Δ*opi3*	59.86 ± 1.39*	0.94 ± 0.13*	-	5.42 ±1.89*	10.23 ± 0.96	2.02 ± 0.43*	21.53 ± 1.39*	32.25 ± 8.07	67.75 ± 8.07
Δ*erg3*	12.49 ± 0.01*	4.66 ± 0.34*	28.34 ± 2.96	21.26 ± 3.97	18.45 ± 0.70*	14.80 ± 1.37*	-	18.25 ± 4.38	81.75 ± 4.38
Δ*erg6*	19.72 ± 0.28	4.46 ± 0.63	20.65 ± 0.32*	17.84 ± 0.90*	27.69 ± 0.57*	9.65 ± 0.92*	-	30.50 ± 6.66	69.50 ± 6.66
Δ*dpl1*	8.42 ± 2.11*	7.02 ± 0.19	39.74 ± 2.30*	23.38 ± 1.53	nq	nq	-	20.62 ± 1.08	79.38 ± 1.08
Δ*lcb3*	10.89 ± 5.47	7.24 ± 1.15	39.15 ± 4.69	26.28 ± 3.25	nq	nq	-	22.51 ± 4.54	77.49 ± 4.54
Δ*lcb4*	13.51 ± 1.03*	6.09 ± 0.41	41.94 ± 3.77*	20.70 ± 3.53	nq	nq	-	29.96 ± 1.84*	70.04 ± 1.84*
*ho*QA23 pGREG	10.34 ± 1.27	6.78 ± 0.76	48.61 ± 3.12	22.16 ± 1.57	3.07 ± 2.79	9.04 ± 1.84	-	29.06 ± 2.67	70.94 ± 2.67
pGREG *PSD1*	7.69 ± 0.61	7.31 ± 0.80	46.70 ± 3.25	27.30 ± 2.96	4.31 ± 0.36	6.70 ± 0.25	-	29.24 ± 2.43	70.76 ± 2.43
pGREG *OPI3*	16.15 ± 4.32	7.64 ± 0.26	28.51 ± 3.46*	24.34 ± 3.69	20.25 ± 5.50	3.10 ± 0.68	-	44.46 ± 1.75*	55.54 ± 1.75*
pGREG *CHO2*	3.46 ± 0.03*	8.85 ± 0.07*	35.79 ± 2.07*	31.98 ± 2.21*	18.30 ± 6.67*	3.25 ± 0	-	29.70 ± 4.70	70.30 ± 4.70
pGREG *ERG3*	10.10 ± 0.55	6.87 ± 0.25	42.27 ± 6.42	19.58 ± 3.51	13.84 ± 1.68*	7.34 ± 0.42	-	31.53 ± 5.45	68.47 ± 5.45
pGREG *ERG6*	15.73 ± 0.28*	6.38 ± 0.63	40.49 ± 5.74	30.58 ± 0.91	4.65 ± 0.57	2.17 ± 0.92*	-	23.51 ± 4.25	76.49 ± 4.25
pGREG *IDI1*	8.87 ± 3.27	5.05 ± 0.66*	40.55 ± 0.96*	28.39 ± 2.83*	4.05 ± 0.71	13.09 ± 0.87*	-	21.83 ± 3.27	78.17 ± 3.27
pGREG *OLE1*	23.01 ± 6.77*	8.00 ± 0.64	46.55 ± 4.90	6.41 ± 1.50*	9.91 ± 0.59*	6.11 ± 0.32	-	12.51 ± 0.27*	87.49 ± 0.27*
pGREG *DPL1*	12.05 ± 1.79	8.82 ± 0.44	53.26 ± 2.99	9.96 ± 0.38*	nq	nq	-	17.88 ± 3.63*	82.12 ± 3.63
pGREG *LCB3*	3.16 ± 0.73*	5.24 ± 0.19*	36.63 ± 2.65*	41.48 ± 2.66*	nq	nq	-	29.50 ± 3.67	70.50 ± 3.67
pGREG *LCB4*	7.58 ± 0.00*	5.79 ± 0.33*	37.83 ± 3.92*	36.35 ± 9.85*	nq	nq	-	30.80 ± 7.31	69.20 ± 7.31*

