Highlights:

- The punicic acid biosynthetic pathway was successfully integrated into the world's 2nd largest oilseed crop *Brasscia napus* (canola) via metabolic engineering.
- Seeds of the transgenic canola can accumulate 11% of total seed oil as punicic acid.
- Levels of punicic acid in the transgenic canola lines were stable over two generations without affecting seed germination and viability.

Punicic acid production in *Brassica napus*

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Abbreviations

ALA, α-linolenic acid; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FAD, fatty acid desaturase; FADX, fatty acid conjugase; FAME, fatty acid methyl ester; LA, linoleic acid; LR-NMR, low-resolution nuclear magnetic resonance spectroscopy; NCJD, binary vector containing *PgFADX* and *PgFAD2*; OA, oleic acid; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PL, polar lipids; PuA, punicic acid; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; TLC, thin layer chromatography; WT, wild-type.

Abstract:

Punicic acid (PuA; 18:3$\Delta^{9cis,11trans,13cis}$), a conjugated linolenic acid isomer bearing three conjugated double bonds, is associated with various health benefits and has potential for industrial use. The major nature source of this unusual fatty acid is pomegranate (*Punica granatum*) seed oil, which contains up to 80% (w/w) of its fatty acids as PuA. Pomegranate seed oil, however, is low yielding with unstable production and thus limits the supply of PuA. Metabolic engineering of established temperate oil crops for PuA production, therefore, has the potential to be a feasible strategy to overcome the limitations associated with sourcing PuA from pomegranate. In this study, the cDNAs encoding a pomegranate fatty acid conjugase and a pomegranate oleate desaturase were co-expressed in canola-type *Brassica napus*. Transgenic *B. napus* lines accumulated up to 11% (w/w) of the total fatty acids as PuA in the seed oil, which is the highest level of PuA reported in metabolically engineered oilseed crops so far. Levels of seed oil PuA were stable over two generations and had no negative effects on seed germination. The transgenic *B. napus* lines with the highest PuA levels contained multiple transgene insertions and the PuA content of *B. napus* seed oil was correlated with efficiency of oleic acid desaturation and linoleic acid conjugation. In addition, PuA accumulated at lower levels in polar lipids (5.0-6.9%) than triacylglycerol (7.5-10.6%), and more than 60% of triacylglycerol-associated PuA was present at the *sn*-2 position. This study provides the basis for the commercial production of PuA in transgenic oilseed crops and thus would open new prospects for the application of this unusual fatty acid in health and industry.

Keywords: Punicic acid; conjugated linolenic acid; fatty acid conjugase; fatty acid desaturase; oilseed crop; triacylglycerol

1. Introduction

Conjugated linolenic acids are fatty acids of 18-carbon chain length with three conjugated double bonds, which have great value in nutraceutical and industrial applications [for a review, see (Holic et al., 2018)]. These unusual fatty acids are naturally present as the major component of seed oil in several plant species, and the most commonly found isomers include punicic acid (PuA; 18: 3$\Delta^{9cis,11trans,13cis}$; Fig. 1A) from pomegranate (*Punica granatum*) and snake gourd (*Trichosanthes kirilowii*), α-eleostearic acid (18: 3$\Delta^{9cis,11trans,13trans}$) from tung (*Aleurites fordii*) and bitter gourd (*Momordica charantia*), calendic acid (18: 3$\Delta^{8trans,10trans,12cis}$) from marigold (*Calendula officinalis*), jacaric acid (18: 3$\Delta^{8cis,10trans,12cis}$) from jacaranda (*Jacaranda mimosifiola*), and catalpic acid (18:3$\Delta^{9trans,11trans,13cis}$) from catalpa (*Catalpa bignonoides* and *Catalpa ovata*) (Holic et al., 2018; Smith, 1971). PuA is the dominant fatty acid in pomegranate seed oil [66% in our sample analysis (Fig. 1B) and up to 80% according to Takagi and Itabashi (1981)] and has attracted considerable attention due to its various beneficial bioactivities, including anti-cancer, anti-obesity, anti-diabetes, hypolipidemic, and anti-inflammatory properties [for reviews, see (Aruna et al., 2016; Holic et al., 2018)]. In addition, PuA is susceptible to auto-oxidation and subsequent polymerization and thus can be used in the production of high-quality alkyd resins, paints, varnishes and polymers. PuA-enriched oils show faster drying rates and provide more resistance to water when compared to unconjugated drying oils, such as flax (*Linum usitatissimum*) or soybean (*Glycine max*) oil. Due to the structural similarity of PuA to α-eleostearic acid, it would be reasonable to assume that the quality of punicic acid-enriched oil would be similar to tung oil as a drying oil. The nutraceutical and industrial applications of PuA, however, remain largely unexplored due to the high cost and limited production of pomegranate seed oil. Although pomegranate and other plant species

naturally produce PuA, these plants, however, are not suitable to large-scale agronomic production for seed oils due to restricted cultivation conditions and low yields (Joh et al., 1995; Takagi and Itabashi, 1981). As a result, the retail price of pomegranate seed oil has been reported to be as high as up to $100,000 USD per metric tonne (Holic et al., 2018). In order to keep up with the rising demands, there is a growing interest in producing PuA in existing oilseed crops via metabolic engineering (Holic et al., 2018).

In developing seeds of oleaginous plants, production of fatty acids, including monounsaturated fatty acids, occurs in plastids. Fatty acids are then exported from plastids and activated to acyl-CoAs to serve as acyl donors in triacylglycerol (TAG) biosynthesis, which occurs in the endoplasmic reticulum (ER). In developing seeds producing oils containing polyunsaturated fatty acids (PUFAs), such as PuA, TAG biosynthesis involves a complex interplay between the *sn*-glycerol-3-phosphate pathway (Kennedy pathway; Kennedy, 1961) leading to TAG and membrane acyl lipid metabolism [for reviews, see (Chapman and Ohlrogge, 2012; Chen et al., 2015)]. In this metabolic interplay, PUFAs are formed at the *sn*-2 position of phosphatidylcholine (PC) in the ER and are then incorporated into TAG through various mechanisms involving acyl chain editing. As examples, PUFA may be transferred from PC to *sn*-1, 2-diacylglycerol (DAG) via the catalytic ation of phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000; Pan et al., 2013) or the reverse reaction catalyzed by lysophosphatidylcholine acyltransferase producing PUFA-CoA which in turn may serve as an acyl donor for diacylglycerol acyltransferase (DGAT) and possibly other acyltransferases of the Kennedy pathway (Lager et al., 2013; Pan et al., 2015; Stymne and Stobart, 1984). The conjugated double bonds in *sn*-2 PuA-PC are synthesized via the subsequent catalytic actions of fatty acid desaturase 2 (FAD2, Δ12-oleate desaturase) and fatty acid conjugase (FADX, a

divergent form of FAD2) (Cahoon et al., 1999), which catalyze the desaturation of oleic acid (OA, 18:1Δ^{cis9}) to form linoleic acid (LA, 18: 2$\Delta^{9cis,\ 12\ cis}$) and the conversion of the *cis*-Δ^{12} double bond of LA into *cis*-Δ^{11} and *trans*-Δ^{13} conjugated double bonds, respectively (Fig. 1A). (Hornung et al., 2002; Iwabuchi et al., 2003; Mietkiewska et al., 2014a).

cDNAs encoding functional FADXs have been isolated from *P. granatum* and *T. kirilowii*; the encoded enzymes are bifunctional enzymes with both conjugase and FAD2 activities (Hornung et al., 2002; Iwabuchi et al., 2003). Proof-of-concept production of PuA in transgenic plants has been reported in the model plant *Arabidopsis thaliana* (hereafter Arabidopsis). Over-expression of *P. granatum FADX* (*PgFADX*) or *T. kirilowii FADX* (*TkFADX*) in Arabidopsis resulted in modest accumulation of PuA in the total acyl lipids of seed oil at levels up to 4.4% (w/w) and 10.2% (w/w), respectively (Iwabuchi et al., 2003). The low PuA accumulation appears to result from the poor availability of substrates for FADX (Mietkiewska et al., 2014b). Indeed, wild-type (WT) Arabidopsis seeds only contain less than 27% of total fatty acids as LA, not all of the 27% LA can be recruited as substrates for FADX due to the competition from the endogenous FAD3, which catalyzes the conversion of LA to α-linolenic acid (ALA, 18:3$\Delta^{9cis,12\ cis,15cis}$). To address this issue, we previously co-expressed *PgFADX* and *P. granatum FAD2* in an Arabidopsis *fad3/fae1* mutant, which accumulates high amount of LA (>50% of the total fatty acids) due to the lack of FAD3 and fatty acid elongase 1 activities (Smith et al., 2003). The PuA content in the resulting transgenic lines reached up to 21% (Mietkiewska et al., 2014b).

Efforts to produce PuA in oilseed crops have been much more limited; up to now, there has only been one report about producing PuA in canola-type *Brassica napus*, but the PuA content was only 2.5% (Koba et al., 2007). Based on our previous study in Arabidopsis, here, we further explored the production of PuA in a double haploid canola-type *B. napus* line via metabolic

engineering. The combined expression of *PgFADX* and *PgFAD2* in *B. napus* resulted in up to 11% PuA accumulation of the total acyl lipids of seed oil. PuA levels in transgenic canola lines were stable over T_2 and T_3 generations and there were no observed negative effects on seed germination. In addition, the relationship of PuA production and transgene insertions/expression levels, OA desaturation and LA conjugation, as well as PuA distribution in polar lipids (PL), TAG and DAG, were also analyzed. These analyses provided valuable information for further improving PuA content in *B. napus* or other oil crops

2. **Material and methods**

2.1. Construct preparation, plant transformation, and plant growth

The binary vector containing *PgFADX* and *PgFAD2* (designated NCJD construct; Fig. 1C) used for canola expression was previously constructed by our group (Mietkiewska et al., 2014b). In brief, the cDNAs encoding *PgFADX* and *PgFAD2* were cloned down-stream of the seed specific *Napin* promoter and up-stream of the *NOS* transcriptional terminator, respectively. Subsequently, *Napin*: *PgFADX*: *NOS* and *Napin*: *PgFAD2*: *NOS* DNA fragments were inserted into the pRD400 vector to generate the binary vector NCJD (Datla et al., 1992). The resulting NCJD construct was then introduced into *Agrobacterium tumefaciens* strain GV3101 via electroporation.

A double haploid canola-type *B. napus* line (DH12075, provided by D. Lydiate, Agriculture Agri-Food Canada, Saskatoon, Saskatchewan, Canada) was transformed with the vector NCJD using the method described previously (Bondaruk et al., 2007). It should be noted that double haploid lines are routinely used in *B. napus* breeding (Möllers and Iqbal, 2009). More

specifically, DH12075 has been used in genomic studies, genetic engineering and breeding (Lock et al., 2009; Snowdon and Iniguez Luy, 2012; Taylor et al., 2009; Yu et al., 2012). The transformed calluses were selected on growth media containing kanamycin (20 mg/L). The binary vector was designed to carry an *NPT II* selection marker gene in tandem with the *Napin*: *PgFADX*: *NOS* - *Napin*: *PgFAD2*: *NOS* transgene cassette, and thus the successful transgenic callus could survive kanamycin selection. Benzyladenine (4.5 mg/L) and naphthalene acetic acid (0.1 mg/L) were then added to induce shoot and root formation, respectively. When roots were established, these T_0 transgenic canola were transferred to soil and cultivated in the greenhouse.

All *B. napus* plants, including T_0, T_1 and T_2 transgenic lines which produce T_1, T_2 and T_3 seeds, respectively, WT lines, and null segregated lines, were grown in growth chambers with the following parameters: 16 h day/8 h night cycle, 25/20ºC day/night temperature, 60% relative humidity, and 250 μmol m^{-2} s^{-1} light intensity.

