


# Metabolic engineering of *Schizosaccharomyces pombe* to produce punicic acid, a conjugated fatty acid with nutraceutical properties

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**Abstract** Punicic acid (PuA) is a conjugated linolenic acid (C18:3 $\Delta^{9c,11t,13c}$ ) with a wide range of nutraceutical effects with the potential to reduce the incidence of a number of health disorders including diabetes, obesity, and cancer. It is the main component of seed oil from *Punica granatum* and *Trichosanthes kirilowii*. Previously, production of relatively high levels of this unusual fatty acid in the seed oil of transgenic *Arabidopsis thaliana* plant was accomplished by the use of *A. thaliana fad3/fae1* mutant high in linoleic acid (18:2 $\Delta^{9c,12c}$ ) and by co-expression of *P. granatum FATTY ACID CONJUGASE (PgFADX)* with  $\Delta 12$ -DESATURASE (*FAD2*). In the current study, *P. granatum* cDNAs governing PuA production were introduced into the yeast *Schizosaccharomyces pombe*. Expression of PgFADX alone resulted in production of PuA at the level of 19.6% of total fatty acids. Co-expression PgFADX with PgFAD2, however, further enhanced PuA content to 25.1% of total fatty acids, the highest level reported to date for heterologous

expression. Therefore, microbial systems can be considered as a potential alternative to plant sources for a source of PuA for nutraceutical applications.

**Keywords** Yeast · *Punica granatum* · Fatty acid conjugase · Fatty acid desaturase · Conjugated linolenic acid

## Introduction

Punicic acid (PuA; C18:3 $\Delta^{9c,11t,13c}$ ) belongs to the family of conjugated linolenic acids (CLNA) which naturally occur in the seed oils of plant species from several families including *Cucurbitaceae*, *Punicaceae*, *Bignoniaceae*, *Rosaceae*, *Chrysobalanaceae*, *Lythraceae*, *Balsaminaceae*, and *Euphorbiaceae* (Badami and Patil 1980; Rawat et al. 2012; Smith 1971). The major sources of PuA are seed oils of pomegranate (*Punica granatum*) [60–80% PuA (w/w)] and Chinese snake gourd (*Trichosanthes kirilowii*) [40% PuA (w/w)]; however, the availability of these oils is very limited (Joh et al. 1995; Koba et al. 2007; Takagi and Itabashi 1981).

Recently, it was reported that natural CLNA-enriched oils have nutraceutical properties with notable health benefits (for review, see Aruna et al. 2016). The antioxidant (Saha and Ghosh 2009), anti-diabetic (Arao et al. 2004; Koba et al. 2007), anti-cancer (Grossmann et al. 2010; Kohno et al. 2004; Suzuki et al. 2001; Tanaka et al. 2011; Wang et al. 2014), and anti-inflammatory (Boussetta et al. 2009; Costantini et al. 2014) activities of CLNA-enriched sources are currently under intensive investigation. In addition to health benefits, seed oils containing CLNA have other applications including the production of dyes, inks, coatings, resins, and cosmetics (Cahoon et al. 2006; Homung et al. 2002; Mietkiewska et al. 2014).

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Limited natural sources of PuA combined with a high demand for oils enriched in PuA has led to a growing interest in the metabolic engineering of temperate plants to produce PuA in their seed oils (Mietkiewska et al. 2014). The fatty acid (FA) precursors oleic acid (OA; 18:1 $\Delta^9$ <sup>c</sup>, hereafter 18:1) and linoleic acid (LA; 18:2 $\Delta^9$ <sup>c,12</sup><sup>c</sup>, hereafter 18:2) lead to PuA (Fig. 1a). This sequential conversion to PuA, however, takes place while OA is attached to phosphatidylcholine (Fig. 1b) (Mietkiewska et al. 2014). OA is converted to LA by the catalytic action of  $\Delta$ 12-fatty acid desaturase (FAD2), and subsequent conversion of LA to PuA is catalyzed by a divergent form of FAD2 often referred to as fatty acid conjugase (FADX) (Cahoon et al. 2006; Vanhercke et al. 2013). This enzyme catalyzes the conversion of the  $\Delta$ 12-double bond of LA into two conjugated double bonds at positions  $\Delta$ 11t and  $\Delta$ 13c (Hornung et al. 2002; Iwabuchi et al. 2003). The activity of native FADX required for the biosynthesis of PuA in *P. granatum* (PgFADX) and *T. kirilowii* (TkFADX) was initially characterized in the yeast *Saccharomyces cerevisiae* by Hornung et al. (2002) and Iwabuchi et al. (2003). In both studies, the heterologous production of PuA in yeast reached less than 2% of total FA. Recombinant PgFADX was also capable of catalyzing the formation of LA from OA indicating that the enzyme also had some FAD2 activity (Hornung et al. 2002; Iwabuchi et al. 2003) (Fig. 1b).

A few research groups have also been successful in the heterologous production of PuA in temperate plants. Upon over-expression of *PgFADX* or *TkFADX* in *Arabidopsis thaliana*, accumulation in seeds reached up to 4.4% (w/w) or 10% PuA (w/w), respectively (Iwabuchi et al. 2003). Over-expression of *TkFADX* in *Brassica napus* resulted in 2.5% accumulation of PuA in the seed oil (Koba et al. 2007). Combined expression of *PgFADX* and *PgFAD2* in the *A. thaliana fad3/fae1* mutant (high in LA), however, led to 21% accumulation of PuA of the total acyl lipids in the seed oil (Mietkiewska et al. 2014). Recently, the lignin promoter

was shown to be very effective, relative to other plant promoters, in raising PuA levels in *A. thaliana* expressing *PgFADX* (Song et al. 2016). Limited accumulation of PuA in transgenic plant seeds indicates that additional pomegranate genes might be required to further enhance PuA content in temperate plants (Mietkiewska et al. 2014).