(cont.)	STEROLS				FATTY ACIDS				
Strains	**Squalene**	**Lanosterol**	**Ergosterol**	**Sterol esters**	**C14:1**	**C16**	**C16:1**	**C18**	**C18:1**
*ho*QA23	6.69 ± 1.07	4.09 ± 0.69	30.80 ± 4.30	58.43 ± 4.68	-	13.62 ± 0.64	32.55 ± 0.19	3.66 ± 0.51	50.18 ± 0.90
Δ*psd1*	1.69 ± 0.09*	8.98 ± 0.25*	33.92 ± 2.28	55.41 ± 1.94	-	13.18 ± 0.88	33.07 ± 1.47*	6.57 ± 2.88	47.19 ± 2.60
Δ*opi3*	12.19 ± 3.95	25.00 ± 4.19*	36.86 ± 2.83	25.94 ± 6.16*	-	16.53 ± 0.37*	43.44 ± 1.54	2.66 ± 2.35	37.37 ± 0.54*
Δ*erg3*	4.73 ± 1.08	5.36 ± 2.47	45.16 ± 5.55	44.74 ± 7.41	-	17.08 ±1.37*	41.92 ± 1.20*	3.23 ± 1.15	35.78 ± 1.43*
Δ*erg6*	10.15 ± 0.72	1.88 ± 2.62	45.78 ± 10.35	42.20 ± 13.69	12.39 ± 4.95*	15.22 ± 0.99	33.45 ± 1.73*	2.35 ± 0.37	34.61 ± 2.78*
Δ*dpl1*	4.19 ± 0.98	3.11 ± 0.65	34.46 ± 1.89	58.24 ± 2.06	-	17.93 ± 1.09*	35.14 ± 1.08*	3.78 ± 0.24	42.62 ± 0.15*
Δ*lcb3*	10.71 ± 3.03	2.85 ± 0.75	30.05 ± 3.07	56.39 ± 4.22	-	19.30 ± 3.31	41.62 ± 2.53*	-	39.08 ± 0.78*
Δ*lcb4*	7.47 ± 2.95	3.60 ± 1.55	43.47 ± 3.49*	45.45 ± 3.01*	-	22.06 ± 1.46*	37.37 ± 1.06*	2.22 ± 1.92	38.35 ± 2.48*
*ho*QA23 pGREG	6.50 ± 1.08	-	15.95 ± 0.12	77.55 ± 1.20	-	22.53 ± 1.02	33.88 ± 1.70	5.37 ± 1.87	35.81 ± 1.09
pGREG *PSD1*	9.59 ± 2.39	-	15.39 ± 3.06	75.02 ± 0.67	-	22.43 ± 0.93	32.89 ± 0.84	4.13 ± 0.10	38.24 ± 1.57
pGREG *OPI3*	3.73 ± 0.67*	1.72 ± 0.11*	24.11 ± 1.21*	70.44 ± 1.99	-	17.65 ± 1.25*	41.50 ± 2.12*	1.05 ± 1.81*	39.81 ± 0.36*
pGREG *CHO2*	3.69 ± 1.44	2.56 ± 0.90*	20.19 ± 4.31	73.57 ± 6.42	-	17.60 ± 0.87*	36.45 ± 1.07	3.31 ± 0.22	42.65 ± 0.03*
pGREG *ERG3*	3.68 ± 0.48*	0.30 ± 0.16	12.95 ± 1.87	83.08 ± 1.94*	-	17.11 ± 0.29*	37.19 ± 1.16*	3.34 ± 0.32	40.30 ± 1.62
pGREG *ERG6*	2.85 ± 0.00*	-	14.44 ± 2.03	82.71 ± 2.03	-	18.35 ± 0.99*	35.51 ± 0.67	3.12 ± 3.03	43.02 ± 2.83*
pGREG *IDI1*	3.97 ± 0.32*	-	11.26 ± 2.83	84.77 ± 2.88*	-	20.29 ± 0.99*	36.47 ± 0.67*	3.31 ± 3.03	39.93 ± 2.83
pGREG *OLE1*	4.61 ± 1.10	2.28 ± 0.52	13.14 ± 1.39	79.98 ± 3.02	-	12.25 ± 0.82*	41.93 ± 0.39*	3.95 ± 0.30	41.88 ± 1.36*
pGREG *DPL1*	3.18 ± 0.60*	0.89 ± 0.12*	10.05 ± 0.46	85.87 ± 0.30*	-	16.80 ± 0.34*	35.77 ± 1.74	3.96 ± 0.35	43.20 ± 1.39*
pGREG *LCB3*	8.01 ± 2.52	9.34 ± 4.55	12.57 ± 1.70	70.08 ± 8.70	-	19.91 ± 0.34	36.37 ± 1.74	3.97 ± 0.35	39.75 ± 1.39*
pGREG *LCB4*	1.90 ± 0.36*	2.23 ± 0.95	11.45 ± 1.89	84.42 ± 2.05*	-	21.35 ± 0.44*	33.58 ± 2.41	5.06 ± 1.40	38.90 ±1.80*

(-) 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.