2.2. Embryo assay

Embryo assay was performed to check the expression of the *NPT II* selection marker gene as described previously (Bondaruk et al., 2007). The embryo assay is used to confirm the presence of the transgene cassette in the transgenic plants and for segregation analysis to estimate the number of transgenic loci. Segregation analyses were performed on T_1 seeds by screening on kanamycin (50 mg/L) containing growth media. Chopped embryos of T_1 canola seeds were grown on growth media containing kanamycin for callusing, and the kanamycin resistant seeds carrying the transgene grow dark green while the non-transgenic seed grow pale due to the sensitivity to kanamycin.

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157 2.3. Quantitative RT-PCR analysis

158 Gene expression level and estimated number of transgene copies were analyzed using
159 quantitative RT-PCR (qPCR) on a StepOnePlus Real-Time PCR System (Applied Biosystems,
160 USA) using the Platinum SYBR Green qPCR Master Mix (Invitrogen) as described previously
161 (Xu et al., 2017).

162 The number of transgene copies was estimated using the standard curve method. In brief,
163 total genomic DNA was extracted from the mature canola seeds using TRIzol reagent
164 (Invitrogen) according to the manufacturers' instructions. The copy number of transgene was
165 quantified by targeting a section of the *PgFADX* gene (with primers 5'-
166 AGATATTCAACTTGAGAGAGCG-3' and 5'-GGCTAGCCGGTAGAGGATGT-3') and
167 comparing to a genomic target of the single copy *BnHMGI/Y* gene (NCBI accession number:
168 AF127919; with primers 5'-GGTCGTCCTCCTAAGGCGAAAG-3' and 5'-
169 CTTCTTCGGCGGTCGTCCAC-3'). The coding sequence of *BnHMGI/Y* gene was cloned into
170 the pRD400 binary vector (designated HMG construct) and a serial dilution of the plasmid
171 mixtures containing *HMG* and *NCJD* constructs at 1:1 ratio was used to generate standard curves
172 for both *PgFADX* and *BnHMGI/Y*.

173 The expression level of *PgFADX* in different transgenic *B. napus* lines was analyzed
174 using the comparative Ct method ($2^{-\Delta\Delta Ct}$ method). In brief, total RNA was isolated from canola
175 developing embryos at the 10 day after pollination using TRIzol reagent (Invitrogen) according
176 to the manufacturers' instructions. The extracted RNA was further treated with the TURBO
177 DNA-free Kit (Invitrogen) and was used to synthesize first-strand cDNA using the SuperScript
178 IV first-strand cDNA synthesis kit (Invitrogen). The relative expression levels of *PgFADX* (with

179 the primers described above for copy number analysis) was normalized to the expression of an
180 internal reference gene *UBC21* (with primers 5'-CCTCTGCAGCCTCCTCAAGT-3' and 5'-
181 CATATCTCCCCTGTCTTGAAATGC-3') as previously described (Chen et al., 2010) and
182 cDNA extracted from *B. napus* developing embryos NCJD-15A was used as a calibrator to
183 normalize for plate-to-plate variation.

184

185 2.4. Lipid content analysis of mature *B. napus* seeds

186 Total lipid content of mature *B. napus* seeds was analyzed by low-resolution nuclear magnetic
187 resonance spectroscopy (LR-NMR) as described previously (Taylor et al., 2009; Weselake et al.,
188 2008). For each sample, approximately 5 ~ 6 g *B. napus* seeds were weighed out and transferred
189 into a 16×150 mm test tube to reach a height of 4 cm for NMR analysis. The total lipid content
190 of each sample was determined using a Bruker Minispec mq20 instrument (Bruker Optik GmbH,
191 76275 Ettlingen, Germany), which was calibrated with mature canola seed of known oil content
192 (obtained from the Grain Research Laboratory of the Canadian Grain Commission, Winnipeg,
193 MB, Canada).

194

195 2.5. Lipid extraction from *B. napus* and *P. granatum* seeds

196 *P. granatum* seeds were obtained from *P. granatum* fruits purchased in local market (Edmonton,
197 Canada). Total lipid extraction from *B. napus* and *P. granatum* seeds was performed as described
198 previously (Weselake et al., 2008). Seeds were homogenized at 30 000 rpm using a Fisher
199 Scientific Power Gen 1000 homogenizer (Fisher Scientific, Pittsburgh, PA) in a 10 mL screw cap
200 glass tube with 3 mL of chloroform: isopropanol (2:1, v/v). After homogenization, another 3 mL

of chloroform: isopropanol (2:1, v/v) and 1 mL of 0.9% NaCl solution were added to the mixture. After vortexing for 1 min, the organic phase (bottom phase) from each sample was separated from the aqueous phase by centrifugation and the samples were re-extracted with 4 mL of chloroform: isopropanol (2:1, v/v). The organic phases of both extractions were combined and evaporated under nitrogen. The total lipid extracts were directly trans-methylated for fatty acid analysis as described below or dissolved in 100 µL of chloroform for further analysis.

2.6. Triacylglycerol, diacylglycerol and polar lipid analysis

The separation of individual lipid classes was performed as described previously (Mietkiewska et al., 2014b). In brief, the total lipid extracts from 6 *B. napus* seeds were separated on thin layer chromatography (TLC) plate (0.25 mm Silica gel, 216 DC-Fertigplatten, Macherey-Nagel, Germany) using hexane/diethyl ether/acetic acid (80:20:1 or 70:30:1, v/v/v). Lipid bands were visualized under UV light after spraying with 0.05% primulin solution. The corresponding TAG, DAG and PL bands were then scraped into screw cap tubes for trans-methylation or positional analysis.

2.7. Positional analysis of triacylglycerol and diacylglycerol

Fatty acid distribution between *sn*-2 and *sn*-1/3 TAG and fatty acid composition of the *sn*-2 position of *sn*-1,2-DAG were analyzed using the method of Luddy et al. (1964). TAG or DAG was first recovered from the silica gel by extracting with 4 mL of diethyl ether, and then was transferred to a new screw cap tube and dried under nitrogen. One milliliter of 1 mM Tris-HCl buffer (pH 8.0), 100 µL of 2.2% $CaCl_2$ and 250 µL of 0.1% deoxycholate were added to each

TAG or DAG sample, and the mixture was sonicated for 60 s to emulsify the lipid. The mixture was incubated in a water bath at 40°C for 30 s, and then 20 mg pancreatic lipase (pancreatic lipase type II, Sigma) were added to initiate hydrolysis. The mixture was further incubated for 3 min at 40°C, and the reaction was terminated by adding 500 µL of 6 M HCl. The lipids were then extracted twice with 2.5 mL of diethyl ether, and the extracts were combined and concentrated under nitrogen. The lipids samples were separated on a silica gel coated TLC plate with hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The *sn*-2 MAG was visualized with 0.05% primulin solution under UV light based on the migration of the 2-monolein standard (Sigma) and then scraped into screw cap tubes for trans-methylation. Individual fatty acid proportion at the *sn*-2 position of TAG was calculated as:

$$\text{Proportion of individual fatty acid at the } sn-2 \text{ position of TAG (\%)} = \frac{\text{Proportion at the } sn-2 \text{ position of TAG}}{\text{Proportion in TAG } \times 3} \times 100$$

2.8. Positional analysis of polar lipid

Positional analysis of PL was performed by cleaving the fatty acids at the *sn*-1 position of PL using phospholipase A_1 according to Vikbjerg et al. (2006) with modifications. In brief, PL was recovered from the silica gel by extracting with 4 mL of diethyl ether, and then was transferred to a new screw cap tube and dried under nitrogen. The extracted PL was dissolved in 2 mL of diethyl ether and then was mixed with 200 µL of Lecitase ultra (Sigma) dissolved in 800 µL of water. The mixture was then vortexed at maximum speed for 5 min and the reaction was terminated by evaporation of diethyl ether under nitrogen. The hydrolyzed lipids were extracted with chloroform: methanol (2:1, v/v) and applied to the TLC plates for separation using

chloroform: methanol: water (65:35:5, v/v/v). The cleaved fatty acids were visualized with 0.05% primulin solution under UV light and then scraped into screw cap tubes for trans-methylation. Individual fatty acid proportion at the *sn*-2 position of PL was calculated as:

$$\text{Proportion of individual fatty acid at the } sn-2 \text{ position of PL (\%)} = 100 - \frac{\text{Proportion at the } sn-1 \text{ position of PL}}{\text{Proportion in PL } \times 2} \times 100$$

2.9. Fatty acid analysis using gas chromatography-mass spectrometry

Fatty acid analysis was performed as described previously (Mietkiewska et al., 2014b). The lipid samples were trans-methylated in screw-cap glass tubes with 1 mL of 5% sodium methoxide in methanol for 30 min at room temperature. The resulting fatty acid methyl esters (FAMEs) were extracted twice with hexane and dried under nitrogen. The FAMEs were then resuspended with 200 µL or 1 mL of iso-octane and then analyzed on an Agilent 6890N Gas Chromatograph equipped with a 5975 inert XL Mass Selective Detector (Agilent Technologies). The FAMEs were separated on a capillary column DB 23 (30 m×0.25 mm×0.25 µm, Agilent Technologies, Wilmington, DE, USA) using the following temperature program: 165 °C for 4 min, increased to 180 °C at 10 °C/min and held for 5 min, and increased to 230 °C at 10 °C/min and held for 5 min.

2.10. Statistical analysis

Statistical analysis was carried out using the SPSS statistical package (SPSS 16.0, Chicago, IL, U.S.A.). The Pearson correlation test was performed to test for significant correlations.

3. Results

3.1. Overexpression of *PgFADX* and *PgFAD2* in *B. napus* led to accumulation of punicic acid in T_1 segregating seeds

An earlier attempt to produce PuA in canola by over-expressing *TkFADX* met with limited success, with only 2.5% of PuA content in the seed oil (Koba et al., 2007). A possible reason for the low PuA accumulation is the limited level of LA (20%) in *B. napus* seed oil (Fig. 1B); LA is the substrate for FADX to produce PuA (Fig. 1A). In the current study, *PgFAD2* was co-expressed with *PgFADX* in canola under seed specific promoters to enhance LA availability for FADX (Fig. 1C) and thus to improve the PuA production. Kanamycin resistant T_0 canola plants were self-pollinated and were grown to seed maturity. The mature T_1 seeds were collected and the extracted oil was subjected to fatty acid composition analysis and estimation of transgene copy number using segregation and qPCR analyses (Table 1 and Fig. 2).

Co-expression of *PgFADX* and *PgFAD2* in *B. napus* resulted in accumulation of PuA in total acyl lipids from seed oil, ranging from 2.3% to 6.2% (six-seed sample) with an average of 4.86% in the selected transgenic lines. No PuA was detected in oils from the WT control lines (Table 1). When compared to the control lines, the increased PuA content in the transgenic lines was concomitantly accompanied by decreases in OA and ALA and an increase in LA (Table 1). It should also be noted, however, that the seed oils of individual transgenic plants contained broad ranges of PuA due to the presence of segregating seeds (Fig. 2). For instance, the PuA content ranged from 3.3% to 10.1% in the NCJD-13 segregating seeds (Fig. 2). Furthermore, PuA content was correlated with the copy number of the transgene insert in *B. napus*. The transgenic lines containing PuA at high levels (6.4%-8.5%), such as NCJD-3, 7, 11, 13, and 15A,

had two or more transgene inserts, whereas the two transgenic lines (NCJD-4 and 5B) with low levels of PuA (2-3%) had a single transgene insert (Fig. 2).

3.2. Punicic acid content was correlated to the efficiency of oleic acid desaturation and linoleic acid conjugation

Homozygous T_1 plant with single transgene insert or homozygous/heterozygous T_1 plants with multiple transgene inserts were grown alongside with null segregant T_1 plants for further study. Oils from the mature T_2 seeds from 33 individual transgenic lines and three null segregant lines were harvested and subjected to fatty acid composition analysis. The transgenic lines accumulated PuA in seed at levels ranging from 0.8%-9.4% (Fig. 3A). Moreover, the increased PuA content in the transgenic lines was accompanied by an increase in LA content and a decrease in OA content (Fig. 3A), which is likely due to enhanced FAD2 activity resulting from *PgFAD2* co-expression.