Microorganisms have shown great promise in production of value-added compounds (Garaiova et al. 2014; Holic et al. 2012; Ledesma-Amaro and Nicaud 2016; Meesapyodsuk et al. 2015). With appropriate gene combinations, it may be possible to metabolically engineer microorganisms to produce PuA.

Here, we report on metabolic engineering of PuA in *Schizosaccharomyces pombe*, a yeast with high OA content. The heterologous expression of *PgFADX* alone resulted in the production of PuA at the level of 19.6% of total FA. Co-expression of *PgFADX* and *PgFAD2*, however, resulted in a further increase in PuA content to 25.1% of total FA, the highest level reported to date for heterologous expression. Our investigation suggests that metabolically engineered yeast may potentially represent an alternate source of PuA to plant oils for nutraceutical applications of this conjugated FA.

## Materials and methods

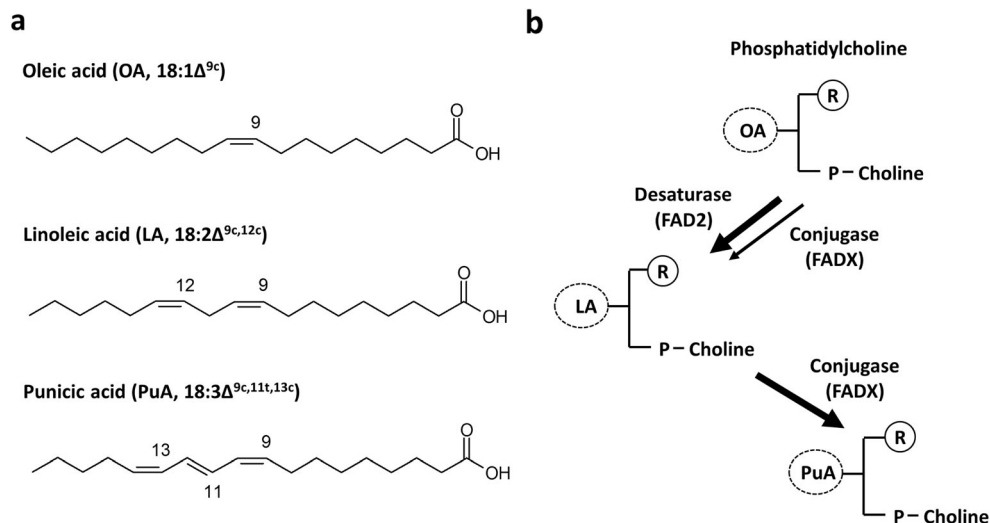
### Materials

Media components were obtained from Becton Dickinson (USA) or BioLife (Italy). Fine chemicals were mostly from MP Biomedicals or Sigma-Aldrich (USA).

### Strain, cultivation conditions, and yeast transformation

Wild-type *S. pombe* strain ED666 (*h+* *ade6-M210 ura4-D18 leu1-32*) was obtained from Bioneer collection (USA). It was

**Fig. 1** Illustration of **a** oleic acid (OA), linoleic acid (LA), and punicic acid (PuA). **b** The biosynthesis of LA and PuA. Additional abbreviations: *FAD2*,  $\Delta$ -12 fatty acid desaturase; *FADX*, fatty acid conjugase; *R*, acyl chain



grown and maintained on the YES agar plates and liquid medium at 30 °C. Strain ED666 was used as a host strain for heterologous expression of codon optimized *PgFAD2* cDNA (GenBank# KY996743) and/or codon-optimized *PgFADX* cDNA (GenBank# KY996742). *S. pombe* strains containing plasmids were grown in Edinburgh minimal medium (EMM) without supplements depending on the selective pressure required to maintain the plasmids. Solid plates were prepared with 2% agar. Growth in liquid media was performed at 30 °C with continuous shaking. Cell growth was estimated by measuring the OD at 600 nm (Spectrophotometer UV-2401 PC, Shimadzu, Japan). Optical density unit equal to 1 (OD = 1) corresponded approximately to  $1 \times 10^7$  cells/mL. Growth in liquid media was monitored by measuring turbidity of the cells at OD<sub>600</sub>. For a detection of the growth in liquid media, strains grown in EMM overnight were inoculated into fresh EMM at OD<sub>600</sub> = 0.2, and the growth curves were obtained by measuring the turbidity of the cells at 600 nm. Yeast transformation was performed using the lithium acetate method (Gietz et al. 1992).