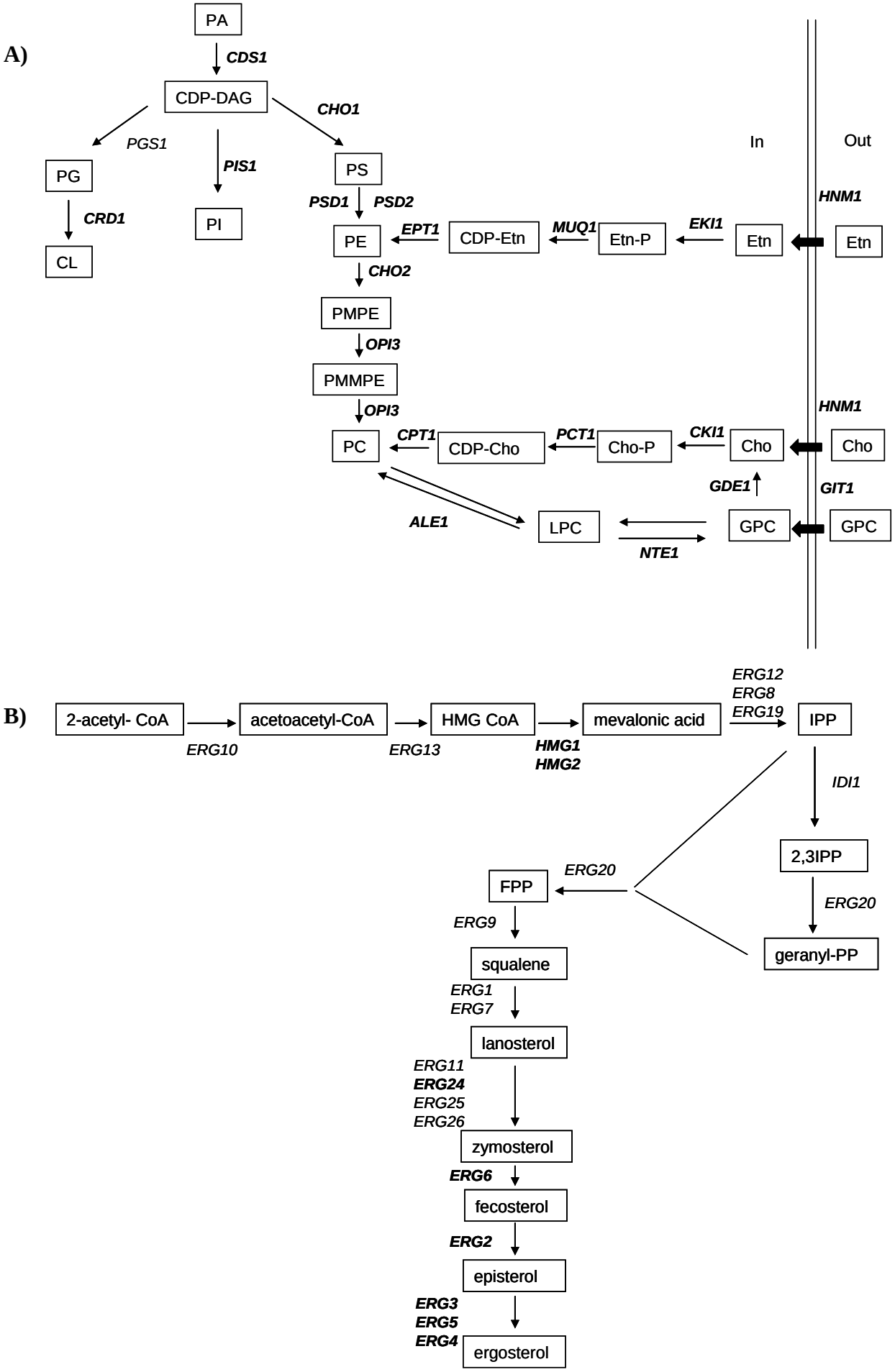
Figure 3. Growth of lipid mutant and overexpressing strains compared with their control strains. Generation time of **A)** mutant and **B)** overexpressing strains grown at 12 ºC (black bars) and at 28 ºC (grey bars). The GT of the mutant and overexpressing strains compared to GT of their control strains *ho*QA23 and *ho*QA23 pGREG (normalized as value 1). The GT for control strains was the following: 15.94 h and 2.58 h for *ho*QA23 and 12.18 h and 3.13 h for *ho*QA23 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, *ho*QA23 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 1


A)
PA
CDS1
CDP-DAG
CHO1
PGS1
PIS1
PG
PI
PS
CRD1
CL
PSD1
PSD2
PE
EPT1
CDP-Etn
MUQ1
Etn-P
EKI1
Etn
In
Out
HNM1
Etn
CHO2
PMPE
OPI3
PMMPE
OPI3
PC
CPT1
CDP-Cho
PCT1
Cho-P
CKI1
Cho
HNM1
Cho
GDE1
GIT1
ALE1
LPC
GPC
GPC
NTE1
B)
2-acetyl- CoA
ERG10
acetoacetyl-CoA
ERG13
HMG CoA
HMG1
HMG2
mevalonic acid
ERG12
ERG8
ERG19
IPP
IDI1
2,3IPP
ERG20
FPP
ERG20
ERG9
squalene
ERG1
ERG7
lanosterol
ERG11
ERG24
ERG25
ERG26
zymosterol
ERG6
fecosterol
ERG2
episterol
ERG3
ERG5
ERG4
ergosterol
geranyl-PP

C)