The cumulative effects of FAD2 activities from endogenous *B. napus* FAD2 and exogenous PgFAD2 in the transgenic plants were then assessed by calculating the efficiency of OA desaturation with the method being described by Singh et al. (2001), which represents the ratio of the OA desaturation products (i.e., LA, ALA and PuA) to the total amount of available OA substrate (i.e., OA desaturation products and the remaining OA). The null segregated *B. napus* lines exhibited a ratio of around 0.2-0.35, indicating that about 20-35% of OA was further desaturated. The transgenic lines co-expressing *PgFAD2* with *PgFADX* exhibited an increased OA desaturation efficiency, wherein up to 50% of OA was further converted (Fig. 3B). PuA content was positively correlated to the OA desaturation efficiency in the transgenic plants

311 (R^2=0.53, P<0.001), indicating that the accumulation of PuA could be enhanced by improving
312 the availability of LA substrate for FADX.

313 The efficiency of LA conjugation was also evaluated using a similar method by
314 calculating the ratio of the LA conjugation product (i.e., PuA) to the total amount of available
315 LA substrate (i.e., PuA, ALA and remaining LA). As expected, a strong positive correlation
316 (R^2=0.97, P<0.001) between PuA content and LA conjugation efficiency was observed (Fig. 3C).
317 Furthermore, the increased PuA content was correlated with a concomitant reduction in ALA
318 (Fig. 3A) suggesting the presence of a potential competition for LA precursor between the
319 exogenous PgFADX and endogenous *B. napus* FAD3. Indeed, a negative correlation (R^2=0.61,
320 P<0.001) was observed between the contents of PuA and ALA (Fig. 3D). Despite the dynamic
321 changes in the proportions of different C18 unsaturated fatty acids (i.e., OA, LA, ALA and PuA),
322 the total amount of all C18 unsaturated fatty acids in the transgenic lines remained at a stable
323 level of 85-90% of total fatty acids (Fig. 3A).

324

325 3.3. Relationship between punicic acid content and seed oil content

326 The oil contents of T_2 seeds from individual transgenic canola lines, null segregant lines and WT
327 control lines were analyzed using LR-NMR. The average oil content of the 9 null segregant lines
328 and the 9 WT lines was 42.4% ± 7.1% (±SD) and 42.4% ± 3.6% (±SD), respectively, whereas an
329 average oil content of 39.3% ± 6.0% (±SD) was observed from the 33 transgenic lines (Fig. 4A
330 and Supplemental Table S1). Individual transgenic lines and null segregant lines showed large
331 fluctuations in the oil content, ranging from 29.4% to 51.0% and from 31.7% to 51.8%,
332 respectively (Fig. 4B). Whether the altered oil content is directly correlated with PuA content,
333 however, is unclear, though there was a weak negative correlation between oil content and PuA

content (R^2= 0.27, P<0.001, Fig. 4B) in support of some possible effect. Indeed, the NCJD-15A-11 line that accumulated the highest level of PuA (9.4%) had considerably reduced oil content compared to the controls, whereas two other high PuA-accumulating lines, NCJD-11-6 (PuA content, 6.6%) and NCJD-11-7 (PuA content, 7.2%), exhibited no decrease in oil content (Fig. 4C). In addition, considerable reductions in oil content (10-20%) were also found in two null segregant line and two transgenic lines NCJD-5B-6 and NCJD-5B-11 that accumulated low levels of PuA (~2%, Fig. 4C).

3.4. The punicic acid trait is stable over two generations

The T_2 seeds of the transgenic line NCJD-15A-11 with the highest level of PuA (9.4%, Fig. 4C) was grown to T_3 generation to assess the trait stability over two generations. As shown in Fig. 5, PuA levels in the T_3 seeds of the transgenic lines were around 7% to 11%, with the highest PuA content accounting for up to 11.1% of total fatty acids on average from six seeds in the best transgenic line NCJD-15A-11-10. These results are consistent with the PuA content of oils from single T_2 transgenic seeds (NCJD-15A-11) ranging from 6% to 11% (Fig. 5). Thus, the observed PuA content was stable from the second to the third generation, although the PuA content in the T_2 segregating lines had large fluctuations due to the presence of multiple transgene inserts (Figs. 2 and 5 and Supplemental Fig. S1). It was estimated that three and two transgene copies were present in the T_1 seeds of NCJD-15A and T_1 seeds of NCJD-15A-11, respectively, whereas the relative expression levels of transgene in the developing seeds of both generations appeared to be comparable (Supplemental Fig. S1). Furthermore, the accumulation of PuA in transgenic *B. napus* seeds had no visually negative effects on seed germination and viability, and the resulting plants were indistinguishable from the WT plants.

357

358 3.5. Distribution of punicic acid in polar and neutral lipids

359 To examine the distribution of PuA in polar and neutral lipids, total lipids were extracted from
360 mature seeds of WT *B. napus* plants and mature T_3 seeds of three transgenic lines (NCJD-15A-
361 11-1, NCJD-15A-11-2, and NCJD-15A-10). The total lipids were then separated into PL, DAG
362 and TAG fractions on a TLC plate for fatty acid composition analysis (Fig. 6). The PL fraction
363 contained 5.0-6.9% PuA, which was lower than levels in the TAG fraction (7.5-10.6% PuA) but
364 higher than levels in the DAG fraction (4.6-5.1% PuA, Fig. 6A, D and G and Supplemental
365 Table S2). The increase in PuA content in the PL, TAG and DAG fractions of the transgenic
366 lines was accompanied by decreases in OA and ALA, with an increase in LA in all fractions.
367 Furthermore, the ratio of LA content in PL to TAG was reduced from 2-fold in the WT control
368 line to 1.4-fold in the transgenic lines suggesting the effective conversion of LA to PuA in PL
369 catalyzed by FADX.

370 Positional analysis of TAG in the WT and transgenic *B. napus* lines showed the dominant
371 presence of C18 unsaturated fatty acids including OA, LA, ALA, and/or PuA at the *sn*-2 position
372 of TAG, whereas no or neglectable saturated fatty acid was present at that position (Fig. 6B and
373 Supplemental Table S2). In the WT *B. napus*, the *sn*-2 position of TAG was composed of 59.6%
374 OA, 23.7% LA and 16.4% ALA. Upon *PgFADX* and *PgFAD2* co-expression, OA and ALA at
375 the *sn*-2 position of TAG were decreased around 1.3-fold and 10-fold to ~44% and ~1.6%,
376 respectively, with substantial increases in PuA and LA accumulating up to 17.5% and 39.1%,
377 respectively (Fig. 6B). In addition, up to 60% of the PuA in the seed TAG from the transgenic *B.*
378 *napus* lines was located at the *sn*-2 position of TAG with the remaining 40% at the *sn*-1 and *sn*-3
379 positions collectively (Fig. 6C). By contrast, ~30% of the OA in TAG from the seeds of

transgenic and WT canola lines was found at the *sn*-2 position of TAG, suggesting a more even distribution of OA in TAG. Positional analysis of PL indicated that OA and LA were the major fatty acids occupying the *sn*-1 position of PL in the WT and transgenic *B. napus* lines, the latter of which are estimated to accumulate around 5-10% of PuA at the *sn*-2 position of PL suggesting up to 50-74% of PuA in the PL being located at that position (Fig. 6E and F; Supplemental Table S2). PuA accounted for 4.6-5.1% of the total fatty acids in the DAG, while the PuA content at the *sn*-2 position of *sn*-1,2-DAG was around 4.3-6.0% (Fig. 6G and H; Supplemental Table S2), suggesting a considerable amount of PuA was retained at the *sn*-2 position of the *sn*-1,2-DAG.

4. Discussion

PuA-enriched seed oils have great potential for use as nutraceuticals and in industrial applications (Holic et al., 2018; Transparency Market Research, 2018). The supply of PuA-enriched oils, however, is limited as they are exclusively extracted from pomegranate seeds which are not readily available. Therefore, it is attractive to develop viable alternative sources of PuA. Here, we demonstrate the metabolic engineering of canola-type *B. napus* to produce oil containing a substantial level of PuA. By introducing *PgFADX* and *PgFAD2* from pomegranate, resulting transgenic *B. napus* lines accumulated PuA up to 11% of the total acyl lipids of seed oil. It is interesting to point out that canola-type *B. napus* represents a type of *B. napus* wherein the gene encoding elongase was mutated and knocked out through the process of breeding to eliminate formation of erucic acid ($22{:}1\Delta^{13cis}$) thereby resulting in higher levels of OA (Fig. 1B; Katavic et al., 2002; Weselake, 2011). Essentially, this would provide more substrate for FAD2 which acts on *sn*-2 OA-PC to produce LA-PC which in turn is the substrate of FADX to fuel *sn*-2 PuA-PC production. This mutation in canola-type *B. napus* would be equivalent to the *fae1*

403 mutation in Arabidopsis resulting in reduced fatty acid elongation (Mietkiewska et al., 2014b;
404 Smith et al., 2003).

405 The strategy for metabolic engineering of *B. napus* by co-expressing *PgFADX* and
406 *PgFAD2* resulted in 9-11% of PuA in T_2 and T_3 seeds (Figs. 3 and 5) suggesting that the LA
407 level in canola seeds might be one of the major factors affecting the production of PuA in the
408 transgenic lines. Indeed, co-expressing *PgFAD2* resulted in up to 30% LA content in the
409 transgenic canola seeds (Fig. 3A), which was two-fold higher than the LA content (~15%)
410 observed in the *TkFADX*-expressing *B. napus* (cv. Westar) producing 2.5% of PuA in seed oil
411 (Koba et al., 2007). Moreover, the PuA content of transgenic *B. napus* lines was correlated with
412 the OA desaturation efficiency (Fig. 3B). It appears that the enhanced activity of FAD2,
413 especially FAD2 from pomegranate, was efficient in providing LA substrate for FADX, thus
414 boosting the production of PuA in our transgenic *B. napus* lines. Similar observations of
415 increased LA availability leading to increased production of LA-derived fatty acids were also
416 reported for α-eleostearic, calendic acid and vernolic acid (12,13-epoxy-18:1Δ^{9cis}) (Cahoon et al.,
417 2006; Zhou et al., 2006). Furthermore, increased PuA was correlated with increased LA
418 conjugation efficiency and decreased ALA content (Fig. 3C and D), suggesting that the
419 competition between exogenous PgFADX and endogenous *B. napus* FAD3 for LA substrate also
420 affected the extent of PuA accumulation. PgFADX appears to be more effective in utilizing *sn*-2
421 LA-PC than *B. napus* FAD3 (Fig. 3D). A specific substrate channeling process may be operative
422 between PgFAD2 and PgFADX which enables the rapid conversion of OA to PuA via LA. It is
423 also possible that PgFAD2 and PgFADX co-localize or even physically interact with each other
424 to facilitate this process. Indeed, plant FAD2 and FAD3 have been shown to physically interact
425 and participate in substrate channeling in the conversion of *sn*-2 OA-PC to *sn*-2 ALA-PC (Lou et

al., 2014). Similarly, the importance of LA availability in affecting the production of PuA in the transgenic lines has also been observed from our previous proof-of-concept work on PuA production using an Arabidopsis *fad3/fae1* mutant with a high LA (>50%) background in which up to 21% of PuA was achieved by co-expressing *PgFADX* and *PgFAD2* (Mietkiewska et al., 2014b). Therefore, it may be worthwhile to further improve FAD2 action to enhance LA availability in the transgenic *B. napus* lines by introducing more transgene copies of *FAD2*. Regardless of the fact that PgFADX seems to be more effective in acting on LA than *B. napus* FAD3 (Fig. 3D), it might still be useful to reduce the potential competition between PgFADX and *B. napus* FAD3 for LA by knocking out the endogenous *B. napus FAD3* using CRISPR Cas 9 (Bortesi and Fischer, 2015; Okuzaki et al., 2018; Subedi et al., 2020) or crossing with *Brassica FAD3* knocked out mutant lines (Rahman et al., 2013).

