Improving the production of punicic acid in baker's yeast by engineering genes in acyl channeling processes and adjusting precursor supply

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ABSTRACT

Punicic acid (PuA) is a high-value edible conjugated fatty acid with strong bioactivities and has important potential applications in nutraceutical, pharmaceutical, feeding and oleochemical industries. Since the production of PuA is severely limited by the fact that its natural source (pomegranate seed oil) is not readily available on a large scale, there is a considerable interest in understanding the biosynthesis and accumulation of this plant-based unusual fatty acid in transgenic microorganisms to support the rational design of biotechnological approaches for PuA production via fermentation. Here we tested the effectiveness of genetic engineering and precursor supply in PuA production in the model yeast strain *Saccharomyces cerevisiae*. The results revealed that the combination of precursor feeding and co-expression of selected genes in acyl channeling processes created an effective 'Push-Pull' approach to increase PuA content, which could prove valuable in future efforts to produce PuA in industrial yeast and other microorganisms via fermentation.

KEYWORDS

punicic acid, *Saccharomyces cerevisiae*, pomegranate, conjugated fatty acid, fatty acid desaturase, fatty acid conjugase, acyl-channeling

INTRODUCTION

Punicic acid (PuA; 18: 3 $\Delta^{9cis, 11trans, 13cis}$), an edible linolenic acid with three conjugated double bonds, which has great potential in nutraceutical and pharmaceutical applications due to its strong antioxidant, antidiabetic, anticancer, anti-obesity, and anti-inflammatory bioactivities.[1] PuA can also be used in the production of high-quality alkyd resins, paints, varnishes, polymers, and drying oils due to its susceptibility to auto-oxidation and subsequent polymerization, fast drying rates and water resistance. The major natural source of PuA is pomegranate (*Punica granatum*), which contains up to 80% PuA in its seed oil,[2] although a few other plant species also produce this fatty acid, albeit at lower levels . Unfortunately, none of these plants are suitable for the large-scale production of PuA due to low oil yields and restricted cultivation conditions.[1] Although initial efforts are underway to produce PuA in transgenic plants, productivities of this unusual fatty acid have been relatively low thus far.[3-6] As a result of these factors, there is a growing interest to produce PuA using other approaches such as fermentation to fulfill rising demands for this high-value fatty acid.[1]

PuA is a high-value edible conjugated fatty acid with applications in food, feed and nutraceuticals, and therefore it is attractive to produce PuA in Generally Recognized as Safe (GRAS) microorganisms. As a GRAS microorganism with a substantial amount of genetic information, baker's yeast (*Saccharomyces cerevisiae*) has been used as a platform to produce various high-value compounds via metabolic engineering, and the edible purified final products, as well as the edible yeast biomass containing the final products can be easily commercialized for down-stream applications.[7-8] Moreover, *S. cerevisiae* is a model yeast species with a simple fatty acid profile (approximately 40% of palmitoleic acid, 30% of oleic acid, 20% of palmitic acid and 5% of stearic acid).[9] Such characteristics theoretically provide an ideal foundation for

the exploration of metabolic engineering strategies to heterologously produce PuA and other plant-based unusual fatty acids. However, in the few reports describing the synthesis of PuA in baker's yeast, the heterologous expression of the *P. granatum fatty acid conjugase* (*PgFADX*), which catalyzes the synthesis of PuA, only resulted in up to 2% (w/w) PuA (as a percentage of total fatty acids).[3-4] These findings indicate that extensive genetic engineering research will be necessary to generate *S. cerevisiae* strains that can accumulate large quantities of PuA.

In *P. granatum*, the synthesis of PuA from oleic acid (18:1) is generally considered to be catalyzed by Δ12-acyl-lipid-desaturase (PgFAD2) and PgFADX, where the former catalyzes the desaturation of oleic acid to linoleic acid (LA; 18:2 $\Delta^{9cis,\ 12cis}$) at the *sn*-2 position of phosphatidylcholine (PC) and the latter converts LA to PuA (Fig. 1A).[3-5] PuA is then channeled from PC to the storage lipid triacylglycerol (TAG).[1] The efficient synthesis and channeling of PuA and other unusual fatty acids in plants that can accumulate large amounts of these fatty acids often requires the contribution of enzymes with special substrate specificities and selectivities. Some such enzymes have been reported in acyl-editing, lipid biosynthetic and lipid regulatory steps, including phospholipase A_2 (PLA_2), lysophospholipid acyltransferase (LPCAT), phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), phospholipid: diacylglycerol acyltransferase (PDAT) and acyl-CoA: diacylglycerol acyltransferase (DGAT).[1, 10-16] These acyl-editing enzymes may be crucial for enhancing PuA assembly into TAG and thus provide valuble candidates for engineering PuA production. In addition to manipulating TAG assembly ('Pull'), other metabolic engineering strategies for increasing PuA producting in microorganisms include increasing fatty acid biosynthesis ('Push') and preventing TAG turnover ('Protect').[17-18]

Up to now, PuA has been heterologously synthesized in *S. cerevisiae*, *Schizosaccharomyces pombe*, *A. thaliana* and *Brassica napus* [4-6, 19]. However, these studies only evaluated the result of *PgFADX* and *PgFAD2* expressions. Some important factors which may affect PuA production in microorganisms, such as genes in acyl editing, storage lipid accumulation and lipid degradation, transcription factors, the functions of precursor supply and their various combinations, have not been studied in yeast or plants. The lack of such study might partially due to the laborious and time-consuming transgenic process in industrial microorganisms and crops.

The aim of this study is to explore the effectiveness of the combination of different metabolic engineering strategies ('Push', 'Pull' and 'Protect') on the biosynthesis and accumulation of PuA in *S. cerevisiae*. As demonstrated in numerous studies before, this model yeast is an ideal platform to study the mechanism of lipid accumulation in eukaryotes. With a *S. cerevisiae* species with clear genetic background and various available genetic modification tools, the effects of various combinations of gene stacking and precursor supply can be rapidly tested to find the good ones for PuA production in oleaginous yeast and other microorganisms with better industrial potential but challenging and time-consuming process of genetic engineering. The results of this study will be of value for the heterologous production of this high-value fatty acid via fermentation and will also expand our understanding of the biosynthesis of plant-based unusual fatty acids.