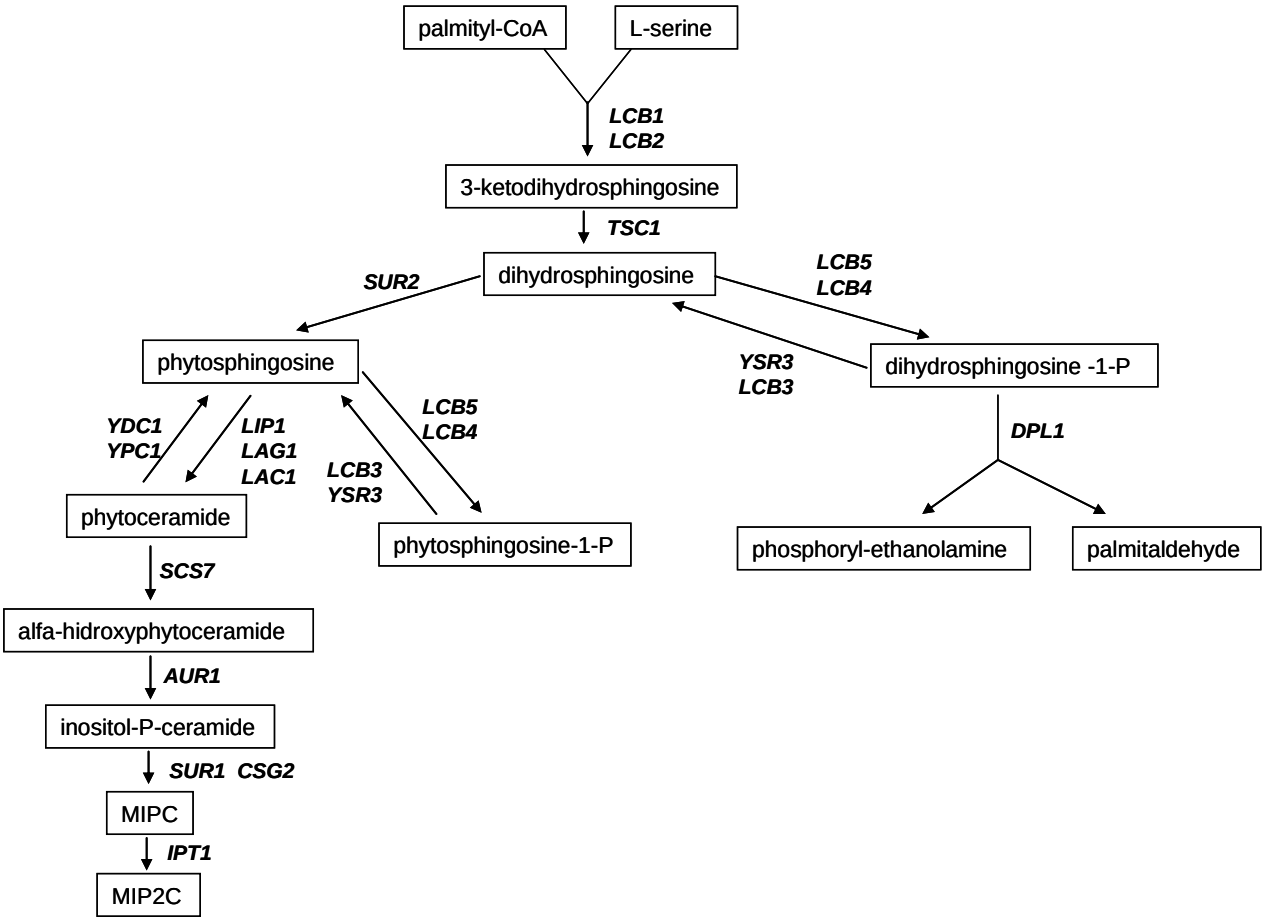
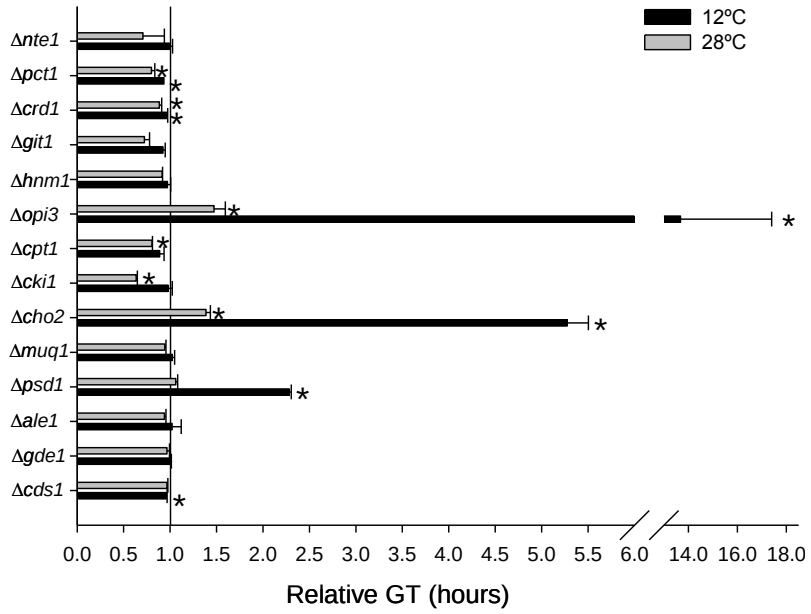
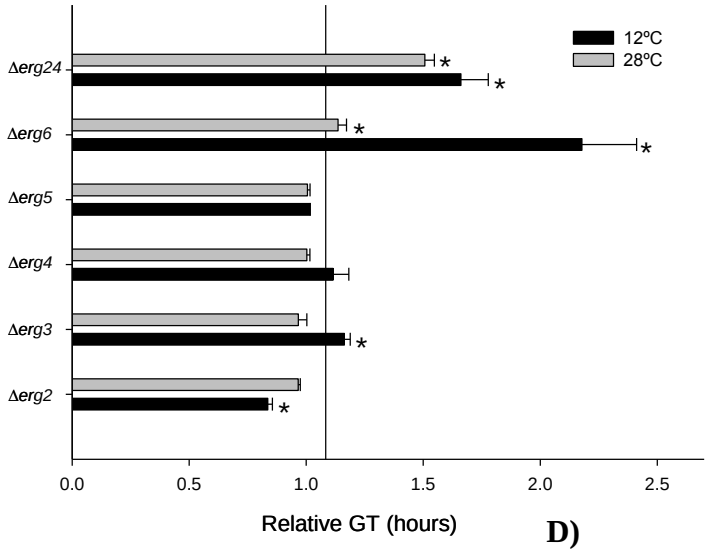