It should be pointed out that the best transgenic *B. napus* line contains multiple transgene insertions (Fig. 5). While expression of multiple copies of a transgene may sometimes be unstable due to gene silencing and genetic segregation (Thompson and Reddy, 2008), our results showed that the PuA trait of the best transgenic canola lines did not show effects of gene silencing and was stable over two generations (Fig. 5). Similar observations have been reported in transgenic canola lines producing “no sat” oil (saturated fatty acid content <3.5%), where plants comprising multiple transgene copies actually had improved traits (Thompson and Reddy, 2008). In addition, the high gene dosage/expression levels may be crucial for the high production of PuA in canola, which is consistent with the production of other unusual fatty acids in transgenic plants, such as docosahexaenoic acid (DHA) and γ-linolenic acid (Kim et al., 2016; Petrie et al., 2012). Nevertheless, since multiple gene insertions may be unstable over time and thus a concern for commercial growth, it would be interesting to further evaluate the stability and

449 applicable potential of our transgenic canola lines by further analyzing the insertions and testing
450 the canola lines in field trial in a follow-up study.

451 The best transgenic *B. napus* line produced up to 11% PuA in the seed oil, but the level of
452 PuA is still much lower than that in the seed oil of *P. granatum* (up to 80 % w/w) or *T. kirilowii*
453 (40 % w/w). Further analysis of fatty acid composition in lipid classes provided some insight into
454 possible metabolic constraints which limited PuA production engineered *B. napus*. In the
455 transgenic *B. napus* lines, similar proportions of PuA (6-8%) were found in TAG and PL
456 fractions (Fig. 6A and D). In contrast, recent analysis of *P. granatum* seed oil indicated that PuA
457 accounts for 60% of the total fatty acids in TAG and only 0.8% of fatty acids in PC
458 (Mietkiewska et al., 2014b). These results suggest that an efficient mechanism of PuA trafficking
459 from the site of synthesis (*sn*-2 position of PC) to TAG, which has evolved in developing *P.*
460 *granatum* seeds, is missing in developing seeds of transgenic *B. napus*.

461 The inefficient trafficking of fatty acids produced at the level of PC, such as hydroxy,
462 epoxy, or conjugated fatty acids, to TAG represent a major obstacle in generating transgenic
463 plants which produce seed oils highly enriched in theses fatty acids (Cahoon et al., 2006; Holic et
464 al., 2018; Napier, 2007; Napier et al., 2014; Singh et al., 2001). Further increases in the
465 transgenic production of modified fatty acids may benefit from the identification and subsequent
466 introduction of the native acyl-trafficking enzymes. Indeed, transgenic production of modified
467 fatty acids such as hydroxy fatty acids and ALA has met with encouraging success by co-
468 expressing cDNAs encoding specialized acyl-editing enzymes, including DGAT, PDAT, and
469 phosphatidylcholine:diacylglycerol cholinephosphotransferase (Burgal et al., 2008; Pan et al.,
470 2013; van Erp et al., 2011; Wickramarathna et al., 2015). To further increase PuA production in
471 *B. napus*, co-expression of *PgPDAT* with *PgFAD2* and *PgFADX* may represent a robust strategy,

especially considering that up to 60% of the PuA in TAG from the transgenic *B. napus* lines was located at the *sn*-2 position (Fig. 6C). Indeed, PDAT action could target PuA to the *sn*-3 position of TAG, which may potentially drive more PuA moieties to TAG. It may also worthwhile co-expressing *PgDGAT* as well, since over-expression of *DGAT* has been shown to boost seed oil production in various plants species (Xu et al., 2018). Although no strong correlation was observed between the PuA content and seed oil content in T_2 *B. napus* seeds, one of the best transgenic T_2 lines exhibited only a slight reduction in seed oil content (Fig. 4). To take advantage of this observation in future metabolic engineering and breeding work, it may be useful to specifically select high PuA lines where seed oil content is not compromised.

In the current study, the total content of OA and its desaturation products (LA, ALA and PuA) was consistent in the range of 85-90%, whereas 16:0, 18:0, 18:1Δ^{11cis} and 20:1Δ^{11cis}, accounted for the remaining 10-15% of the total fatty acids, in individual transgenic and WT *B. napus* lines (Fig. 3A). It should be noted that although co-expression of *PgFAD2* led to considerable increase in the conversion of OA to LA (from 15% to 30%) in the transgenic *B. napus* lines (Fig. 3), the remaining OA content in the transgenic *B. napus* lines was maintained as high as 50% (Fig. 3). One possibility is that a large proportion of OA synthesized in the plastid is directly utilized as oleoyl-CoA in TAG assembly through the Kennedy pathway, instead of being incorporated on the *sn-2* position of PC for the biosynthesis of LA, the precursor of PuA. From this perspective, increasing the biosynthesis of LA in the transgenic *B. napus* lines may further increase PuA content in seed oil. Possible strategies may include facilitating the conversion of 16:0 and 18:0 to OA to increase OA content, improving the efficiency of OA incorporation on the *sn*-2 position of PC, and increasing the reaction efficiency of *sn*-2 OA-PC desaturation to increase LA content in seeds.

The even distribution of PuA between TAG and PL observed in the current study (Fig. 6A and D) is also different from that observed in Arabidopsis *fad3/fae1* co-expressing *PgFAD2* and *PgFADX*, in which a higher relative proportion of PuA was found in PL rather than TAG (Mietkiewska et al., 2014b). These results suggest that *B. napus* is more efficient in trafficking PuA from PC to TAG than Arabidopsis. Moreover, up to 60% and 50-74% of PuA in seed TAG and PL from *B. napus* transgenic lines were detected at the *sn*-2 position of TAG and PL, respectively (Fig. 6B,C, E and F; Supplemental Table S2). Thus, a large portion of PuA synthesized at the *sn*-2 position of PC remained at the same position upon incorporation into TAG. One possibility to account for enrichment of PuA at the *sn*-2 position of TAG would be through phospholipase C action, which would remove the phosphocholine headgroup from PC producing *sn*-1,2-DAG enriched in PuA at the *sn*-2 position. Alternatively, PuA may be cleaved from the *sn*-2 position of PC by the catalytic action of phospholipase A_2, and then activated to acyl-CoA by the catalytic action of long-chain acyl-CoA synthetase before re-incorporation onto the *sn*-2 position of TAG by the catalytic action of lysophosphatidic acid acyltransferase. Indeed, it has been suggested that *B. napus* lysophosphatidic acid acyltransferase displays preference towards substrates containing C18 unsaturated fatty acids but discriminates against saturated acyl-CoA (Brown et al., 2002), which is supported by our observation of no or neglectable saturated fatty acids present on the *sn*-2 position of TAG (Fig. 6B and C). Considering a substantial amount of PuA was retained at the *sn*-2 position of the *sn*-1,2-DAG (Fig. 6G and H; Supplemental Table S2), phospholipase C appears to actively act in the *B. napus* transgenic lines.

It should be noted that the considerable enrichment of PuA at the *sn*-2 position of TAG from transgenic *B. napus* seed may have implications in terms of the potential efficacy of this oil. Indeed, numerous feeding studies with mammals have demonstrated that the absorption of long

chain fatty acids at the *sn*-2 position of TAG are favored over those at the *sn*-1 and *sn*-3 positions (Hunter, 2001; Ramírez et al., 2001). *sn*-2 MAG, resulting from the digestion of dietary TAG in the small intestine, is used to re-synthesize TAG in the intestinal mucosa thus influencing the fatty acid composition of TAGs in chylomicrons which act in lipid delivery in the bloodstream.

In summary, metabolic engineering of *B. napus* with the co-expression of *PgFAD2* and *PgFADX* resulted in the accumulation of PuA up to 11% of total acyl lipids in seed oil. PuA was found in both PL and TAG fractions of seed oil with 60% of this fatty acid present in the *sn*-2 position of TAG. This is the highest reported PuA content for a seed oil from a transgenic crop to date. The PuA content of seed oil from T_2 and T_3 generations was stable and there were no negative effects on seed germination and viability. Therefore, canola-type *B. napus* shows great promise for producing a PuA-enriched seed oil for both nutraceutical and industrial applications.

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Author contributions

R.J.W. conceived the project; G.C. and R.J.W. supervised the experiments; E.M. and S.S. performed the canola transformation, total lipid analysis and plant characterization; Y.X. performed the qRT-PCR analysis, and analyses of total lipid, lipid classes and positional distribution; Y.X. and E.M. analyzed the data; Y.X., G.C. and R.J.W. wrote the manuscript with the contributions of all the authors.

Conflict of interest

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

References:

Aruna, P., Venkataramanamma, D., Singh, A.K., Singh, R., 2016. Health benefits of punicic acid: A review. Compr. Rev. Food Sci. Food Saf. 15, 16–27. doi:10.1111/1541-4337.12171

Bortesi, L., Fischer, R., 2015. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. 33, 41–52. doi: 10.1016/j.biotechadv.2014.12.006

Bondaruk, M., Johnson, S., Degafu, A., Boora, P., Bilodeau, P., Morris, J., Wiehler, W., Foroud, N., Weselake, R., Shah, S., 2007. Expression of a cDNA encoding palmitoyl-acyl carrier protein desaturase from cat's claw (*Doxantha unguis-cati* L.) in *Arabidopsis thaliana* and *Brassica napus* leads to accumulation of unusual unsaturated fatty acids and increased stearic acid content in the. Plant Breed. 126, 186–194. doi:10.1111/j.1439-0523.2007.01316.x

Brown, A.P., Slabas, A.R., Denton, H., 2002. Substrate selectivity of plant and microbial lysophosphatidic acid acyltransferases. Phytochemistry 61, 493–501. doi: 10.1016/S0031-9422(02)00290-X

Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I., Browse, J., 2008. Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. Plant Biotechnol. J. 6, 819–831. doi:10.1111/j.1467-7652.2008.00361.x

Cahoon, E.B., Carlson, T.J., Ripp, K.G., Schweiger, B.J., Cook, G.A., Hall, S.E., Kinney, A.J., 1999. Biosynthetic origin of conjugated double bonds: production of fatty acid components of high-value drying oils in transgenic soybean embryos. Proc. Natl. Acad. Sci. U. S. A. 96, 12935–40. doi:10.1073/pnas.96.22.12935

570 Cahoon, E.B., Dietrich, C.R., Meyer, K., Damude, H.G., Dyer, J.M., Kinney, A.J., 2006.
571 Conjugated fatty acids accumulate to high levels in phospholipids of metabolically
572 engineered soybean and *Arabidopsis* seeds. Phytochemistry 67, 1166–1176.
573 doi:10.1016/j.phytochem.2006.04.013

574 Chapman, K.D., Ohlrogge, J.B., 2012. Compartmentation of triacylglycerol accumulation in
575 plants. J. Biol. Chem. 287, 2288–2294. doi:10.1074/jbc.R111.290072

576 Chen, X., Truksa, M., Shah, S. and Weselake, R.J., 2010. A survey of quantitative real-time
577 polymerase chain reaction internal reference genes for expression studies in *Brassica napus*.
578 Anal. Biochem. 405, 138-140. doi.org/10.1016/j.ab.2010.05.032

579 Chen, G., Woodfield, H.K., Pan, X., Harwood, J.L., Weselake, R.J., 2015. Acyl-trafficking
580 during plant oil accumulation. Lipids 50, 1057–1068. doi:10.1007/s11745-015-4069-x

581 Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, H.,
582 Ståhl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S., Stahl, U.,
583 Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S., Ståhl, U., Lenman,
584 M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S., Stahl, U., Lenman, M., Banas,
585 A., Lee, M., Sandager, L., Ronne, H., Stymne, H., 2000. Phospholipid:diacylglycerol
586 acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of
587 triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. U. S. A. 97, 6487–6492.
588 doi:10.1073/pnas.120067297

589 Datla, R.S.S., Hammerlindl, J.K., Panchuk, B., Pelcher, L.E., Keller, W., 1992. Modified binary
590 plant transformation vectors with the wild-type gene encoding NPTII. Gene 122, 383–384.
591 doi:10.1016/0378-1119(92)90232-E