### Preparation of *S. pombe* expression plasmids of *PgDADX* and *PgFAD2*

Standard techniques of DNA manipulation used in this study are described in Sambrook and Russell (2001). *PgFAD2* (GenBank# AY178447) and *PgFADX* (GenBank# AY178446) (Mietkiewska et al. 2014) were re-designed using yeast preference codon usage, and the cDNA were chemically synthesized by GenScript USA, Inc. (USA). The synthesized open reading frames of *PgFAD2* (GenBank# KY996743) or *PgFADX* (GenBank# KY996742) were excised from *Escherichia coli* plasmid pUC57-Kan-*PgFAD2* or pUC57-Kan-*PgFADX*, respectively, as *NdeI*–*Bam*HI fragments by using artificially added restriction enzyme sites at the 5' and 3' ends of the cDNA, and they were inserted into the *NdeI*–*Bam*HI multiple cloning sites of multicopy-type plasmids pREP1 (*LEU2*) or pREP2 (*ura4+*), respectively. *PgFAD2* and *PgFADX* were expressed from plasmids under the control of the *nmt1* promoter of *S. pombe*. Gene expression under the *nmt1* promoter was repressed by 15 μM thiamine (5 μg/mL). The control strain contained the empty vectors pREP1 and pREP2.

### Fatty acid analysis

Extraction of yeast lipids was performed as described by Mietkiewska et al. (2014) with minor modifications. Briefly, 25–35 mg of yeast cells was suspended in 1 mL of a mixture of chloroform and isopropanol (2:1, v/v) containing the antioxidant butylated hydroxytoluene at a final concentration of 0.01%. Cells were disrupted using the FastPrep disintegrator

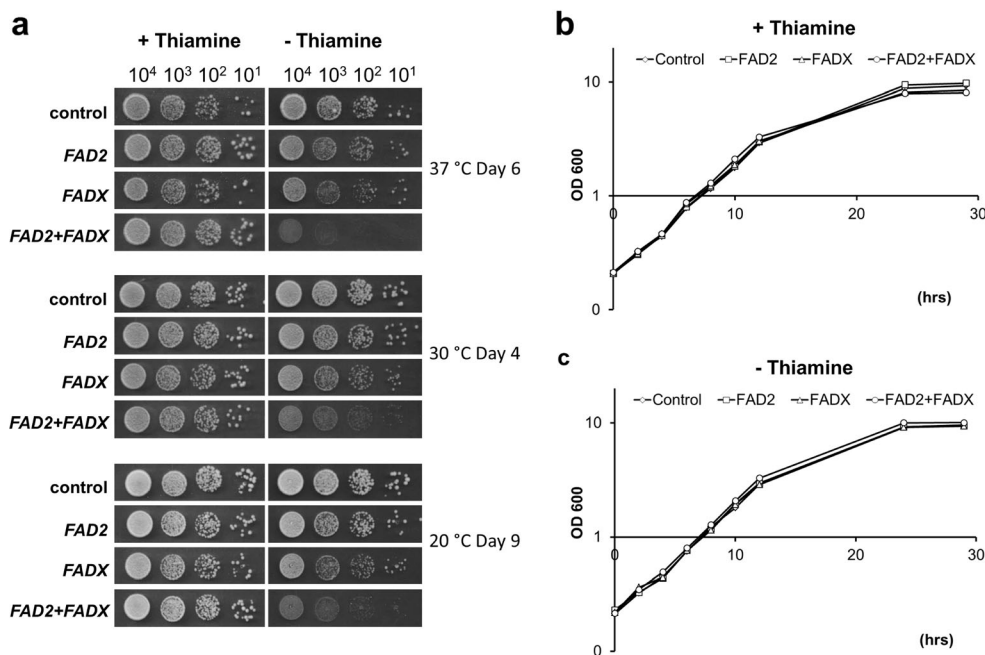
(MP Biomedicals) using glass beads (diameter 0.4 mm) at  $3 \times 40$  s, at the highest speed (6.5 m/s), with 5 min cooling on ice between cycles. The homogenate was then dried under N<sub>2</sub> gas and suspended in 1 mL of chloroform and isopropanol (2:1, v/v). An aliquot of 100 μL was transferred to a glass tube, dried under N<sub>2</sub> gas, and transmethylated with 1 mL of 5% sodium methoxide (Na-OCH<sub>3</sub>) in methanol at 30 °C for 30 min. To stop the reaction, 1.5 mL of 0.9% (w/v) sodium chloride was added to the samples, and fatty acid methyl esters (FAME) were extracted with 1 mL of hexane as described by Iwabuchi et al. (2003). Samples were centrifuged at 1000×g for 5 min, and the hexane layers were transferred to glass vials. Analysis of FAME was performed by the injection of 1-μL aliquots to a gas chromatography (GC) apparatus (GC-2010 Plus, Shimadzu, Japan) equipped with a BPX70 capillary column (30 m × 0.25 mm × 0.25 μm, SGE Analytical Science, Australia) under temperature programming (165 °C hold for 4 min, 10 °C/min to 180 °C hold 5 min, and 10 °C/min to 230 °C hold 5 min) (Mietkiewska et al. 2011). Individual FAME were identified by comparison with the authentic standards of the C<sub>4</sub>–C<sub>24</sub> FAME mixture (Supelco, USA). PuA methyl ester was identified by comparison with PuA (Larodan, Sweden) methylated by a method allowing free FA methylation (Stumpe et al. 2001) and by methylation of pomegranate oil (80 mg) (Bioshop, Biopurus). Quantification of individual FA was performed using the heptadecanoic acid methyl ester internal standard (Sigma-Aldrich, USA).