MATERIALS AND METHODS

Strain and culture conditions

All strains used in this study are listed in Table 1. In brief, *S. cerevisiae* BY4741 (*MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) was used as the background strain and *Escherichia coli* DH5α was used for plasmid construction and amplification. *Yarrowia lipolytica* E122 and *Lipomyces starkeyi* NRRL Y11557 were cultured for RNA isolation and cDNA synthesis. *E. coli* cultures were grown in Luria-Bertani (LB) medium containing 50 mg/L ampicillin at 37 °C with constant shaking at 225 rpm. For PuA production, individual colonies of transformed yeast cells were first grown in yeast nitrogen base (YNB) (Sigma Y-0626) medium supplemented with the appropriate amino acid drop out mix and 2% raffinose for 24 h at 30 ºC with shaking at 225 rpm. Cells were then inoculated into induction medium consisting of 10 mL YNB, 1.2 g/L ammonium sulfate, 1% raffinose, and 2% galactose in 50 mL tubes at an initial OD600 of 0.2. Inoculum for PuA accumulation experiments was grown for 48 h at 20 ºC in an incubated shaker with constant shaking at 225 rpm. For LA feeding experiments, LA was first dissolved in ethanol and then mixed with induction medium containing 0.1 % (v/v) tyloxapol to assist with its distribution.

Genes, plasmids and yeast transformation

Genes encoding various desaturases, including pomegranate derived *PgFADX*, *PgFAD2*, and *Acheta domesticus AdFAD2*, were synthesized by Twist Bioscience (San Francisco, USA) and codon-optimized for yeast expression. In order to obtain the template for cloning *FAD2*s from *Y. lipolytica* and *L. starkeyi*, total RNA was isolated from yeast cells at the mid-log phase using the RNeasy kit (Qiagen, Toronto, Canada), and cDNA was synthesized using the SuperScript IV first-strand cDNA synthesis kit (Invitrogen, Burlington, Canada). Putative gene sequences encoding pomegranate acyl-editing and TAG assembly enzymes were

predicted through searches of deep sequencing data (unpublished data) of *P. granatum* seeds or by performing Blast searches using the publicly available draft genome of *P. granatum*,[20] and then synthesized by Twist Bioscience. The resulting coding sequences were then inserted into the multiple cloning sites of pESC-Ura or pESC-Leu using conventional restriction-based cloning methods or the ClonExpress One Step Cloning Kit (Vazyme Biotech). Control strains used in this study consist of empty vectors pESC-Ura or pESC-Ura and pESC-Leu, respectively. Yeast transformations were performed using the lithium acetate and PEG3350 method as described previously.[21]

Lipid extraction and separation of lipid classes

Total lipids were extracted from yeast samples as described in our previous study.[5] Briefly, yeast biomass was collected by centrifugation, washed, lyophilized, and suspended in 800 μL of cold lipid extraction mix consisting chloroform, isopropanol (2:1, v/v) and antioxidant butylated hydroxytoluene at a final concentration of 0.01 %. Cells were then disrupted (3 × 1 min) using a bead beater (Biospec, Bartlesville, OK) with glass beads (diameter 0.5 mm) and 2 min cooling between each cycle. The organic phase from each sample was separated by centrifugation and collected. The extraction procedure for each sample was performed twice. The combined organic phase containing lipids was dried under a stream of nitrogen and resuspended in 200 μL cold chloroform. For the separation of lipid classes, single yeast colonies were inoculated into 25 mL induction medium in 250 mL flasks for 48 h. Total lipids were extracted from lyophilized biomass and then separated on thin layer chromatography (TLC) plates (0.25 mm Silica gel, DCFertigplatten, Macherey-Nagel, Germany) with a solvent system containing hexane/ diethyl ether/ acetic acid (80: 20: 1).[5] Lipid fractions on the TLC plate were visualized through primulin

staining, and bands corresponding to TAG and PC were scraped off, methylated, and analyzed by gas chromatography.

Fatty acid analysis

Lipid transmethylation was carried out using the base-catalyzed method with 1 mL of 5 % sodium methoxide dissolved in methanol with C17:0-TAG as the internal standard.[5-6] After incubation at 30 °C for 30 min, 1.5 mL of 0.9% (w/v) sodium chloride solution was added to stop the reaction, and fatty acid methyl esters (FAME) were extracted with 1 mL of chromatographic grade hexane. The hexane phase was collected, dried and FAMEs were then resuspended in 100 µL hexane and analyzed on an Agilent 6890N Gas Chromatograph (GC) equipped with a 5975 inert XL Mass Selective Detector (MS, Agilent Technologies) and flame ionization detector (FID) using the method described in our previous study.[6] Briefly, FAMEs were separated on a capillary column DB23 (30 m×0.25 mm×0.25 µm, Agilent Technologies, Wilmington, DE, USA) using the following program: 5:1 split ratio, 1 µL injection. 4 min at 165 °C, then increased to 180 °C (10 °C/ min) and held for 5 min, and increased to 230 °C and held for 5 min. Individual FAME peaks were identified by MS and further confirmed with the comparison with the authentic standards of FAMEs with various chain length and unsaturation degree. Quantification of lipid was performed using the C17:0 triacylglycerol as internal standard with the peaks measured with GC-FID.

Statistical analysis

Unless otherwise mentioned, all data represent the mean values of biological replicates ± SD (n=3). Statistical analyses were conducted with Student's *t*-test using the GraphPad Prism (v8) software.

RESULTS AND DISCUSSION

Establishment of punicic acid synthesis in *Saccharomyces cerevisiae* via the heterologous expression of various *fatty acid desaturase 2* genes and exogenous linoleic acid supplement

In order to produce baker's yeast containing PuA, we first expressed *PgFAD2* and *PgFADX* in *S. cerevisiae* BY4741 using single plasmid pESC-Ura under the control of the inducible promoters *GAL1* and *GAL10*, respectively, resulting in yeast strain PA1 (Table 1). As shown in Fig. 1B, PA1 accumulated up to 6% and 0.3% of total fatty acids as LA and PuA, respectively, whereas the control strain harboring the corresponding empty plasmid did not synthesize LA and PuA. To further enhance PuA production in baker's yeast, we then assessed the performance of other *FAD2* genes in this context. To achieve this, we replaced *PgFAD2* in the pESC-Ura-*PgFADX*-*PgFAD2* construct with *FAD2*s originating from the insect *A. domesticus* as well as the oleaginous yeasts *Y. lipolytica* and *L. starkeyi*, and generated yeast strains PA2, PA3 and PA4, respectively (Table 1). The PA3 strain hosting *YlFAD2* was found to accumulate 8.5% of total fatty acids as LA, which was significantly higher than the other *FAD2*s; however, this construct did not lead to a higher PuA content (Fig. 1B). As an alternative approach, we also evaluated the effect of supplying a yeast strain bearing *PgFADX* with exogenous LA since this fatty acid serves as the precursor for PuA synthesis. In this case, we found that supplying BY4741 transformed with the pESC-Ura-*PgFADX* vector (strain designated as PA0) with 0.05% exogenous LA increased cellular LA to 60% and resulted in 1.3% of total fatty acids as PuA,