592 Holic, R., Xu, Y., Caldo, K.M.P., Singer, S.D., Field, C.J., Weselake, R.J., Chen, G., 2018.
593 Bioactivity and biotechnological production of punicic acid. Appl. Microbiol. Biotechnol.
594 102, 3537–3549. doi: 10.1007/s00253-018-8883-y

595 Hornung, E., Pernstich, C., Feussner, I., 2002. Formation of conjugated $\Delta^{11}\Delta^{13}$-double bonds by
596 Δ^{12}-linoleic acid (1,4)-acyl-lipid-desaturase in pomegranate seeds. Eur. J. Biochem. 269,
597 4852–4859. doi:10.1046/j.1432-1033.2002.03184.x

598 Hunter, J.E., 2001. Studies on effects of dietary fatty acids as related to their position on
599 triglycerides. Lipids 36, 655-668. doi: 10.1007/s11745-001-0770-0

600 Iwabuchi, M., Kohno-Murase, J., Imamura, J., 2003. Δ^{12}-oleate desaturase-related enzymes
601 associated with formation of conjugated *trans*-Δ^{11}, *cis*-Δ^{13} double bonds. J. Biol. Chem.
602 278, 4603–4610. doi:10.1074/jbc.M210748200

603 Joh, Y.G., Kim, S.J., Christie, W.W., 1995. The structure of the triacylglycerols, containing
604 punicic acid, in the seed oil of *Trichosanthes kirilowii*. J. Am. Oil Chem. Soc. 72, 1037–
605 1042. doi:10.1007/BF02660718

606 Katavic, V., Mietkiewska, E., Barton, D.L., Giblin, E.M., Reed, D.W., Taylor, D.C., 2002.
607 Restoring enzyme activity in nonfunctional low erucic acid *Brassica napus* fatty acid
608 elongase 1 by a single amino acid substitution. Eur. J. Biochem. 269, 5625–5631.
609 doi:10.1046/j.1432-1033.2002.03270.x

610 Kennedy, E.P., 1961. Biosynthesis of complex lipids. Fed. Proc. 20, 934-940. PMID: 14455159.

611 Kim, S.H., Roh, K.H., Lee, K.R., Kang, H.C., Kim, H.U., Kim, J.B., 2016. Metabolic
612 engineering to produce γ-linolenic acid in *Brassica napus* using a Δ6-desaturase from pike
613 eel. Plant Biotechnol. Rep. 10, 475–481. doi:10.1007/s11816-016-0415-6

614 Koba, K., Imamura, J., Akashoshi, A., Kohno-Murase, J., Nishizono, S., Iwabuchi, M., Tanaka,
615 K., Sugano, M., 2007. Genetically modified rapeseed oil containing *cis*-9, *trans*-11, *cis*-13-
616 octadecatrienoic acid affects body fat mass and lipid metabolism in mice. J. Agric. Food
617 Chem. 55, 3741–3748. doi:10.1021/jf063264z

618 Lager, I., Yilmaz, J.L., Zhou, X.-R., Jasieniecka, K., Kazachkov, M., Wang, P., Zou, J.,
619 Weselake, R., Smith, M.A., Bayon, S., Dyer, J.M., Shockey, J.M., Heinz, E., Green, A.,
620 Banas, A., Stymne, S., 2013. Plant acyl-CoA:lysophosphatidylcholine acyltransferases
621 (LPCATs) have different specificities in their forward and reverse reactions. J. Biol. Chem.
622 288, 36902–36914. doi:10.1074/jbc.M113.521815

623 Lock, Y.Y., Snyder, C.L., Zhu, W., Siloto, R.M., Weselake, R.J. and Shah, S., 2009. Antisense
624 suppression of type 1 diacylglycerol acyltransferase adversely affects plant development in
625 *Brassica napus*. Physiol. Plant. 137, 61-71. doi: 10.1111/j.1399-3054.2009.01258.x

626 Lou, Y., Schwender, J., Shanklin, J., 2014. FAD2 and FAD3 desaturases form heterodimers that
627 facilitate metabolic channeling *in vivo*. J. Biol. Chem. 289, 17996–18007.
628 doi:10.1074/jbc.M114.572883

629 Luddy, F.E., Barford, R.A., Herb, S.F., Magidman, P., Riemenschneider, R.W., 1964. Pancreatic
630 lipase hydrolysis of triglycerides by a semimicro technique. J. Am. Oil Chem. Soc. 41, 693–
631 696. doi:10.1007/BF02661412

632 Mietkiewska, E., Lin, Y., Weselake, R.J., 2014a. Engineering production of C18 conjugated fatty
633 acids in developing seeds of oil crops. Biocatal. Agric. Biotechnol. 3, 44–48.
634 doi:10.1016/j.bcab.2013.11.003

635 Mietkiewska, E., Miles, R., Wickramarathna, A., Sahibollah, A.F., Greer, M.S., Chen, G.,

636 Weselake, R.J., 2014b. Combined transgenic expression of *Punica granatum* conjugase
637 (FADX) and FAD2 desaturase in high linoleic acid *Arabidopsis thaliana* mutant leads to
638 increased accumulation of punicic acid. Planta 240, 575–583. doi:10.1007/s00425-014-
639 2109-z

640 Möllers, C. and Iqbal, M.C., 2009. Doubled haploids in breeding winter oilseed rape. In:
641 Advances in Haploid Production in Higher Plants; (eds Touraev A., Forster B.P., Jain
642 S.M.). Springer, Dordrecht, pp. 161-169. doi:10.1007/978-1-4020-8854-4_13

643 Napier, J.A., 2007. The production of unusual fatty acids in transgenic plants. Annu. Rev. Plant
644 Biol. 58, 295–319. doi:10.1146/annurev.arplant.58.032806.103811

645 Napier, J.A., Haslam, R.P., Beaudoin, F., Cahoon, E.B., 2014. Understanding and manipulating
646 plant lipid composition: Metabolic engineering leads the way. Curr. Opin. Plant Biol. 19,
647 68–75. doi:10.1016/j.pbi.2014.04.001

648 Okuzaki, A., Ogawa, T., Koizuka, C., Kaneko, K., Inaba, M., Imamura, J. and Koizuka, N.,
649 2018. CRISPR/Cas9-mediated genome editing of the *fatty acid desaturase 2* gene in
650 *Brassica napus*. Plant Physiol. Biochem. 131, 63–69. doi: 10.1016/j.plaphy.2018.04.025.

651 Pan, X., Chen, G., Kazachkov, M., Greer, M.S., Caldo, K.M.P., Zou, J., Weselake, R.J., 2015. *In*
652 *vivo* and *in vitro* evidence for biochemical coupling of reactions catalyzed by
653 lysophosphatidylcholine acyltransferase and diacylglycerol acyltransferase. J. Biol. Chem.
654 290, 18068–18078. doi:10.1074/jbc.M115.654798

655 Pan, X., Siloto, R.M.P., Wickramarathna, A.D., Mietkiewska, E., Weselake, R.J., 2013.
656 Identification of a pair of phospholipid:diacylglycerol acyltransferases from developing flax
657 (*Linum usitatissimum* L.) seed catalyzing the selective production of trilinolenin. J. Biol.

Chem. 288, 24173–24188. doi:10.1074/jbc.M113.475699

Petrie, J.R., Shrestha, P., Zhou, X.R., Mansour, M.P., Liu, Q., Belide, S., Nichols, P.D., Singh, S.P., 2012. Metabolic engineering plant seeds with fish oil-like levels of DHA. PLoS One 7, 2–8. doi:10.1371/journal.pone.0049165

Rahman, H., Singer, S.D. and Weselake, R.J., 2013. Development of low-linolenic acid Brassica oleracea lines through seed mutagenesis and molecular characterization of mutants. Theor. Appl. Genet. 126, 1587-1598. doi: 10.1007/s00122-013-2076-y

Ramírez, M., Amate, L., Gil, A., 2001. Absorption and distribution of dietary fatty acids from different sources. Early Hum Dev. 65 Suppl, S95-S101. doi: 10.1016/s0378-3782(01)00211-0

Singh, S., Thomaeus, S., Lee, M., Stymne, S., Green, A., 2001. Transgenic expression of a Δ12-epoxygenase gene in *Arabidopsis* seeds inhibits accumulation of linoleic acid. Planta 212, 872–879.

Smith, C.R., 1971. Occurrence of unusual fatty acids in plants. Prog. Chem. Fats Other Lipids 11, 137139–131177. doi:10.1016/0079-6832(71)90005-X

Smith, M.A., Moon, H., Chowrira, G., Kunst, L., 2003. Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. Planta 217, 507–516. doi:10.1007/s00425-003-1015-6

Snowdon, R.J. and Iniguez Luy, F.L., 2012. Potential to improve oilseed rape and canola breeding in the genomics era. Plant Breed. 131, 351-360. doi:10.1111/j.1439-0523.2012.01976.x

Stymne, S., Stobart, A.K., 1984. Evidence for the reversibility of the acyl-CoA :

lysophosphatidylcholine acyltransferase in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons and rat liver. Biochem. J. 223, 305–314. doi:10.1042/bj2230305

Subedi, U., Jayawardhane, K., Pan, X., Ozga, J., Chen, G., Foroud, N.A., Singer, S.D., The potential of genome editing for improving seed oil content and fatty acid composition in oilseed crops. Lipids 2020, Accepted.

Takagi, T., Itabashi, Y., 1981. Occurrence of mixtures of geometrical isomers of conjugated octadecatrienoic acids in some seed oils: Analysis by open-tubular gas liquid chromatography and high performance liquid chromatography. Lipids 16, 546–551. doi:10.1007/BF02535054

Taylor, D.C., Zhang, Y., Kumar, A., Francis, T., Giblin, E.M., Barton, D.L., Ferrie, J.R., Laroche, A., Shah, S., Zhu, W.W., Snyder, C.L., Hall, L., Rakow, G., Harwood, J.L., Weselake, R.J., 2009. Molecular modification of triacylglycerol accumulation by over-expression of *DGAT1* to produce canola with increased seed oil content under field conditions. Botany 87, 533–543. doi:10.1139/B08-101

Thompson, M.A., Reddy, A.S., 2008. Certain plants with “no saturate” or reduced saturate levels of fatty acids in seeds, and oil derived from the seeds. U.S. Patent Application No. 11/576,750

Transparency Market Research, 2018. Pomegranate Oil Market - Global Industry Analysis, Size, Share, Growth, Trends, and Forecast 2018 – 2026. Albany, NY: Transparency Market Research. https://www.transparencymarketresearch.com/pomegranate-oil-market.html (accessed 29 June 2020).

van Erp, H., Bates, P.D., Burgal, J., Shockey, J., Browse, J., 2011. Castor phospholipid:diacylglycerol acyltransferase facilitates efficient metabolism of hydroxy fatty acids in transgenic *Arabidopsis*. Plant Physiol. 155, 683–693. doi:10.1104/pp.110.167239

Vikbjerg, A.F., Mu, H. and Xu, X., 2006. Elucidation of acyl migration during lipase-catalyzed production of structured phospholipids. J. Am. Oil Chem.' Soc. 83, 609-614. doi: 10.1007/s11746-006-1246-3

Weselake, R.J., 2011. Genetic engineering approaches for trait development in *Brassica* oilseed species. In: Canola: Description, Variety Development, Agronomy, Composition, and Utilization; (eds Daun J.L., Hickling D., Eskin N.A.M.) AOCS Press, Urbana, IL, pp 57-91. doi: 10.1016/B978-0-9818936-5-5.50007-3

Weselake, R.J., Shah, S., Tang, M., Quant, P.A., Snyder, C.L., Furukawa-Stoffer, T.L., Zhu, W., Taylor, D.C., Zou, J., Kumar, A., Hall, L., Laroche, A., Rakow, G., Raney, P., Moloney, M.M., Harwood, J.L., 2008. Metabolic control analysis is helpful for informed genetic manipulation of oilseed rape (*Brassica napus*) to increase seed oil content. J. Exp. Bot. 59, 3543–3549. doi:10.1093/jxb/ern206