## Results

### Heterologous expression of *PgFAD2* and *PgFADX*

cDNA-encoding *PgFAD2* and *PgFADX* were previously identified as necessary factors for effective metabolic engineering of PuA biosynthesis in the model plant *A. thaliana* (Mietkiewska et al. 2014). To achieve the heterologous production of PuA in yeast, a *S. pombe* strain was transformed with the same cDNA under the control of the *nmt1* promoter (repression by thiamine). The control strain contained the corresponding empty vectors.

Previous work on metabolic engineering of ricinoleic acid (12-OH 18:1Δ<sup>9c</sup>) production in *S. pombe* cells showed that cultivation temperature highly affects the cell growth and the activity of the heterologously produced *Claviceps purpurea* oleate Δ12-hydroxylase enzyme (Holic et al. 2012). Therefore, first, we tested the growth of cells expressing *PgFAD2* and/or *PgFADX* at various cultivation temperatures. Cells were spotted on agar plates with or without thiamine and incubated at 20, 30, and 37 °C. As shown in Fig. 2a, the control strain grew well in all tested temperatures. In the presence of thiamine (repressed condition), the growth of all strains was similar. In the absence of thiamine (induced



**Fig. 2** Growth of *S. pombe* strains expressing *P. granatum*  $\Delta$ -12 FATTY ACID DESATURASE (*PgFAD2*) and/or *P. granatum* FATTY ACID CONJUGASE (*PgFADX*) and the control strain. The control strain contained the corresponding empty vectors (pREP1 and pREP2). **a** Growth on EMM (Edinburgh minimal medium) agar plates at various temperatures. Cells were grown in EMM in the presence of thiamine overnight at 30 °C until an OD<sub>600</sub> of about 6 was reached. Cells were washed in sterile water and 10  $\mu$ L of 10-fold serial dilutions of the strains were spotted on EMM plates in the presence (repressed condition) or

absence (induced condition) of 15  $\mu$ M thiamine. The plates were incubated at 37, 30, and 20 °C, respectively, for 4 to 9 days as indicated depending on the growth at tested temperatures. Liquid growth of the strains in the **b** presence (repressed condition) of 15  $\mu$ M thiamine or **c** absence (induced condition) of thiamine with starting OD<sub>600</sub> of 0.2 after pre-culturing the cells in EMM in the presence of thiamine at 30 °C overnight. Cells were grown in EMM at 30 °C, and the cell turbidity was monitored spectrophotometrically (UV-2401 PC, Shimadzu, Japan)

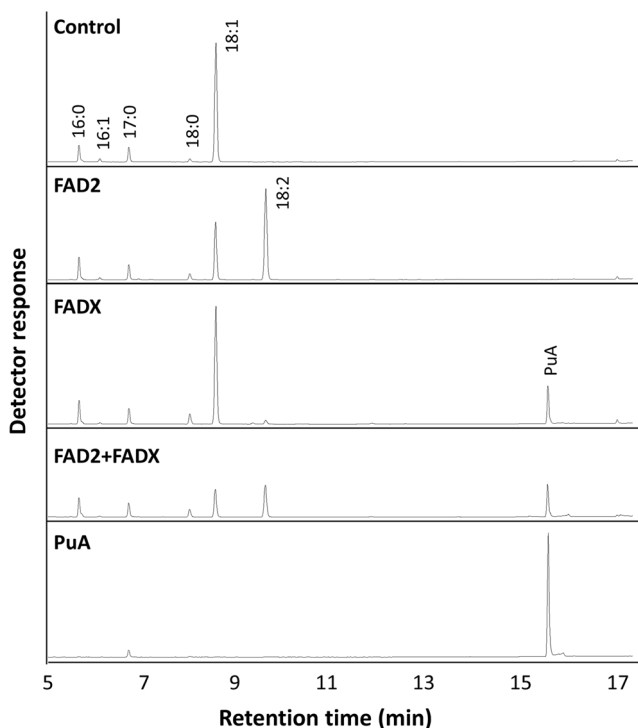
condition), the growth of *PgFADX*-containing strain was affected at all tested temperatures. The growth of transformed yeast was strongly reduced when *PgFADX* was co-expressed together with *PgFAD2*, but *PgFAD2* expression alone did not affect the growth. Since growth inhibition was not dependent on temperature, as in the case of heterologous expression of a cDNA-encoding *C. purpurea* oleate  $\Delta$ 12-hydroxylase (Holic et al. 2012), a standard growth condition of 30 °C was adopted. In addition, an earlier study showed that *S. cerevisiae* transformed with *PgFADX* exhibited more efficient production of PuA when cultured at 30 °C (Hornung et al. 2002). Next, the growth in liquid media was examined. Under either repressed or induced conditions, all strains showed similar growth (Fig. 2b, c, respectively).

### Heterologous production of punicic acid

To confirm the activity of *PgFAD2* and/or *PgFADX* in transformed *S. pombe*, the fatty acid methyl esters (FAME) from cells expressing *PgFAD2* and *PgFADX* under induced conditions (without thiamine) were analyzed by GC (Fig. 3). In the case of *PgFAD2* expression, production of LA was detected. In the case of *PgFADX* expression, peak for both LA and PuA was detected.

It has been shown earlier that maximal expression driven by the *nmt1* promoter after the thiamine wash is achieved after 18 h of cultivation (Maundrell 1990). Thus for the FA analysis, to release the thiamine repression, pre-culture cells were incubated overnight in media lacking thiamine as shown in Fig. 4a. Since the serial dilution test showed that the cell growth is affected upon heterologous co-expression of *PgFADX* with *PgFAD2* (Fig. 2a), the culture cells for GC analysis were inoculated to high initial OD equal to 1. By this approach, only three generations were needed to reach the cell confluency. After 1 day of incubation, all cultures reached similar OD to the control except in transformed yeast co-expressing *PgFADX* with *PgFAD2* in which growth was highly affected (Supplementary Table S1).