192 which is more than 3-fold higher than the PA1-PA4 strains (Fig. 1B). Since the PA0 strain
193 with LA supplementation enhanced LA and PuA levels, and PA3 was found to accumulate a
194 relatively high proportion of LA, the two corresponding constructs (pESC-Ura-*PgFADX* and
195 pESC-Ura-*PgFADX*-*YlFAD2*) were selected for further analysis.

196 The expression of *FAD2* is considered to be important in this context since LA is a
197 precursor for PuA synthesis and *S. cerevisiae* does not have the inherent ability to synthesize
198 this polyunsaturated fatty acid. However, the co-expression of *PgFAD2* and *PgFADX* led to
199 the production of low amounts of PuA, but relatively high levels of LA in yeast cells (Fig. 1),
200 which were below the levels observed in fission yeast *S. pombe* when *PgFAD2* and *PgFADX*
201 were coexpressed.[19] Although this study used yeast codon-optimized coding gene sequences,
202 strong inducible promoters and a high copy number plasmid for the expression of *PgFAD2*
203 and *PgFADX*, the PgFADX could still be subjected to strong post-translational regulation. For
204 example, when *Arabidopsis thaliana FAD2* and *FAD3* were expressed in yeast, AtFAD3 was
205 more efficiently degraded by the yeast proteasome than AtFAD2.[20] Moreover, yeast post-
206 translational regulation of *Brassica napus* and *Vernicia fordii* FAD3 has been found to be
207 temperature-sensitive, where the half-life of FAD3 was much longer under low temperatures.[22]

208 Comparison of three alternative FAD2s with PgFAD2 indicated that the heterologous
209 expression of *YlFAD2* led to the production of the highest levels of LA (Fig. 1B), which
210 suggests that the FAD2 from this oleaginous yeast strain performed better than PgFAD2 in *S.*
211 *cerevisiae*. Among the four FAD2s tested, AdFAD2 is known to be able to convert oleyl-CoA
212 to linoleoyl-CoA in the acyl-CoA pool.[23-25] Since acyl chains esterified to various head groups
213 are subject to dynamic acyl-editing,[26] we hypothesized that LA produced in the acyl-CoA pool
214 might be quickly moved to PC and used for PuA synthesis by PgFADX. However, AdFAD2

resulted in the lowest LA content (Fig. 1B), indicating that newly synthesized linoleoyl-CoA might not be efficiently converted to 18:2-PC for PuA synthesis. It should be also noted here that the level of LA in *S. cerevisiae* was far below what we have achieved previously in fission yeast over-expressing *PgFAD2*.[19] This suggests that the production of LA is inefficient in budding yeast *S. cerevisiae*, which might be caused by significant difference in the palmitoleic acid and oleic acid levels of these two yeasts or enhanced post-translational degradation of PgFAD2 protein in *S. cerevisiae* cells.

Although the co-expression of *FAD2* with *PgFADX* did lead to the production of LA and PuA in *S. cerevisiae*, LA supplementation led to a much higher accumulation of these two fatty acids (Fig. 1B), indicating that precursor feeding is an effective approach to increase PuA production, which is consistent with previous reports focused on yeast *S. cerevisiae*.[3-4] Similar findings were obtained by Dyer *et al.* (2002) whereby the heterologous co-expression of tung tree *VfFAD2* and *VfFADX* in yeast led to significantly lower levels of α-eleostearic acid (the major unusual fatty acid in tung tree seed oil) than when *VfFADX* was expressed alone with supplementation with exogenous fatty acid precursor (0.3% vs. 2.1%, respectively).[27]

Effects of *Saccharomyces cerevisiae* transcription factors ('push') and triacylglycerol mobilization ('protect') genes on punicic acid synthesis

Fatty acid biosynthesis and accumulation in *S. cerevisiae* could be regulated by transcription factors such as *snf1*, which encodes an ADP-activated serine/threonine kinase, *snf2*, which encodes a general transcription factor involved in the regulation of lipid accumulation, and *ira2*, which encodes a GTPase-activating protein involved in glucose-induced signaling.[28-33] In this study, we tested the functions of *snf1, snf2* and *ira2* in PuA synthesis. Yeast strains bearing

deletions of *ira2*, *snf2,* and *snf1*, respectively, were transformed with the plasmid pESC-Ura-*PgFADX*-*YlFAD2*, resulting in strain PA5, PA6 and PA7, respectively (Table 1). The *snf2* or *snf1* mutation led to higher PuA content in strain PA6 and PA7 (Fig. 2). In order to evaluate the effects of these transcription factors under LA feeding, we also constructed yeast strains PA8, PA9, and PA10 by transforming *ira2*, *snf2,* and *snf1* mutants with plasmid pESC-Ura-*PgFADX*. With supplementation of 0.05% LA, PA8 and PA9 also accumulated higher amounts of PuA than the control strain PA0 (Fig. 2). PA9 possessing *PgFADX, snf2* deletion and 0.05% LA feeding accumulated the highest PuA content (2.7%).

Since TAG is the final deposition site for various fatty acids including PuA, the deletion of genes related to TAG mobilization may partially block turnover of the PuA product and increase PuA content in yeast cells.[34-35] In light of this, yeast mutants of TAG lipase (encoded by *tgl3*) and peroxisomal long-chain fatty acid importers (encoded by *pxa1* and *pxa2*), were transformed with pESC-Ura-*PgFADX*-*YlFAD2* or pESC-Ura-*PgFADX*, respectively, resulting in six engineered strains (PA11-PA16, Table 1). Somewhat surprisingly, none of the gene deletions benefited overall PuA accumulation, regardless of LA feeding or not. Taken together, our results demonstrate that among the six tested genes encoding transcription factors and TAG mobilization, the *snf2* deletion leads to the greatest improvement of PuA accumulation in *S. cerevisiae*. As such, the remaining experiments were carried out using the *snf2Δ* strain (PA6 and PA9; Table 1).