palmityl-CoA
L-serine
LCB1
LCB2
3-ketodihydrosphingosine
TSC1
dihydrosphingosine
SUR2
LCB5
LCB4
phytosphingosine
YSR3
LCB3
dihydrosphingosine -1-P
YDC1
YPC1
LIP1
LAG1
LAC1
LCB3
YSR3
LCB5
LCB4
DPL1
phytoceramide
phytosphingosine-1-P
phosphoryl-ethanolamine
palmitaldehyde
SCS7
alfa-hidroxyphytoceramide
AUR1
inositol-P-ceramide
SUR1 CSG2
MIPC
IPT1
MIP2C

Figure 2

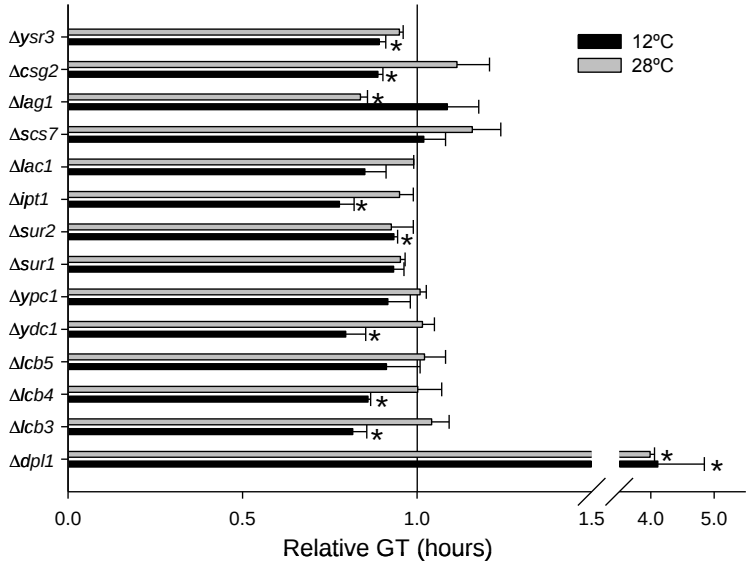
A)



B)



C)



D)

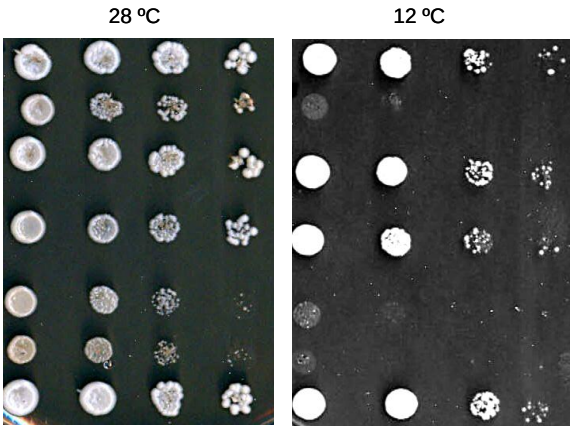
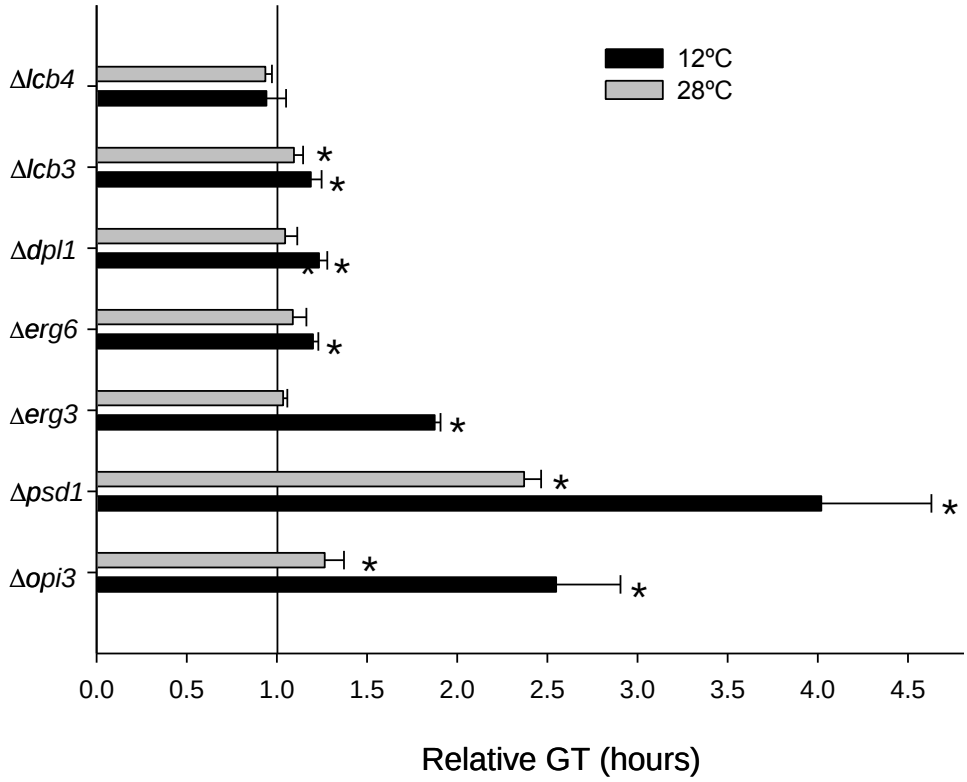