FA analysis of yeast cells with heterologous *PgFAD2* expression under induced conditions (absence of thiamine) revealed high production of LA (49.1%) at 30 °C after 1 day of incubation, and this high level of LA was maintained during 10 days of cultivation (Fig. 4b). Upon heterologous expression of *PgFADX* under induced conditions, PuA (8.5%) was detected at 30 °C after 1 day of incubation and the level of PuA was maintained during 10 days of cultivation (Fig. 4b). In the case of either *PgFADX* expression alone or *PgFADX* with *PgFAD2* expression, the highest level of PuA production

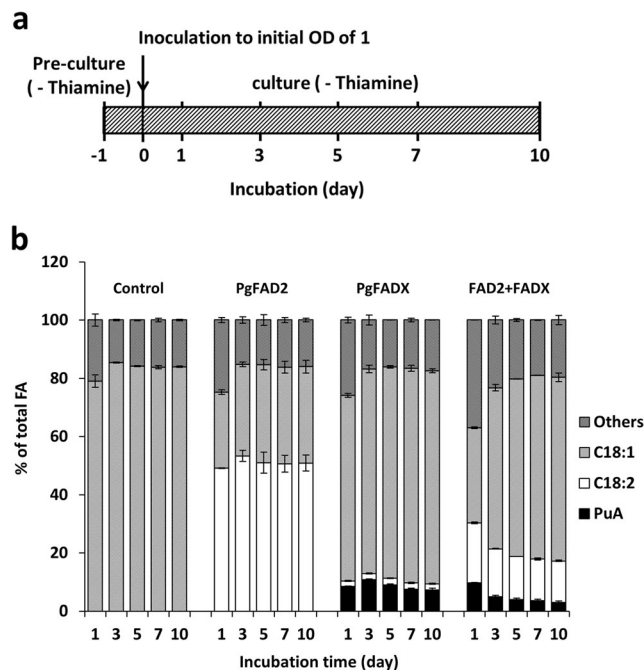


**Fig. 3** Gas chromatographic analysis of fatty acid methyl esters from the lipid extracts of *S. pombe* strains expressing *P. granatum*  $\Delta$ -12 FATTY ACID DESATURASE (*PgFAD2*) and/or *P. granatum* FATTY ACID CONJUGASE (*PgFADX*) and the control strain. The control strain contained the corresponding empty vectors. Cells were grown at 30 °C for 3 days. The bottom panel indicates a gas chromatogram of punicic acid (PuA). Heptadecanoic acid methyl ester was used as an internal standard

occurred within the first 3 days of cultivation. In addition, low production of LA (1.9%), the precursor of PuA, was detected in cells expressing *PgFADX* after 1 day of incubation and this level was maintained during 10 days of cultivation. Cells co-expressing *PgFAD2* and *PgFADX* under induced conditions produced 20.6% LA and 9.8% PuA after 1 day of incubation, respectively (Fig. 4b). More extensive cultivation, beyond 1 day, resulted in further decreases in LA and PuA content (Fig. 4b).

#### Further enhancement of punicic acid synthesis and dynamics of accumulation

Increased PuA accumulation was achieved by change in the pre-culture conditions and by alteration of the initial culture OD. Pre-culture cells were cultivated in the presence of thiamine (repressed condition), inoculated to various initial OD into media lacking thiamine (induced condition), and incubated for 3 days as shown in Fig. 5a. In addition, FA analysis of cells expressing *PgFADX* (Fig. 5b) and cells co-expressing *PgFAD2* with *PgFADX* (Fig. 5c) revealed that maximum accumulation of PuA occurred at the initial inoculation OD equal to 0.2. The highest accumulation of PuA (22.3%) was achieved by co-expression of *PgFAD2* with *PgFADX*.