Wickramarathna, A.D., Siloto, R.M.P., Mietkiewska, E., Singer, S.D., Pan, X., Weselake, R.J., 2015. Heterologous expression of flax *PHOSPHOLIPID:DIACYLGLYCEROL CHOLINEPHOSPHOTRANSFERASE* (*PDCT*) increases polyunsaturated fatty acid content in yeast and *Arabidopsis* seeds. BMC Biotechnol. 15, 1–15. doi:ARTN 63\r10.1186/s12896-015-0156-6

Xu, Y., Caldo, K.M.P., Pal-Nath, D., Ozga, J., Lemieux, M.J., Weselake, R.J., Chen, G., 2018. Properties and biotechnological applications of acyl-CoA:diacylglycerol acyltransferase and

phospholipid:diacylglycerol acyltransferase from terrestrial plants and microalgae. Lipids. 53, 663–688. doi:10.1002/lipd.12081

Xu, Y., Chen, G., Greer, M., Caldo, K.M.P., Ramakrishnan, G., Shah, S., Wu, L., Lemieux, M.J., Ozga, J., Weselake, R.J., 2017. Multiple mechanisms contribute to increased neutral lipid accumulation in yeast producing recombinant variants of plant diacylglycerol acyltransferase 1. J. Biol. Chem. 292, 17819-17831. doi: 10.1074/jbc.M117.811489

Yu, F., Lydiate, D.J., Gugel, R.K., Sharpe, A.G. and Rimmer, S.R., 2012. Introgression of *Brassica rapa subsp. sylvestris* blackleg resistance into *B. napus*. Mol Breeding 30, 1495–1506. doi: 10.1007/s11032-012-9735-6

Zhou, X., Singh, S., Liu, Q., Green, A., 2006. Combined transgenic expression of Δ12-desaturase and Δ12-epoxygenase in high linoleic acid seeds leads to increased accumulation of vernolic acid. Funct. Plant Biol. 33, 585–592. doi: 10.1071/FP05297

738 **Table**

739 **Table 1.** Fatty acid composition of the total acyl lipids in oil extracted from mature T_1 seeds of
740 T_0 *B. napus* lines transformed with the NCJD construct carrying *PgFADX+PgFAD2* genes
741 (NCJD) and wild-type control (WT). Fatty acid composition from each line was analyzed using
742 oil from six seed samples. Data represent mean ± SD from 7 independent transgenic NCJD lines
743 and 6 non-transformed WT control lines. PuA, punicic acid.

744

	Fatty acid composition									
	16:0	16:1Δ^9	18:0	18:1Δ^9	18:1Δ^{11}	18:2$\Delta^{9,12}$	18:3$\Delta^{9,12,15}$	20:0	20:1Δ^{11}	PuA
	Percentage (w/w) of total fatty acids									
NCJD	4.9 ± 0.3	0.13 ± 0.02	3.7 ± 0.8	55.1 ± 4.3	2.0 ± 0.3	23.4 ± 3.3	3.4 ± 0.7	1.3 ± 0.2	1.3 ± 0.1	4.9 ± 1.5
Range	4.5 ~ 5.4	0.10 ~ 0.16	2.6 ~ 4.8	49.9 ~ 61.4	1.7 ~ 2.3	19.5 ~ 28.6	2.8 ~ 4.7	1.0 ~ 1.6	1.1 ~ 1.5	2.3 ~ 6.2
WT	4.8 ± 0.1	0.13 ± 0.01	2.5 ± 0.1	63.6 ± 1.4	2.0 ± 0.2	15.4 ± 0.7	9.6 ± 0.8	0.82 ± 0.07	1.3 ± 0.0	0
Range	4.7 ~ 4.8	0.12 ~ 0.14	2.4 ~ 2.5	61.0 ~ 65.6	1.7 ~ 2.4	14.9 ~ 15.8	7.7 ~ 11.4	0.71 ~ 0.92	1.1 ~ 1.4	0

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Figure legends

Fig. 1. Schematic representation of the two key enzymes engineered in the current study for punicic acid (PuA) production in *Brassica napus*. A. Modification of C18 fatty acids in *Punica granatum* (grey) and *B. napus* (blue). The structures of fatty acids were drawn with ChemDraw Prime software (PerkinElmer Informatics). B. Comparison of major C18 fatty acids (%) in *P. granatum* and *B. napus* seeds. Data represent mean ± SD of triplicates. C. The binary vector used for *B. napus* transformation (Mietkiewska et al., 2014b). 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid; CLNA, conjugated linolenic acid; FAD, fatty acid desaturase; FADX, fatty acid conjugase; P, promoter; T, terminator.

Fig. 2. Punicic acid (PuA) content of mature T_1 seed from T_0 transgenic *Brassica napus* lines co-expressing *PgFADX* and *PgFAD2* genes and wild-type control (WT). Each data point represents the PuA content analyzed from two T_1 seeds per line. Horizontal bars indicate the mean value for each dataset. The copy number of the transgene cassette carrying *PgFADX+PgFAD2* genes was estimated by segregation analysis and qPCR assays and shown in brackets. WT, wild-type *B. napus*.

Fig. 3. Content of major fatty acids from mature T_2 seeds of individual T_1 transgenic *Brassica napus* lines and their correlation analysis. A. Fatty acid composition of mature T_2 seeds. B. Correlation of punicic acid (PuA) content and the desaturation proportion of oleic acid (18:1). C. Correlation of PuA content and the conjugation proportion of linoleic acid (18:2). D. Correlation of PuA content and α-linolenic acid (18:3) content. Lipid analysis was carried out using six seed

per line from 33 transgenic lines and 3 individual null segregant lines (negative control; marked by black dots) and data represent mean ± SD of triplicates. For B, C and D, each dot represents one *B. napus* line.

Fig. 4. Punicic acid (PuA) content in relation to total oil content in mature T_2 seeds. A. Average T_2 seed oil content of seeds from 33 T_1 *B. napus* lines co-expressing *PgFADX* and *PgFAD2* during seed development, 9 null segregant lines and 9 wild-type (WT) lines. B. Correlation of PuA content and seed oil content. C. Seed oil content and PuA content of selected T_1 transgenic *B. napus* lines, null segregant control lines and WT lines. Lipid analysis was carried out using six seeds per line. For A, data represent mean ± SD, n=9 (WT and null segregant lines) or 33 (transgenic T_1 *B. napus* lines). For B, each data point represents one *B. napus* line. Seeds from transgenic T_1 *B. napus* lines, null segregant lines and WT lines are shown as red circle, blue square and grey triangle, respectively. For C, data represent mean ± SD of triplicates.

Fig. 5. Punicic acid (PuA) content in three generations of transgenic *B. napus* lines. The red arrow indicates T_2 seeds from the *B. napus* line NCJD-15A-11, which was transformed with multi copies of the NCJD construct carrying *PgFADX+PgFAD2* genes. Lipids were analyzed from six seeds per line and the results are shown as black dot (T_1), blue dot (T_2 with the exception of NCJD-15A-11), and red dot (T_2 and T_3 seeds from the NCJD-15A-11 line). NCJD-15A-11 was used to analyze the PuA content of single seeds and progressed to produce the T_3 seeds.

Fig. 6. Relative content of punicic acid (PuA) in polar lipid (PL), triacylglycerol (TAG) and diacylglycerol (DAG) isolated from total lipids extracted from mature T_3 transgenic *B. napus* seeds. A. Fatty acid composition of TAG. B. Fatty acid composition at the *sn*-2 position of TAG. C. The content of individual fatty acid at the *sn*-2 position versus the *sn*-1/*sn*-3 positions of TAG. D. Fatty acid composition of PL. E. Fatty acid composition at the *sn*-1 position of PL. F. The content of individual fatty acid at the *sn*-2 position versus the *sn*-1 positions of PL. G. Fatty acid composition of DAG. H. Fatty acid composition at the *sn*-2 position of *sn*-1,2-DAG. For panels A, B, D, E, G and H, data represent mean ± SD of triplicates.

Figures

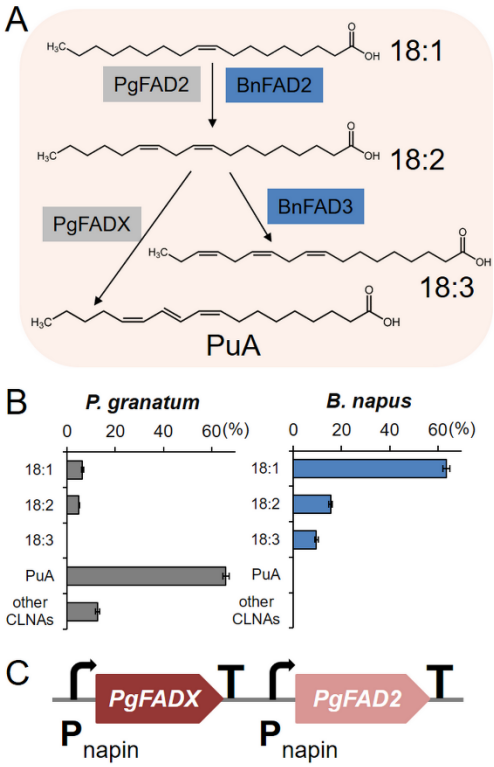
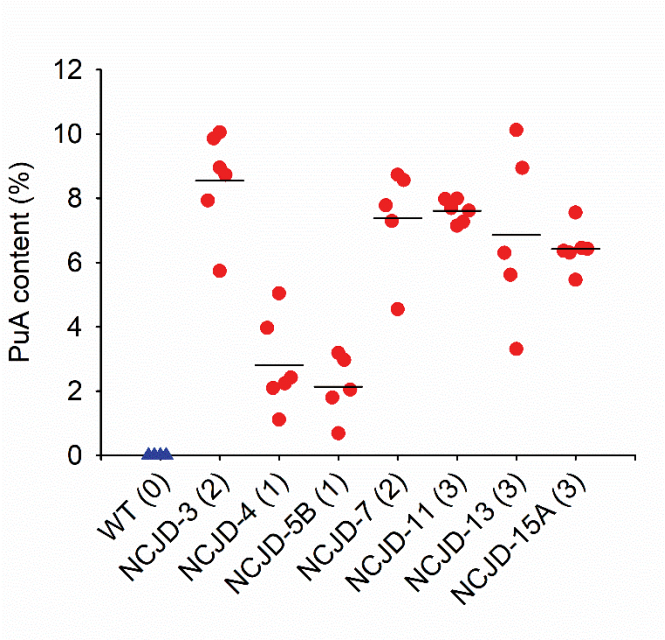


Fig. 1. Schematic representation of the two key enzymes engineered in the current study for punicic acid (PuA) production in *Brassica napus*.



807

808 **Fig. 2.** Punicic acid (PuA) content of oils of mature T_1 seed from T_0 transgenic *Brassica napus*

809 lines co-expressing *PgFADX* and *PgFAD2* genes and wild-type control (WT).