The heterologous biosynthesis of PuA in yeast may be regulated to a substantial degree by the native lipid metabolism of the host strain. Therefore, we further evaluated whether the 'Push' of fatty acid biosynthesis could enhance PuA production. The process of storage lipid accumulation involves complex interactions between carbon and energy relocation. Regulation of transcription

261 factors such as Snf1p and Snf2p, has been proved to be effective in increasing neutral lipid
262 content in yeast in previous studies [31-32]. For instance, *SNF1* knockout in *Y. lipolytica* and *S.*
263 *cerevisiae* significantly increased the lipid accumulation [32, 36]. *SNF1* encodes an ADP-activated
264 serine/threonine kinase and plays an important role in lipid synthesis as a master transcription
265 factor [28, 32, 36]. In yeast cells, this transcription factor controls carbon homeostasis and stress
266 responses by regulating genes involved in lipid synthesis and nitrogen metabolism [37]. Snf1p
267 negatively controls the first committed step of fatty acid synthesis by directly phosphorylating
268 and consequent inactivation of acetyl-CoA carboxylase [38-40].
269 Moreover, *SNF2* is a transcription factor forming part of the SWI/SNF (switching/sucrose
270 nonfermenting chromatin-remodeling) complex and a recent study showed that snf2 knockout
271 resulted in significant increase of lipid content in *S. cerevisiae* [31]. It is assumed that Snf2p might
272 regulate the accumulation of phospholipids and storage lipids, and the incorporation of
273 exogenous fatty acids into the *snf2*Δ mutant was found to be improved, suggesting potential
274 regulation of fatty acid transporters by Snf2p [30]. All of the above characteristics are likely to be
275 the cause of the increased PuA content in either FAD2-harboring strains or strains with
276 exogenous LA feeding. Up to now, many transcription factors in model microorganisms have
277 been annotated, however, only a few of them have been linked to native lipid metabolism, let
278 alone the heterologous synthesis of unusual fatty acids. Therefore, as demonstrated in this study,
279 the use of metabolic engineering to study the function of transcription factors is of great
280 significance to the rational design of recombinant yeast enriched in unusual fatty acids.
281 Our results indicate that among the three tested transcription factors, the deletion of *snf2*
282 increased the LA and PuA content in recombinant yeasts expressing *PgFADX* (Table 2). Our
283 results, along with reports that the *snf2* mutant can effectively incorporate exogenous fatty acids

and increase lipid content in *S. cerevisiae*,[31, 41] demonstrate that the 'Push' of fatty acid production by manipulation of endogenous transcription factors could be an effective approach to improve PuA production in yeast.

Effects of pomegranate acyl-editing and triacylglycerol assembly genes ('pull') on punicic acid synthesis in *Saccharomyces cerevisiae*

Acyl-editing and TAG assembly processes play a pivotal role in the enrichment of unusual fatty acid in higher plants (Fig. 3A).[12-15] The lack of such a specialized metabolic network in yeast could cause the retention of PuA in PC, which may trigger potential feedback inhibition and reduce the accumulation of PuA in storage lipids. To test the functions of related genes in PuA synthesis in yeast, five putative acyl-channeling genes from pomegranate, including *PLA$_2$*, *LPCAT*, *DGAT2*, *PDCT* and *PDAT*, were synthesized and cloned into pESC-Leu in pairs. The resulting plasmids pESC-Leu-*PLA$_2$-LPCAT*, pESC-Leu-*DGAT2-LPCAT*, pESC-Leu-*DGAT2-PLA$_2$*, pESC-Leu-*DGAT2-PDCT*, pESC-Leu-*PDAT-PDCT*, and pESC-Leu-*PDAT-LPCAT* were then transformed into strains PA6 (BY4741-*snf2Δ*/pESC-Ura-*PgFADX-YlFAD2*) and PA9 (BY4741-*snf2Δ*/pESC-Ura-*PgFADX*), respectively, generating strains PA17-PA28 (Table 1).

In strains harboring *YlFAD2* and *PgFADX*, coexpression of acyl-editing and TAG assembly genes generally led to a 67% - 128% increase in PuA content (Fig. 3B, Table 2). Among the genes assessed, strain PA22 bearing *PgPDAT* and *PgLPCAT* produced 17.3% and 1.23% of total fatty acid as LA and PuA, respectively. In contrast, improvements were less obvious when LA was acquired exogenously without the presence of *YlFAD2* (strains PA23-28). When compared to PA9, only PA27 and PA28 led to increases in PuA content (by 20%) with 0.05% LA supplementation (Fig. 3B), accumulating the highest PuA levels of 3.26% and 3.37% of total

fatty acids, respectively. While PuA contents in these two strains are not statistically different, both exhibit levels that are 10 times higher than the starting strain PA1 heterologously expressing *PgFAD2* and *PgFADX* (0.33% of total fatty acids) (Fig. 1 & 3).

Given the importance of PuA channeling from PC to TAG, we further examined the distribution of PuA in lipid fractions in our engineered yeast cells. In PA9, PuA accounted for 6.3% and 1.4% of total fatty acids in polar lipid and TAG fractions, respectively (Fig. 4A). In strain PA27, the relative PuA content in TAG was increased by 50%, comparing with PA9 ($P<$ 0.05; Fig. 4A). When comparing the two strains accumulating the highest amounts of PuA in cells, the co-expression of *PgPDAT* and *PgPDCT* (PA27) led to a higher proportion of PuA in TAG than the co-expression of *PgPDAT* and *PgLPCAT* (PA28) (Fig. 4A). Similar results were also observed in lipid samples extracted from strains heterologously expressing *YlFAD2* without LA feeding (PA6 and PA21). Although PuA contents in both TAG and PL fractions were less than 0.5% of total fatty acids in these strains, the co-expression of *PDAT* and *PDCT* (PA21) still increased PuA proportions by 65% and 87% in TAG and polar lipid fractions, respectively (Fig. 4B).