Figure 3

A)


12ºC
28ºC
Δlcb4
Δlcb3
Δdpl1
Δerg6
Δerg3
Δpsd1
Δopi3
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Relative GT (hours)

B)

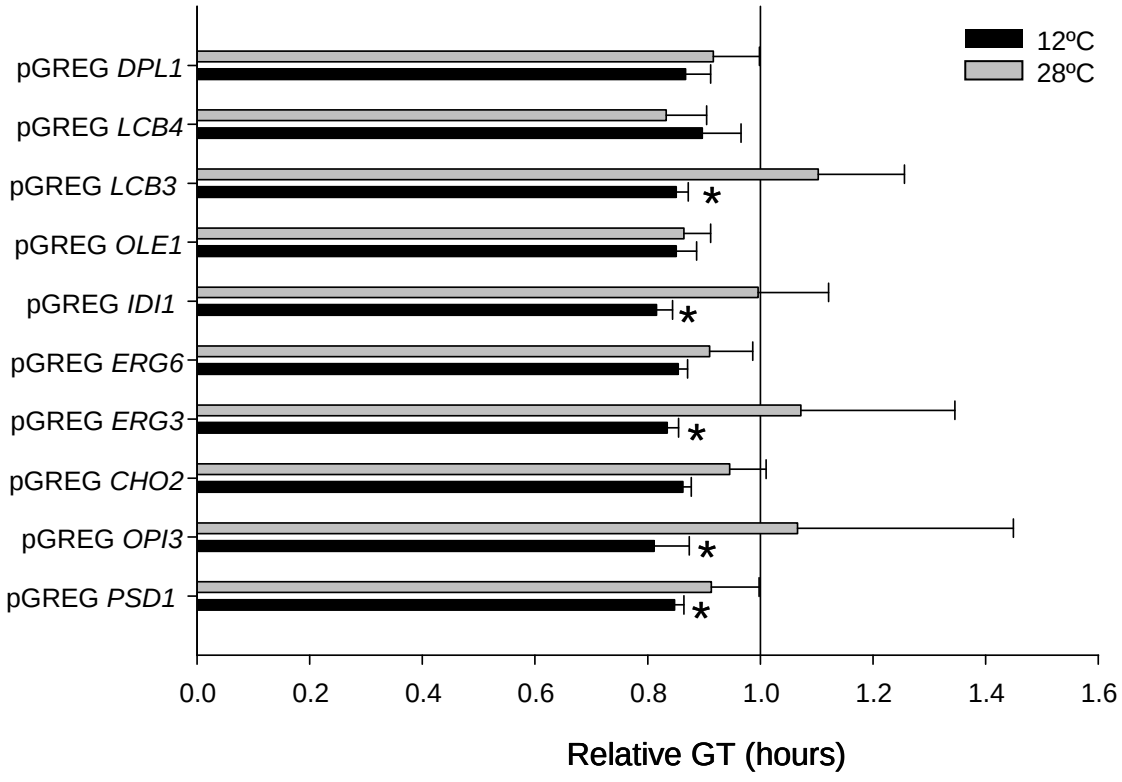
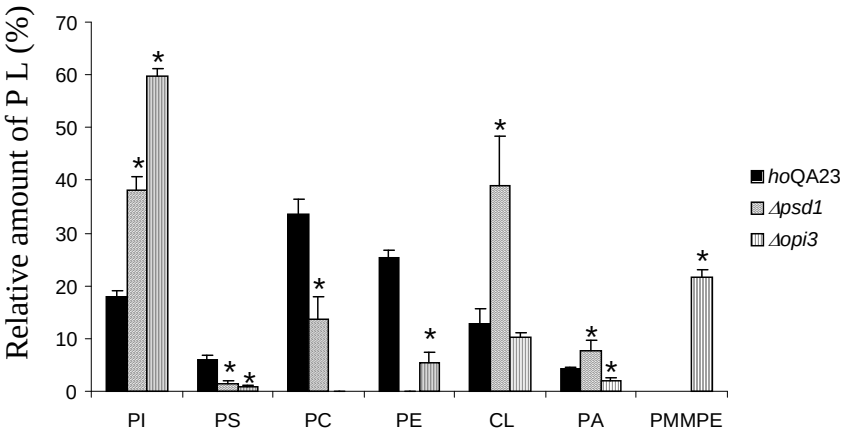
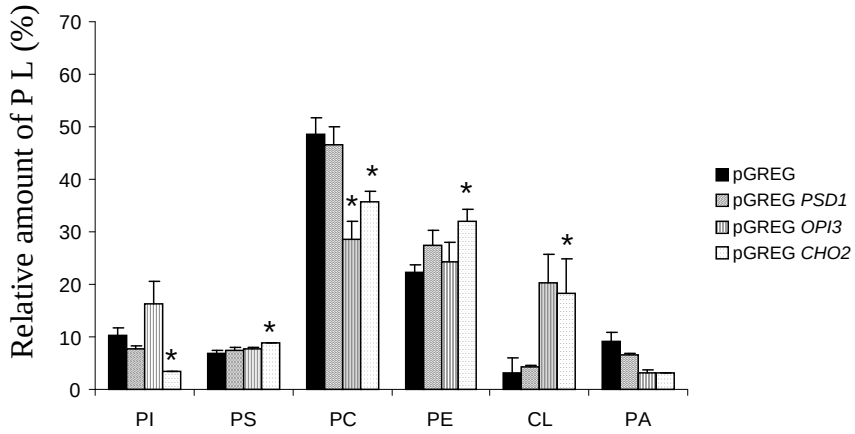