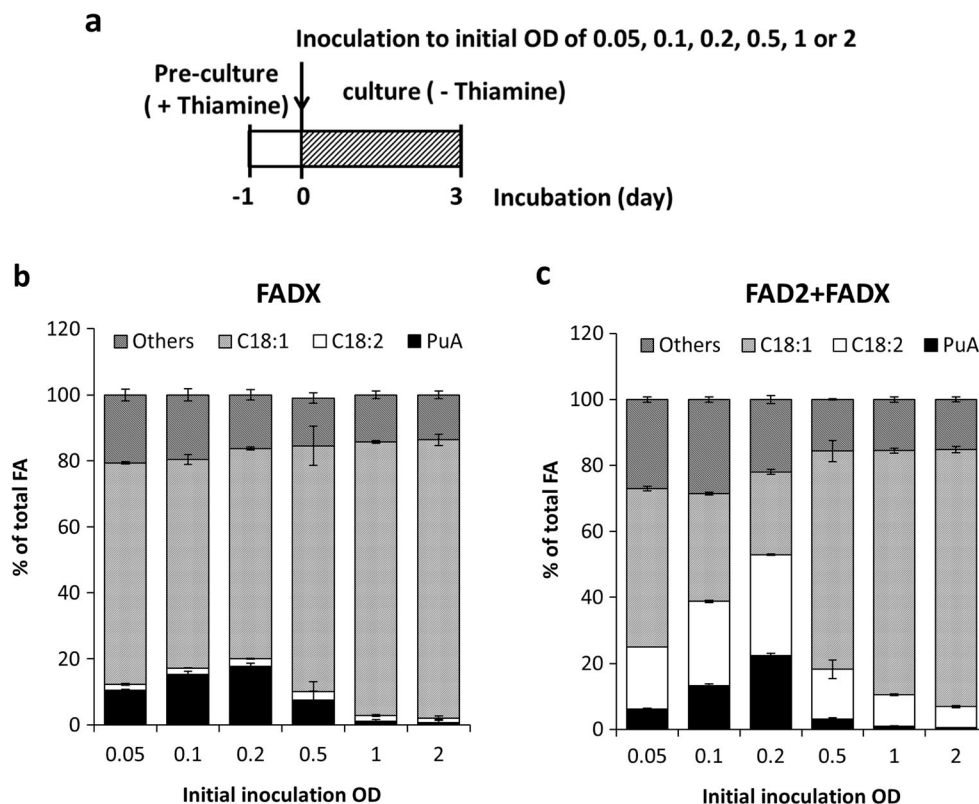


**Fig. 4** Fatty acid composition of *S. pombe* over-expressing *P. granatum*  $\Delta$ -12 FATTY ACID DESATURASE (*PgFAD2*) and/or *P. granatum* FATTY ACID CONJUGASE (*PgFADX*) under induced conditions. **a** Diagram describing the culture process. Yeasts were pre-cultured in EMM in the absence of thiamine at 30 °C for 1 day (hatched box). The pre-cultured cells were inoculated in the same medium at OD<sub>600</sub> of 1, and incubated at 30 °C for 10 days and sampled as indicated. **b** FA composition of the control, *PgFAD2*-expressing yeast, *PgFADX*-expressing yeast, or yeast co-expressing *PgFAD2* with *PgFADX* at different incubation times. The values are the average of two independent experiments  $\pm$  standard deviation. Abbreviations: EMM Edinburgh minimal medium; FA fatty acids; Others FA (16:0, 16:1, and 18:0); PuA punicic acid

In order to further characterize the dynamics of PuA production in *S. pombe* expressing *PgFADX*, or co-expressing *PgFAD2* with *PgFADX*, under more optimized conditions, PuA content of the cells was monitored for 10 days at 30 °C as shown in Fig. 6a. In the case of yeast expressing *PgFADX* alone, the level of PuA was steadily high from day 3 (18.7%) to day 6 (18.9%) with the maximal content of PuA (19.6%) occurring on day 4 (Fig. 6b). This level of PuA on day 4 corresponded to 38.71  $\mu$ g PuA/mL of culture (Supplementary Table S2). A different dynamic in PuA accumulation was observed in the case of yeast co-expressing *PgFAD2* with *PgFADX*. In this case, PuA content was only highest at day 2 (25.1%) and day 3 (24.3%) (Fig. 6c). This highest PuA content observed at day 2 corresponded to 34.33  $\mu$ g PuA/mL of culture (Supplementary Table S2).

## Discussion

The aim of this study was to produce PuA, a CLNA with health benefits, in *S. pombe* by heterologous expression of genes from *P. granatum*. A few groups have attempted to



**Fig. 5** The extent of punical acid (PuA) accumulation in *S. pombe* expressing *P. granatum* FATTYACID CONJUGASE (*PgFADX*) or *PgFADX* with  $\Delta$ -12 FATTY ACID DESATURASE (*PgFAD2*) is dependent on the initial OD of culture. **a** Diagram describing the culture process. Yeasts expressing *PgFADX* or yeasts co-expressing *PgFAD2* with *PgFADX* were pre-cultured in EMM in the presence of thiamine at 30 °C for 1 day (white box). The pre-cultured cells were inoculated into the fresh EMM at

various OD<sub>600</sub> (from 0.05 to 2) and incubated at 30 °C for 3 days (hatched boxes). **b** FA composition of *PgFADX*-expressing yeast analyzed at day 3 after inoculation. **c** FA composition of yeast co-expressing *PgFAD2* with *PgFADX* analyzed at day 3 after inoculation. The values are the average of three independent experiments  $\pm$  standard deviation. Abbreviations: EMM Edinburgh minimal medium; FA fatty acids; Others FA (16:0, 16:1, and 18:0)