PuA is synthesized on the membrane lipid PC but is deposited in the storage lipid TAG in pomegranate seeds,[5] and a similar scenario is seen with the accumulation of many other unusual fatty acids in higher plants.[8, 42] The channeling of unusual fatty acids from PC to TAG (the "Pull" of PuA assembly into TAG) has been considered a major bottleneck in the heterologous synthesis of unusual fatty acids, and the expression of related genes from native producers of the unusual fatty acid in transgenic plants has been shown to be an effective approach to increase production.[8, 42] In this study we assessed whether the co-expression of pomegranate genes involved in acyl-editing and TAG assembly could increase PuA content in engineered yeast. The

expression of all different combinations of the selected genes, along with *YlFAD2* and *PgFADX*, effectively increased PuA content in our system (Fig. 3B), suggesting that this approach also works well for PuA production in yeast. The beneficial effect of acyl-channeling genes on strains supplemented with exogenous LA was less obvious. Only the combination of *PDAT*+*LPCAT* and *PDAT*+*PDCT* increased PuA content (Fig. 3B) by approximately 2- to 4-fold times higher compared to previous reports.[3-4] In future studies it would be interesting to further characterize the channeling of unusual fatty acids from PC to TAG. In addition, a detailed analysis of PuA distribution in lipid classes with lipidomics would expand our understanding of PuA channeling among lipid classes and shed a light on novel strategies to channel PuA to TAG by engineering related genes.

Further analysis of PuA content in TAG and PL lipid classes indicated that although the co-expression of both *PgPDAT*+ *PgPDCT* and *PgPDAT*+ *PgLPCAT*, respectively, can increase PuA content with or without LA supplementation (Fig. 3), the former performed better than the latter in terms of channeling PuA from PC to TAG (Fig. 4). In addition, we noticed that upon LA supplementation, the content of LA in engineered *S. cerevisiae* reached approximately 60% in PL fraction which is much higher compared to LA level in cells over-expressing *FAD2*. Although comparable high level of endogenous LA in *S. pombe* cells can be achieved by over-expressing *PgFAD2*, only moderate increase in PuA (25%) was observed.[19] Taken together, our results indicate that different genes and gene combinations may provide distinct contributions to PuA accumulation in different yeast species. Moreover, many enzymes catalyzing plant lipid biosynthesis actively interact with and influence each other. Therefore, complex plant metabolomes and interactomes may likely work in concert with unusual fatty acid enrichment in TAG.[10, 42-43] Given the multifaceted nature of the acyl-channeling apparatus, single gene

353 expression or a simple combination of genes cannot fully transfer PuA biosynthesis from
354 pomegranate to yeast. Further studies such as the shuffling of plant-derived acyl-channeling
355 genes and interactomes from the native PuA producer to yeast may provide more comprehensive
356 solutions for producing PuA, as well as other unusual fatty acids, in *S. cerevisiae*.

357 PuA has great potential in nutraceutical, pharmaceutical and oleochemical applications due to
358 its three conjugated double bonds and the associated strong antioxidant, antidiabetic, anticancer,
359 anti-obesity, and anti-inflammatory bioactivities. However, its production is severely limited due
360 to the fact that the major natural source of PuA is pomegranate seed oil, which is not readily
361 available on a large scale.[1] Similar to other unusual fatty acids and high value bioproducts, it is
362 thus attractive to provide a stable and economical supply of PuA in engineered microorganisms.
363 To achieve this objective, it will be of critical importance to expand our knowledge of PuA
364 accumulation in yeast, and to use this information in the design of effective metabolic
365 engineering strategies. Therefore, we evaluated the combination of various metabolic
366 engineering strategies for engineering PuA production in *S. cerevisiae* and found that a 'Push-
367 Pull' approach with the combination of *snf2* deletion, along with the heterologous expression of
368 *PgFADX*, *PgPDAT* and *PgPDAT/LPCAT*, and supplementation with 0.05% LA in the culture
369 medium resulted in approximately 3.4% of total fatty acids as PuA. This was a 10-fold increase
370 compared to the background yeast strain heterologously expressing *PgFAD2* and *PgFADX*, and
371 approximately two to four-fold enhancement compared to previous reports focused on *S.*
372 *cerevisiae*.[4] Although the content of LA in strains with precursor feeding (PA23-PA28) largely
373 remains the same, in strains coexpressing *YlFAD2* (PA17-PA22), engineering genes in acyl
374 channeling processes seems to improve the content of both LA and PuA (Table 2,
375 Supplementary Table S1). After incubation, yeast cultures with exogenous LA accumulated

higher lipid content and slightly less biomass compared to yeast coexpressing *YlFAD2*, possibly due to the inhibition from the high concentration of fatty acid feeding (Supplementary Table S1). Taken together, the dry cell weight of strain PA28, which accumulates the highest PuA content, is 1.7mg/ml culture and the total lipid content is around 12.6%, which corresponds to 7.2μg/mL PuA of liquid culture.

Nevertheless, the percentage of PuA in total fatty acids was relatively low and also was lower than LA (Fig. 1), suggesting the conversion rate of LA to PuA by PgFADX is low in *S. cerevisiae*. Since the expression of PgFADX in *Arabidopsis* and fission yeast result in over 20% of total fatty acids as PuA, the catalytic ability of PgFADX itself should be acceptable. The low percentage of PuA might due to other reasons with the baker's yeast platform. The possible reasons can be rather complex, including but not limited to post-transcriptional regulation apparatus targeting FAD2-like enzyme in baker's yeast, rapid PuA turnover, cellular localization of PgFADX, its spatial distance to LA storage site, and the inefficient incorporation of PuA into TAG. In this study, we have investigated the PuA synthesis in baker's yeast mutants to address this issue and found out that the deletion of transcription factors that modulating multiple metabolic pathways can significantly improve the content of PuA.