12ºC
28ºC
pGREG DPL1
pGREG LCB4
pGREG LCB3
pGREG OLE1
pGREG IDI1
pGREG ERG6
pGREG ERG3
pGREG CHO2
pGREG OPI3
pGREG PSD1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Relative GT (hours)

Figure 4

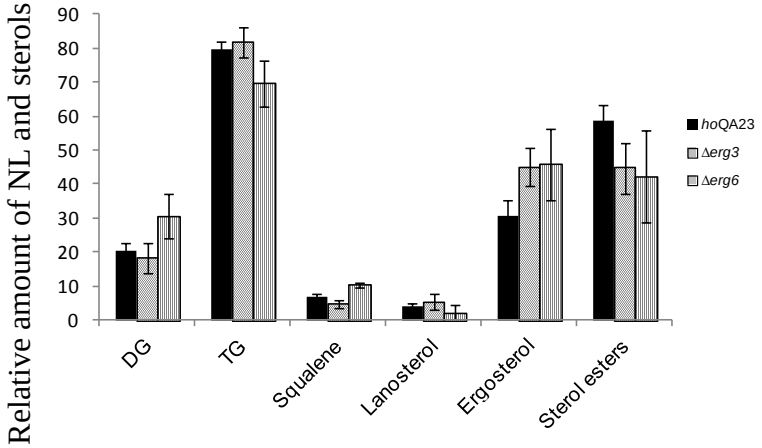
A.1)



A.2)



B.1)



B.2)