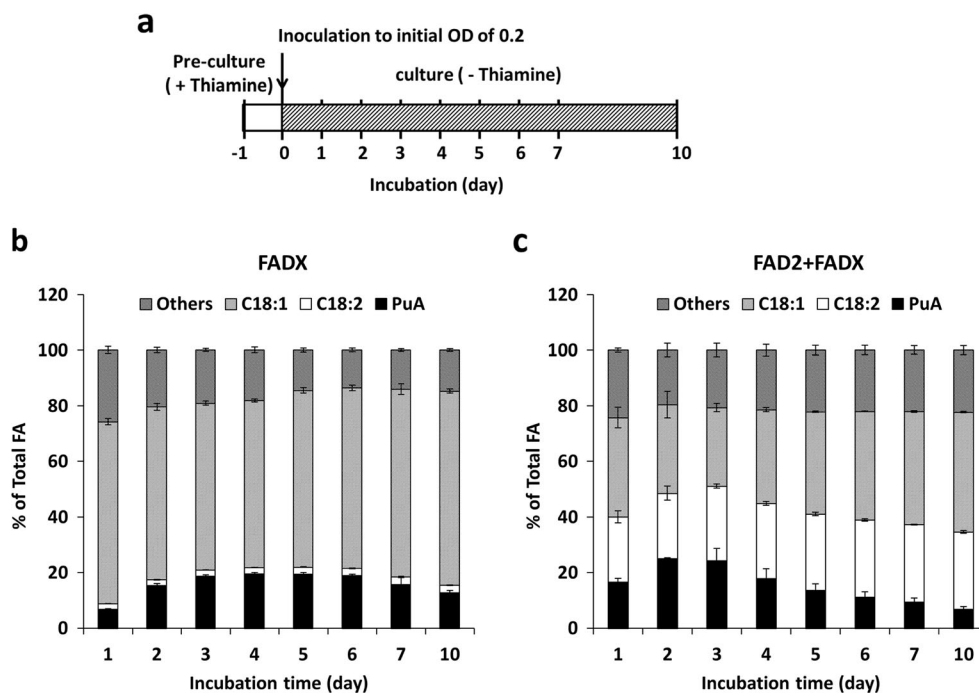
produce this unusual FA in transgenic *A. thaliana* (Iwabuchi et al. 2003; Mietkiewska et al. 2014) or *B. napus* plants (Koba et al. 2007). Only limited accumulation of PuA (4.4–10.2%) was observed in *A. thaliana* when compared with the levels of this CLNA (60–80%) in seeds from plants that naturally produced PuA (Iwabuchi et al. 2003). It is known that conjugated FAs are synthesized by a divergent form of FAD2 known as FADX, either from OA or from LA precursors esterified to the *sn*-2 position of PC (Cahoon et al. 2006; Vanhercke et al. 2013). Recently, our group reported on the production of PuA up to 11.3% of the total FA in the seed oil in transgenic *A. thaliana* over-expressing *PgFADX* in a *fad3/fae1* mutant background containing a high level of LA (Mietkiewska et al. 2014). Further enhancement to 21.2% PuA content in the seed oil was achieved by co-expression of *PgFAD2* with *PgFADX* because expression of the naturally occurring *AtFAD2* was inhibited by transgenic expression of *PgFADX* (Mietkiewska et al. 2014).

In previous studies, *S. cerevisiae* was used to characterize the properties of microsomal recombinant *PgFAD2* or *PgFADX* when the encoding cDNA was expressed under the control of a strong *GAL10* promoter. Enzyme activity was

very low, and only 0.2 and 0.8% of PuA of total FA was produced by growing cells at 30 °C for 3 days on 2% galactose without or with external LA supplementation, respectively (Iwabuchi et al. 2003). So far, the highest accumulation of PuA to 1.6% of total FA was achieved by growing yeast cells at 30 °C in media supplemented with 0.3 mM LA, the substrate for FADX (Hornung et al. 2002). This study also reported that PuA could only be detected after external supplementation of yeast growth media with LA (Hornung et al. 2002).

In the current study, we attempted to produce PuA in *S. pombe* instead of *S. cerevisiae* because of the high level of OA in this yeast. *S. pombe* strains with plasmids containing cDNA-encoding *PgFAD2* and/or *PgFADX* were constructed under the control of a strong *nmt1* promoter. Both cDNAs were codon optimized for yeast expression.

The heterologous expression of *PgFADX* driven by the *nmt1* promoter resulted in a slight growth inhibition effect which was enhanced by co-expression of *PgFAD2* and *PgFADX* (Fig. 2). This growth inhibition effect was also observed when we previously engineered *S. pombe* to produce ricinoleic acid (Holic et al. 2012). Preliminary flow cytometry data with asynchronous yeast culture co-expressing *PgFAD2*



**Fig. 6** Punicic acid (PuA) content as a function of incubation time in *S. pombe* expressing *P. granatum* FATTYACID CONJUGASE (*PgFADX*) or *PgFADX* with  $\Delta$ -12 FATTYACID DESATURASE (*PgFAD2*) under the induced condition (without thiamine). **a** Diagram describing the culture process. The *PgFADX*-expressing yeasts or yeasts co-expressing *PgFAD2* with *PgFADX* were pre-cultured in EMM in the presence of thiamine at 30 °C for 1 day (white box). The pre-cultured cells were inoculated into

the fresh EMM for an initial OD<sub>600</sub> of 0.2 and incubated at 30 °C for 10 days (hatched boxes) and sampled as indicated. FA composition of **b** *PgFADX*-expressing yeast or **c** yeast co-expressing *PgFAD2* with *PgFADX* and analyzed at indicated time points after the culture inoculation. The values are the average of three independent experiments  $\pm$  standard deviation. Abbreviations: EMM Edinburgh minimal medium; FA fatty acids; Others FA (16:0, 16:1, and 18:0)

with *PgFADX* showed that many cells were arrested in the S phase (data not shown). PuA production in our transformed yeast may have exerted a cytotoxic effect on the yeast to some extent. It should be noted, however, that combined expression of *PgFAD2* and *PgFADX* actually led to lower levels of PuA after 4–10 days of induction when compared to the levels of this conjugated FA for expression of *PgFADX* alone (see Fig. 6b versus Fig. 6c). Therefore, there may have been other factors, in addition to possible PuA-mediated cell death, which contributed to the observed growth inhibition. The anti-cancer and cytotoxic properties of PuA, and pomegranate oil, however, have been documented (Costantini et al. 2014; Sharma et al. 2017; Shinohara et al. 2012; Yuan et al. 2014). In addition, conjugated FAs are known to inhibit DNA polymerases and DNA topoisomerases (Mizushima et al. 2004).