In summary, we have engineered *S. cerevisiae* to produce PuA, to expand our knowledge in the context of generating this high-value unusual fatty acid in baker's yeast. Among the genes tested, a 'Push-Pull' approach by combining the deletion of transcription factor *snf2* with the heterologous expression of *PgFADX*, *PgPDAT,* and *PgLPCAT,* along with the feeding of 0.05% LA, resulted in *S. cerevisiae* cells yielding 3.37% of total fatty acids as PuA. To further improve PuA production in baker's yeast, subsequent studies are required, including the evaluation of PgFADX enzymatic activity and enzyme engineering, its subcellular localization

399 and stability, coupled with comprehensive metabolic engineering of PgFADX and other genes in
400 lipid biosynthesis and accumulation and carbon fluxing. Moreover, the strain used in the current
401 proof-of-concept study is a common laboratorial baker's yeast strain with four auxotrophic
402 markers on its genome for easy genetic manipulation. By transferring the knowledge reported by
403 this study to other yeast species, especially oleaginous yeast such as *Y. lipolytica*, we may
404 generate industrial-level PuA-producing yeast strains. Followed by bioprocess optimization, the
405 strains may be used in the industrial production of PuA via fermentation.
406

407 **ABBREVIATIONS**

408 DGAT, acyl-CoA: diacylglycerol acyltransferase; FAD2, Δ12-acyl-lipid-desaturase; FADX,
409 fatty acid conjugase; FAME, fatty acid methyl esters; GRAS, Generally Recognized as Safe; LA,
410 linoleic acid; LB, Luria-Bertani; LPCAT, lysophospholipid acyltransferase; PC,
411 phosphatidylcholine; PDAT, phospholipid: diacylglycerol acyltransferase; PDCT,
412 phosphatidylcholine: diacylglycerol cholinephosphotransferase; PLA_2, phospholipase A_2; PuA,
413 Punicic acid; TAG, triacylglycerol; TLC, thin layer chromatography; YNB, yeast nitrogen base
414

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418

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CONFLICT OF INTEREST:

The authors declare that they have no conflicts of interest with the content of this article.

SUPPORTING INFORMATION:

Dry cell weight and profiles of oleic acid, LA, PuA, and total fatty acids in the recombinant yeast cells with or without LA feeding.

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582

583 **FIGURE CAPTIONS**

584 **Fig. 1.** Reconstitution of the pomegranate derived PuA synthetic pathway in *S. cerevisiae.* (A)
585 Illustration of PuA biosynthesis. Abbreviations: FAD2, Δ12 fatty acid desaturase; FADX, fatty
586 acid conjugase; PuA, punicic acid. (B) Contents of LA and PuA in recombinant yeast strains
587 with different sources of precursor. Strain names were listed in Table 1. Data represent the mean
588 values of biological replicates ± SD (*n*=3).

589 **Fig. 2.** The deletion of transcription factors affecting *S. cerevisiae* lipid metabolism led to
590 improvements in punicic acid (PuA) content. Strain names were listed in Table 1. Data represent
591 means ± SD of three biological replicates.

592 **Fig. 3.** Introduction of pomegranate-derived acyl-channeling genes. (A) Illustration of acyl-
593 channeling process among different substrate pools. Abbreviations: AAPT, choline/ethanolamine
594 phosphotransferase; DGAT, acyl-CoA: diacylglycerol acyltransferase; GPAT, glycerol-3-
595 phosphate acyltransferase; LACS, long chain acyl-CoA synthetase; LPAAT, lysophosphatidic
596 acid acyltransferase; LPC: lysophosphatidylcholine; LPCAT: lysophosphatidylcholine
597 acyltransferase; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDAT,
598 phospholipid: diacylglycerol acyltransferase; PDCT, phosphatidylcholine: diacylglycerol
599 cholinephosphotransferase PLA_2, phospholipase A_2; (B) Introducing pomegranate-derived acyl-
600 channeling genes moderately increased PuA content. Strain names were listed in Table 1. Data
601 represent the mean values of biological replicates ± SD (*n*=3).

602 **Fig. 4.** Relative punicic acid (PuA) content in TAG and polar lipids extracted from recombinant
603 yeast cells with linoleate (LA) supplementation or co-expression with *YlFAD2*. Strain names
604 were listed in Table 1. Data shown represent the mean values biological replicates ± SD (*n*=3). ∗,
605 $P < 0.05$ (Student's t test).