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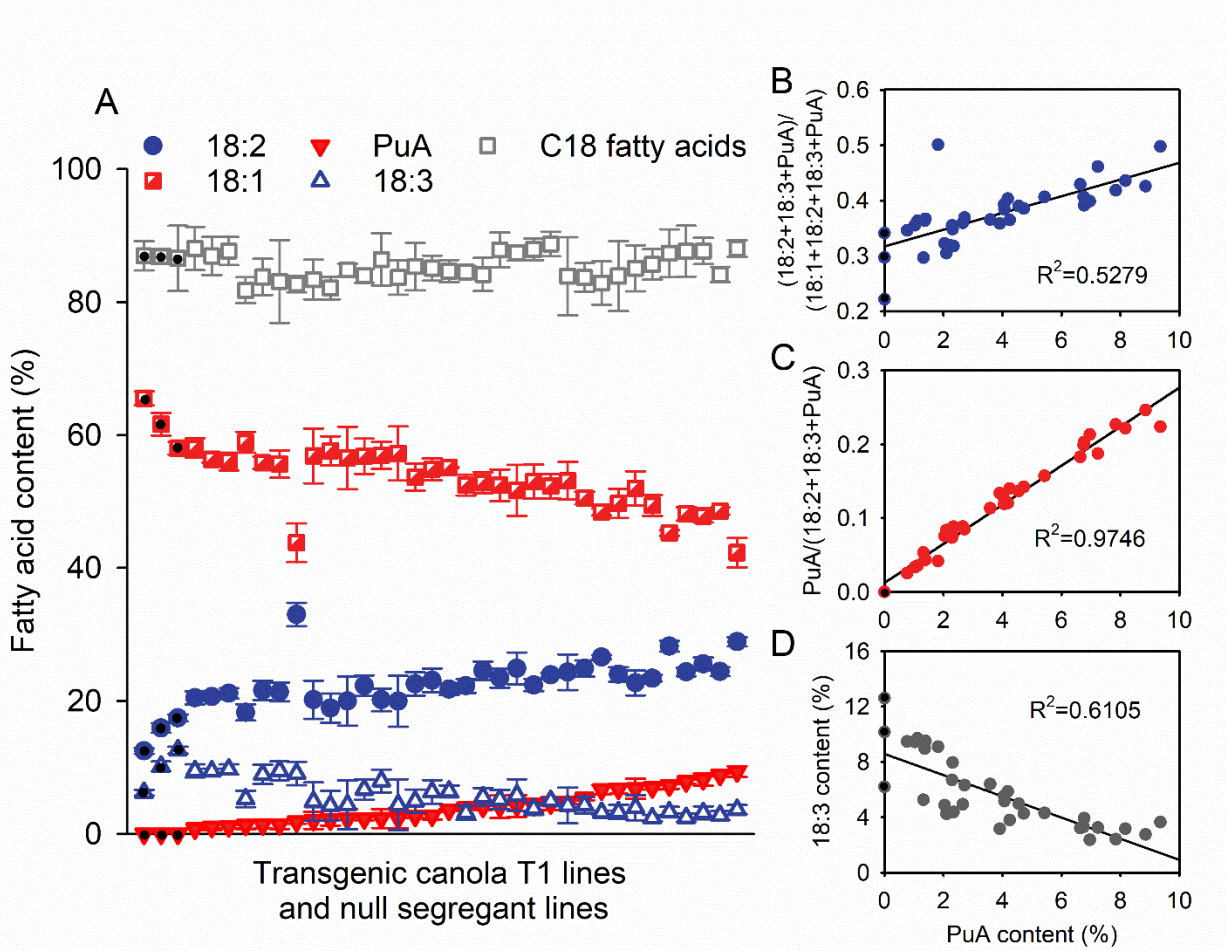
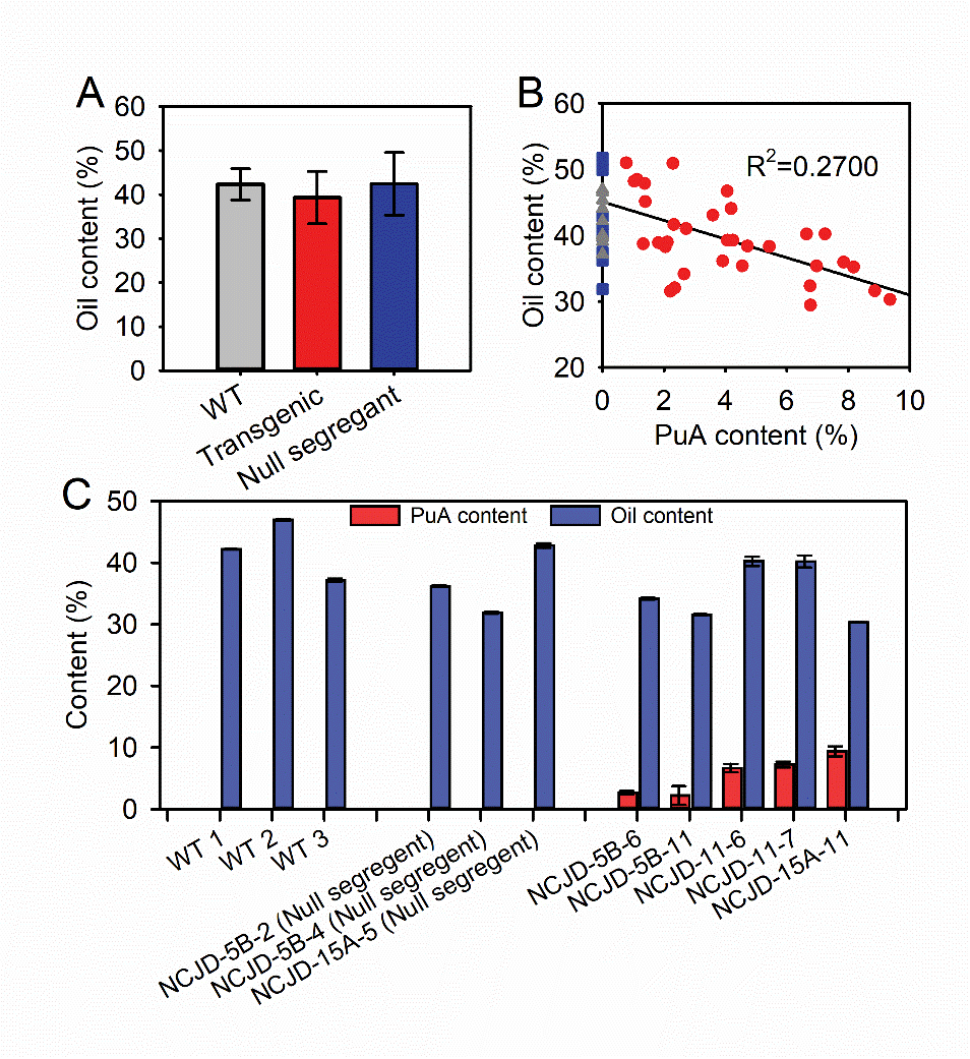


811

812 **Fig. 3.** Content of major fatty acids from mature T_2 seeds of individual T_1 transgenic *Brassica*

813 *napus* lines and their correlation analysis.



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815 **Fig. 4.** Punicic acid (PuA) content in relation to total oil content in mature T_2 seeds.

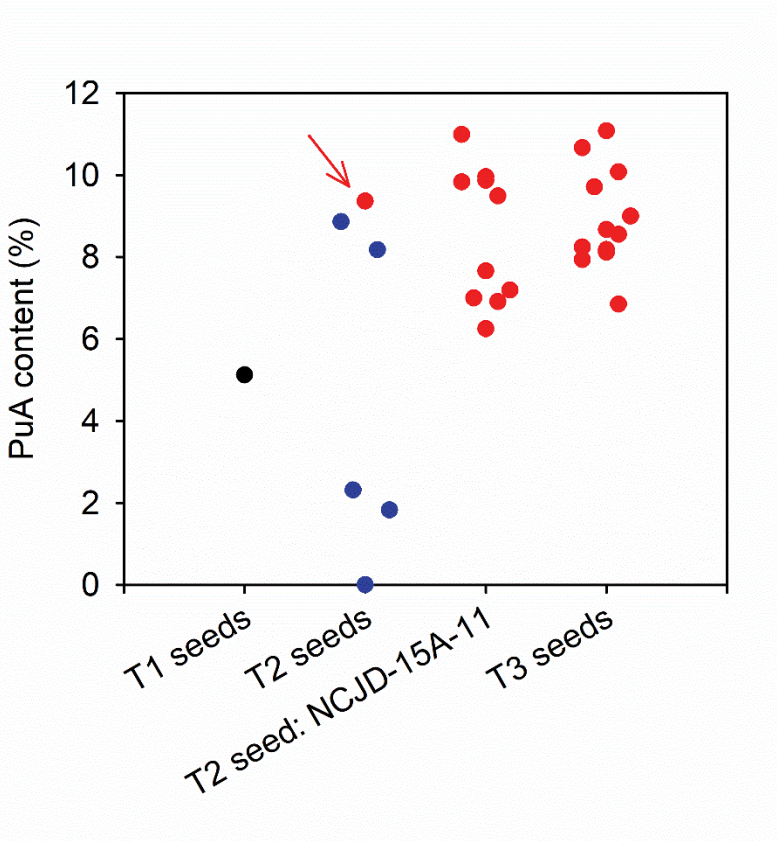
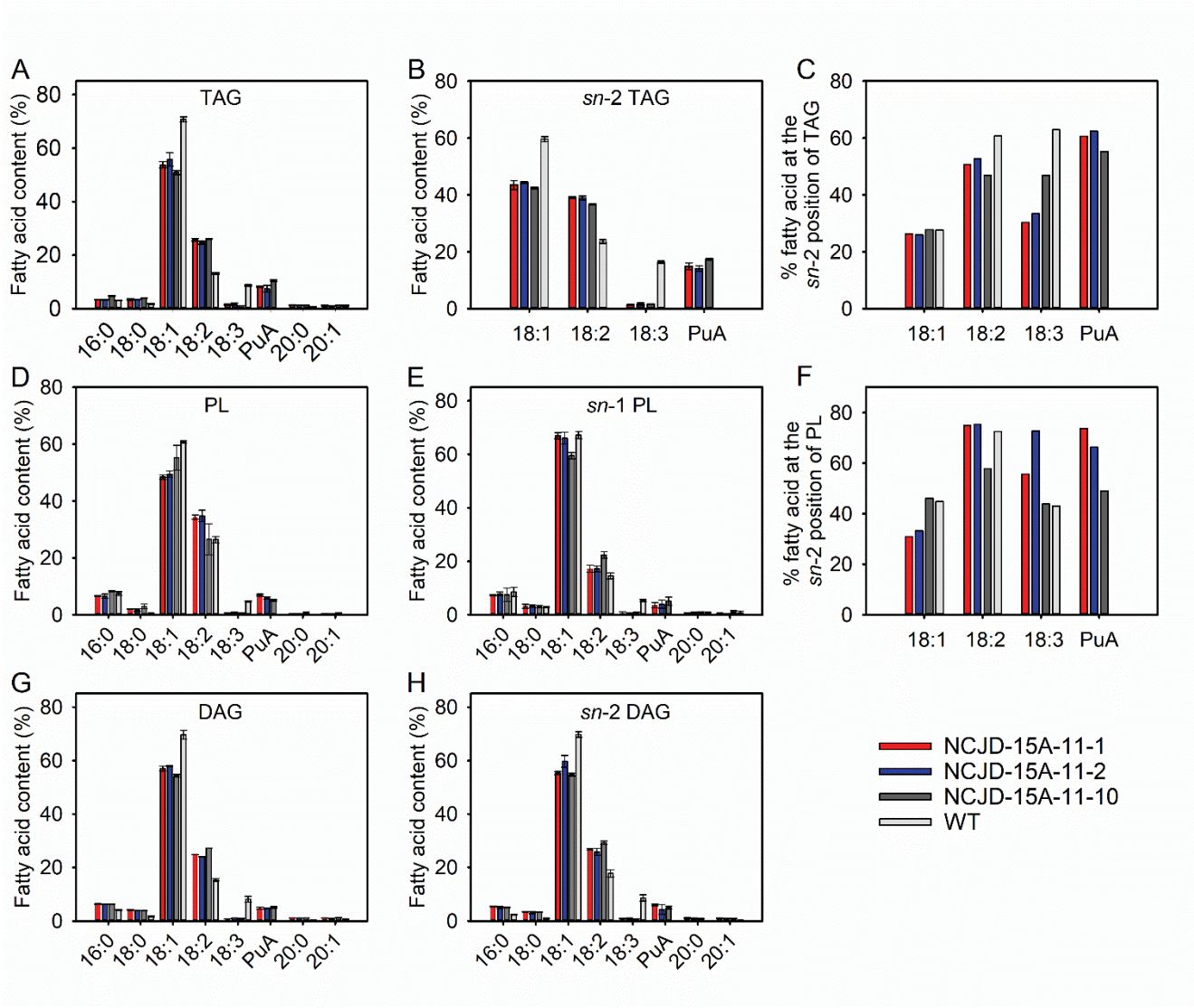


Fig. 5. Punicic acid (PuA) content in three generations of transgenic *B. napus* lines.