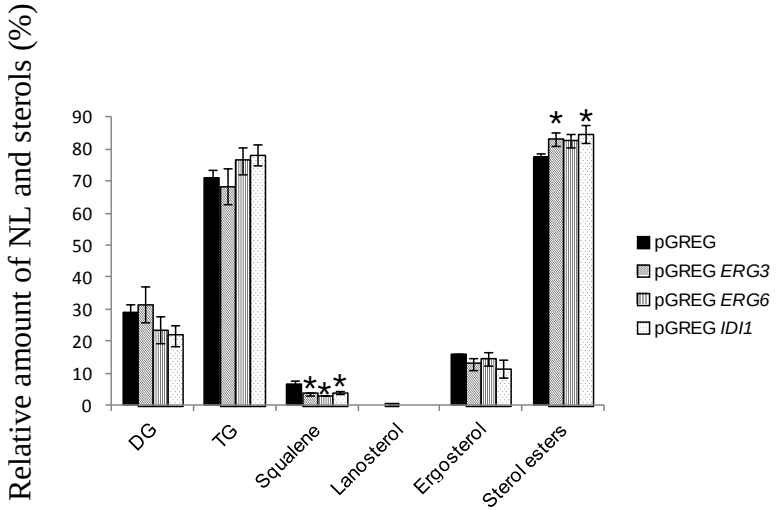


Figure 5

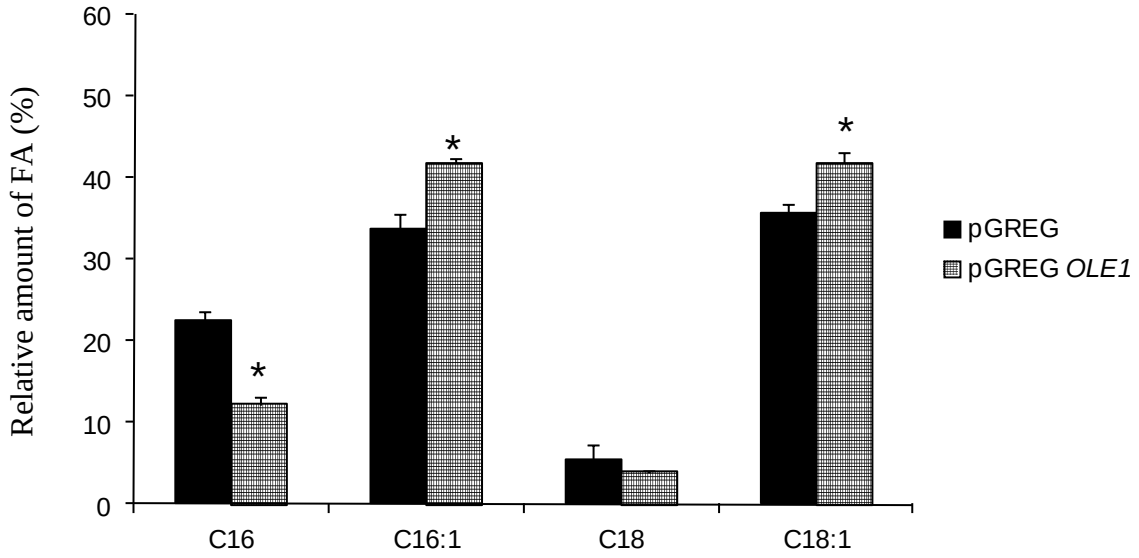
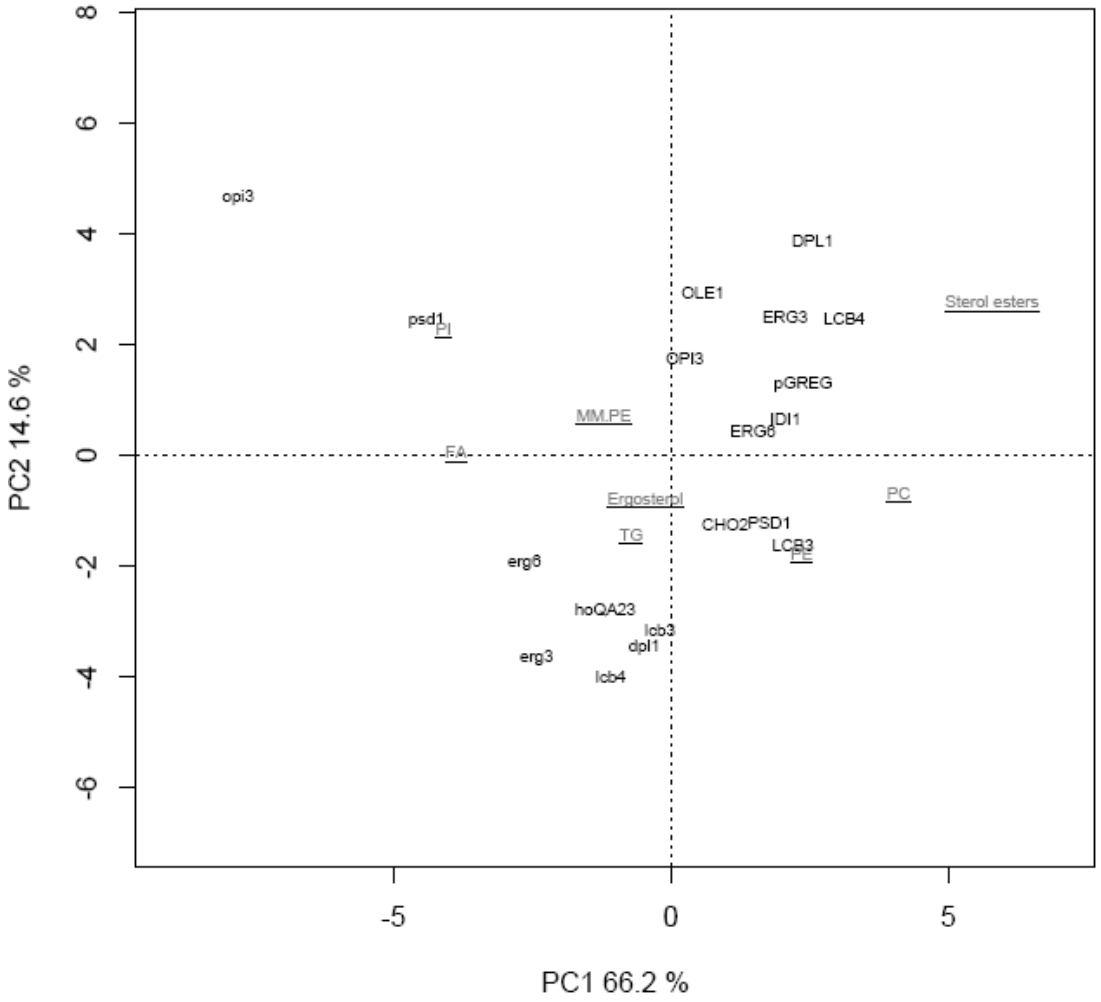

Relative amount of FA (%)
60
50
40
30
20
10
0
*
*
*
C16
C16:1
C18
C18:1
pGREG
pGREG *OLE1*

Figure 6


opi3
DPL1
OLE1
Sterol esters
psd1
PI
ERG3
LCB4
OPI3
pGREG
MM.PE
IDI1
ERG6
FA
Ergosterol
PC
CHO2PSD1
TG
LCB3
PE
erg6
hoQA23
lcb3
dpl1
erg3
lcb4
PC2 14.6 %
PC1 66.2 %
8
6
4
2
0
-2
-4
-6
-5
0
5