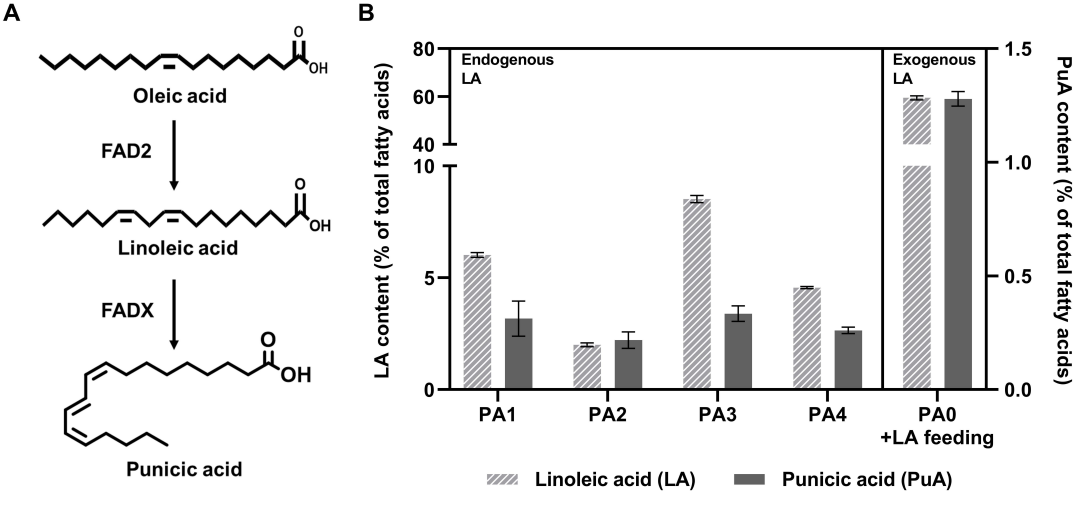
Table 1 Strains used in this study

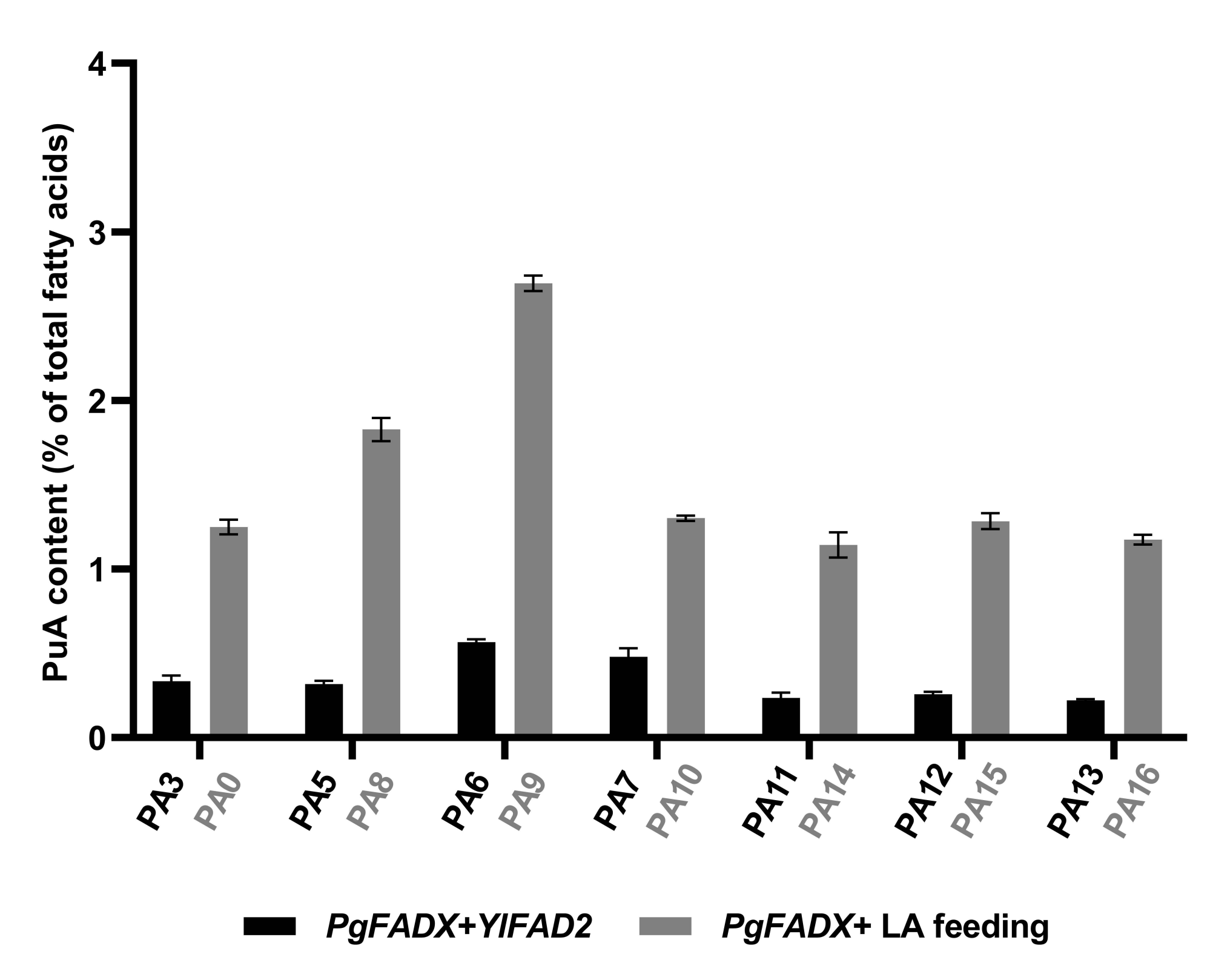
Strain name	Relevant genotype/property	Source
Escherichia coli DH5a	*end*A1, *rec*A1, *gyr*A96, *thi*-1, *hsd*R17, *rel*A1, *sup*E44 ΔlacU169, Φ80d lacZΔM15	Invitrogen
Saccharomyces cerevisiae		
BY4741	*MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	[44]
ira2Δ	BY4741-*ira2::KanMX*	Euroscarf
snf2Δ	BY4741-*snf2::KanMX*	Euroscarf
snf1Δ	BY4741-*snf1::KanMX*	Euroscarf
tgl3Δ	BY4741-*tgl3::KanMX*	Euroscarf
pxa1Δ	BY4741-*pxa1::KanMX*	Euroscarf
pxa2Δ	BY4741-*pxa2::KanMX*	Euroscarf
PA0	BY4741/pESC-Ura-*PgFADX*	This report
PA1	BY4741/pESC-Ura-*PgFADX-PgFAD2*	This report
PA2	BY4741/pESC-Ura-*PgFADX-AdFAD2*	This report
PA3	BY4741/pESC-Ura-*PgFADX-YlFAD2*	This report
PA4	BY4741/pESC-Ura-*PgFADX-LsFAD2*	This report
PA5	BY4741-*ira2Δ*/pESC-Ura-*PgFADX-YlFAD2*	This report
PA6	BY4741-*snf2Δ*/pESC-Ura-*PgFADX-YlFAD2*	This report
PA7	BY4741-*snf1Δ*/pESC-Ura-*PgFADX-YlFAD2*	This report
PA8	BY4741-*ira2Δ*/pESC-Ura-*PgFADX*	This report
PA9	BY4741-*snf2Δ*/pESC-Ura-*PgFADX*	This report
PA10	BY4741-*snf1Δ*/pESC-Ura-*PgFADX*	This report