In the first attempt to produce PuA in *S. pombe*, the strategy of cell growth based on previous experiments with production of ricinoleic acid was adopted and modified. In this strategy, high accumulation of this hydroxy FA was achieved by inoculation of cells to high initial OD. In addition, this strategy reduced the carryover of thiamine (repressing the expression from the *nm1* promoter) by cultivation of the pre-culture in media lacking thiamine (Holic et al. 2012). The strategy used for engineering of ricinoleic acid production, however, was

not applicable for engineering relatively high levels of PuA accumulation (Fig. 4b). Therefore, we optimized the pre-culture condition and tested the effect of various initial OD inoculations on final PuA production. In case of PuA accumulation, it was more effective to cultivate pre-culture cells in repressed condition and inoculate the cultured cells to an initial OD equal to 0.2. This approach greatly enhanced the production of PuA.

*S. pombe* appeared to be a much better host for production of PuA than *S. cerevisiae*. With introduction of recombinant *PgFADX* alone, substantial production of PuA to 19.6% of total FA was achieved. This is considerably greater than what was previously achieved when recombinant *PgFADX* was introduced into *S. cerevisiae*. The very low level of LA accumulation seen in our experiments suggests that PuA formation catalyzed by *PgFADX* kept pace with LA formation catalyzed by the same enzyme. Thus, it is possible that LA produced by the catalytic action of *PgFADX* is more readily used by *PgFADX* than a source of LA produced by the catalytic action of an independent FAD2. This would imply that substrate channeling may be operative in the conversion of OA to PuA catalyzed by *PgFADX*. Recently, substrate channeling was shown to be operative in plant recombinant FAD2 and  $\Delta$ 15-desaturase (FAD3) (sequentially catalyzing the

conversion of 18:1-phosphatidylcholine to 18:3 $\Delta^{9cis,12cis,15cis}$ -phosphatidylcholine) which were shown to interact in the membrane (Lou et al. 2014).

Co-expression of *PgFAD2* with *PgFADX* further increased PuA accumulation to 25.1% of the total FA in *S. pombe*. This exceeds the value of about 20% which was previously reported for the PuA content of the oil of *A. thaliana* co-expressing *PgFAD2* with *PgFADX* (see Mietkiewska et al. 2014). In addition, in the current study, different dynamics of PuA accumulation in yeast over-expressing *PgFADX* when compared to yeast co-expressing *PgFADX* with *PgFAD2* were observed. Elevated PuA content was maintained for a longer period in the case of *PgFADX* alone. In contrast, co-expression of *PgFAD2* with *PgFADX* resulted in a higher percentage of PuA at an earlier incubation time. Although the LA precursor of PuA greatly increased with combined expression of *PgFAD2* and *PgFADX*, the increase in the content of this precursor FA did not appear to be effective in sustaining relatively high levels of PuA beyond 3 days of incubation (Fig. 6c). Perhaps the introduction of recombinant *PgFAD2* resulted in the production of a limited pool of phosphatidylcholine which was accessible to recombinant *PgFADX*.

The difficulty to engineer heterologous organisms with large amounts of an unusual FA is mainly caused by their toxicity in membrane phospholipids (Holic et al. 2012; Yazawa et al. 2013a) and lack of efficient channeling of unusual FA from phospholipid to triacylglycerol (Mietkiewska et al. 2014; Yazawa et al. 2013a; Yazawa et al. 2014). Recently, we demonstrated that the lipotoxicity of accumulated FA can be decreased by their excretion into the growth medium (Sec et al. 2015; Yazawa et al. 2013b). Since the conjugated system of double bonds makes PuA more unstable compared to other non-conjugated polyunsaturated FAs, more effective channeling of PuA into triacylglycerol may be a useful strategy in future work to further boost the PuA content of yeast. Indeed, the storage capacity of lipid droplets is a factor which we have shown to influence the toxicity of accumulated lipid in cells (Valachovic et al. 2016). It would be particularly useful to implement species or strains of yeast capable of accumulating relatively high levels of triacylglycerol for this type of investigation. The lipid content in *S. pombe*, similar to *S. cerevisiae*, is generally not high (5–10% of dry cell weight) (Holic et al. 2012; Kamisaka et al. 2006). Recent studies have shown, however, that by optimization of growth conditions and by modification of metabolic pathways, a much higher lipid content (> 20%) can in fact be achieved using *S. cerevisiae* (Kamisaka et al. 2016; Kamisaka et al. 2013; Kamisaka et al. 2007). Thus, it may also be possible to engineer *S. pombe* to accumulate substantially more lipids. Moreover, the high oil yeast, *Yarrowia lipolytica*, has already been used to produce a range of specialized FA (Ledesma-Amaro and Nicaud 2016).

In conclusion, the heterologous expression of *PgFADX* in *S. pombe* resulted in yeast cells accumulating PuA up to 19.6% of total FAs. Co-expression of *PgFADX* with *PgFAD2* led to a further increase in PuA content up to 25.1% of total FAs, the highest level reported to date in a transgenic organism producing this FA.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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