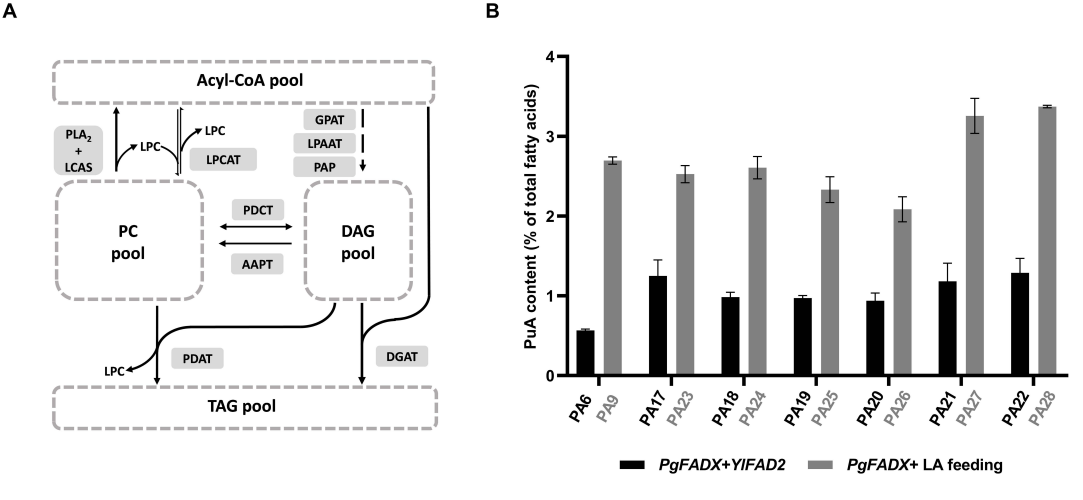
PA11	BY4741-*tgl3Δ*/pESC-Ura-*PgFADX-YlFAD2*	This report
PA12	BY4741-*pxa1Δ*/pESC-Ura-*PgFADX-YlFAD2*	This report
PA13	BY4741-*pxa2Δ*/pESC-Ura-*PgFADX-YlFAD2*	This report
PA14	BY4741-*tgl3Δ*/pESC-Ura-*PgFADX*	This report
PA15	BY4741-*pxa1Δ*/pESC-Ura-*PgFADX*	This report
PA16	BY4741-*pxa2Δ*/pESC-Ura-*PgFADX*	This report
PA17	PA6 with pESC-Leu-*PgPLA$_2$-PgLPCAT*	This report
PA18	PA6 with pESC-Leu-*PgDGAT2-PgLPCAT*	This report
PA19	PA6 with pESC-Leu-*PgDGAT2-PgPLA$_2$*	This report
PA20	PA6 with pESC-Leu-*PgDGAT2-PgPDCT*	This report
PA21	PA6 with pESC-Leu-*PgPDAT-PgPDCT*	This report
PA22	PA6 with pESC-Leu-*PgPDAT-PgLPCAT*	This report
PA23	PA9 with pESC-Leu-*PgPLA$_2$-PgLPCAT*	This report
PA24	PA9 with pESC-Leu-*PgDGAT2-PgLPCAT*	This report
PA25	PA9 with pESC-Leu-*PgDGAT2-PgPLA$_2$*	This report
PA26	PA9 with pESC-Leu-*PgDGAT2-PgPDCT*	This report
PA27	PA9 with pESC-Leu-*PgPDAT-PgPDCT*	This report
PA28	PA9 with pESC-Leu-*PgPDAT-PgLPCAT*	This report

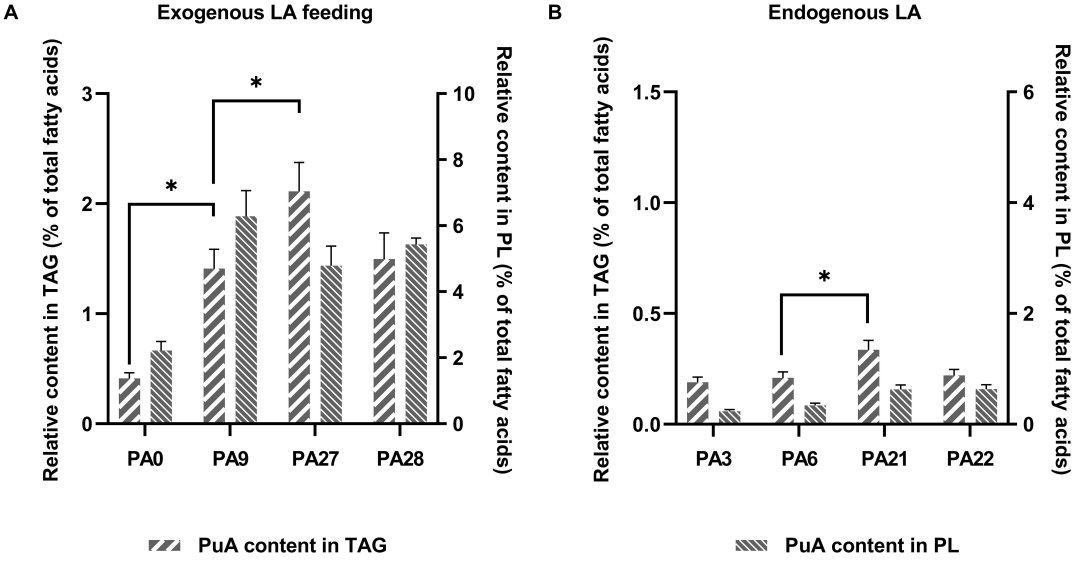
Table 2 Linoleic acid (LA) and punicic acid (PuA) content of recombinant yeast cells.

Strain	LA feeding	LA content	PuA content (% total fatty acids)
PA0	0.05%	59.58 ± 0.99	1.25±0.04
PA9		73.36 ± 0.33	2.70±0.05
PA27		66.4 ± 5.27	3.26±0.22
PA28		64.21 ± 1.59	3.37±0.02
PA3	-	8.52 ± 0.16	0.33±0.03
PA6		8.08 ± 0.21	0.57±0.02
PA21		15.37 ± 1.5	1.2±0.23
PA22		17.33 ± 1.63	1.23±0.11


A
Oleic acid
FAD2
Linoleic acid
FADX
Punicic acid
B
LA content (% of total fatty acids)
PuA content (% of total fatty acids)
Endogenous LA
Exogenous LA
80
60
40
10
5
0
1.5
1.0
0.5
0.0
PA1
PA2
PA3
PA4
PA0 +LA feeding
Linoleic acid (LA)
Punicic acid (PuA)


PuA content (% of total fatty acids)
4
3
2
1
0
PA3
PA0
PA5
PA8
PA6
PA9
PA7
PA10
PA11
PA14
PA12
PA15
PA13
PA16
PgFADX+YlFAD2
PgFADX+ LA feeding


A
Acyl-CoA pool
PLA2 + LCAS
LPC
LPC
LPCAT
GPAT
LPAAT
PAP
PC pool
PDCT
AAPT
DAG pool
LPC
PDAT
DGAT
TAG pool
B
PuA content (% of total fatty acids)
0
1
2
3
4
PA6
PA9
PA17
PA23
PA18
PA24
PA19
PA25
PA20
PA26
PA21
PA27
PA22
PA28
PgFADX+YlFAD2
PgFADX+ LA feeding


A
Exogenous LA feeding
Relative content in TAG (% of total fatty acids)
Relative content in PL (% of total fatty acids)
3
2
1
0
10
8
6
4
2
0
*
*
PA0
PA9
PA27
PA28
PuA content in TAG
B
Endogenous LA
Relative content in TAG (% of total fatty acids)
Relative content in PL (% of total fatty acids)
1.5
1.0
0.5
0.0
6
4
2
0
*
PA3
PA6
PA21
PA22
PuA content in PL