818

819 **Fig. 6.** Relative content of punicic acid (PuA) in polar lipid (PL), triacylglycerol (TAG) and
820 diacylglycerol (DAG) isolated from total lipids extracted from mature T_3 transgenic *B. napus*
821 seeds.

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823

Figure 1

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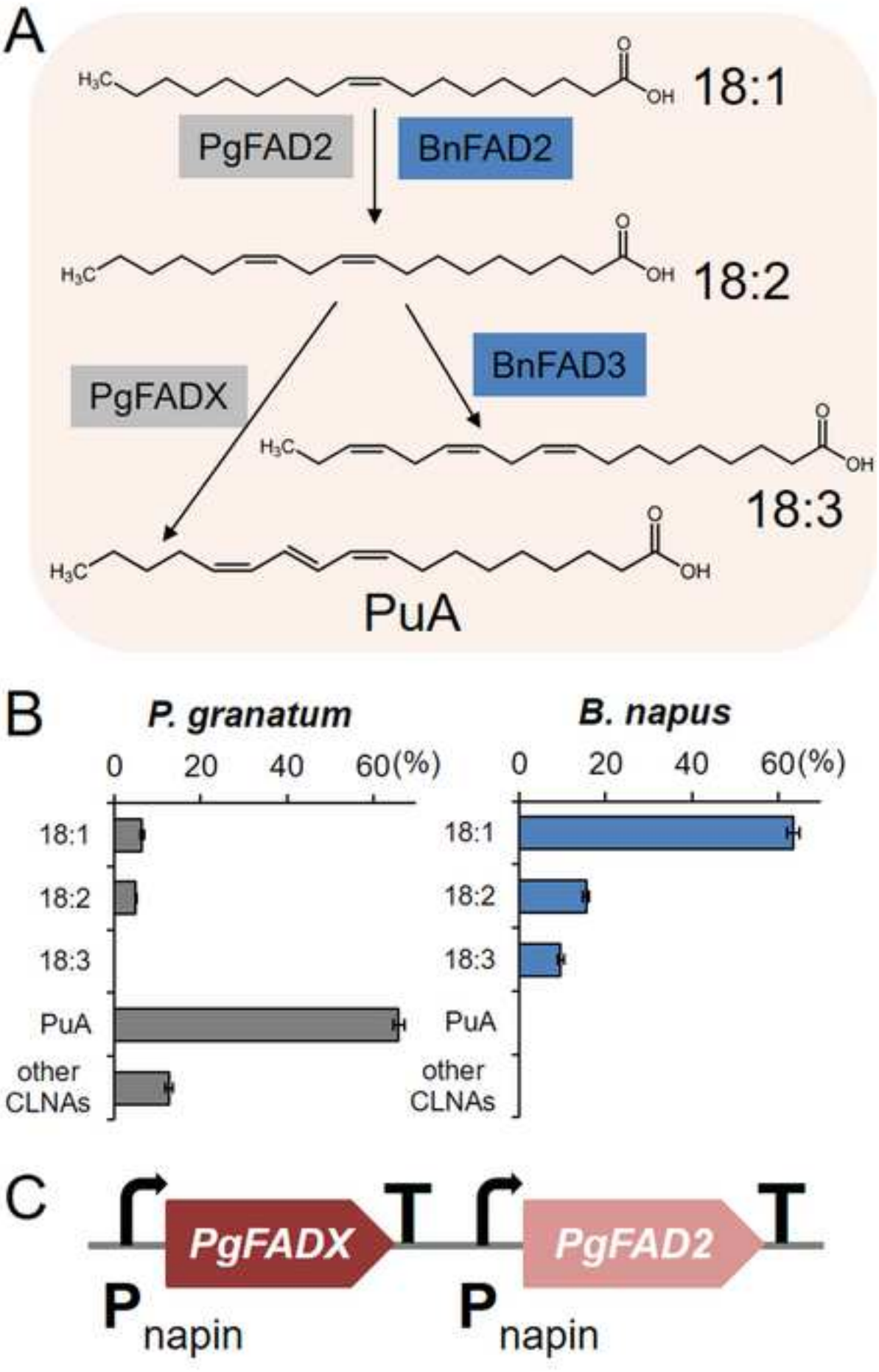

Figure 2

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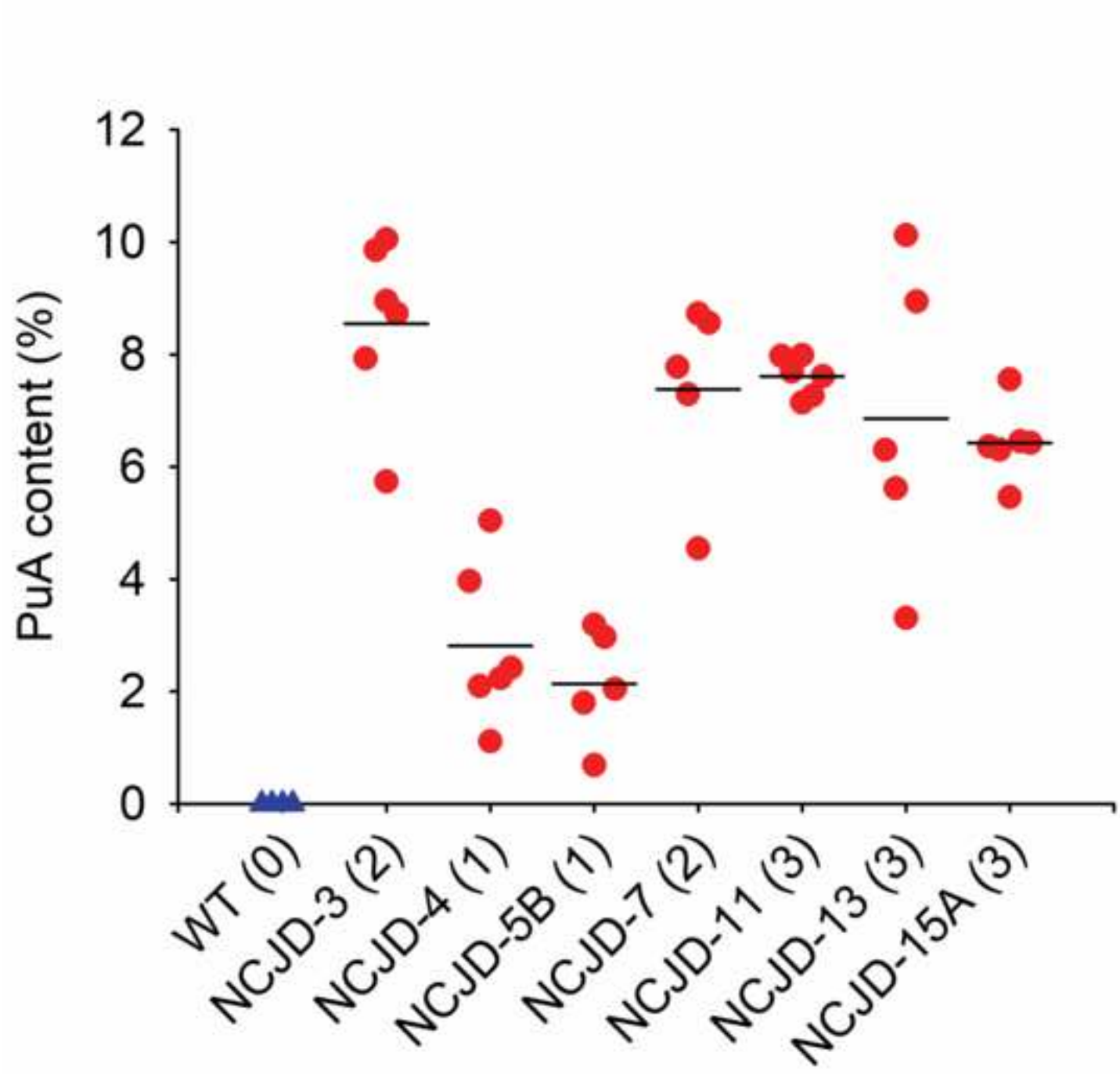

Figure 3

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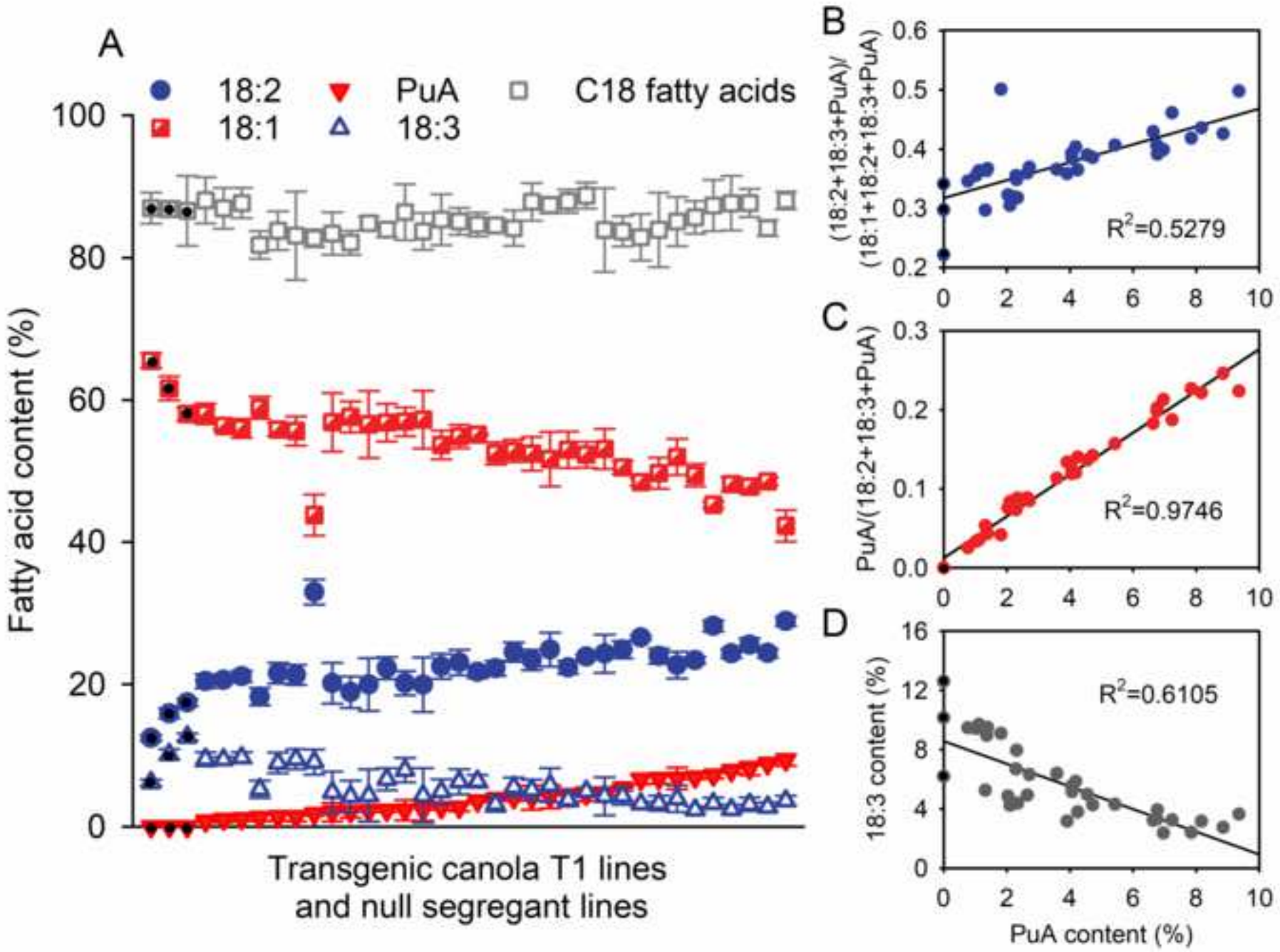

Figure 4

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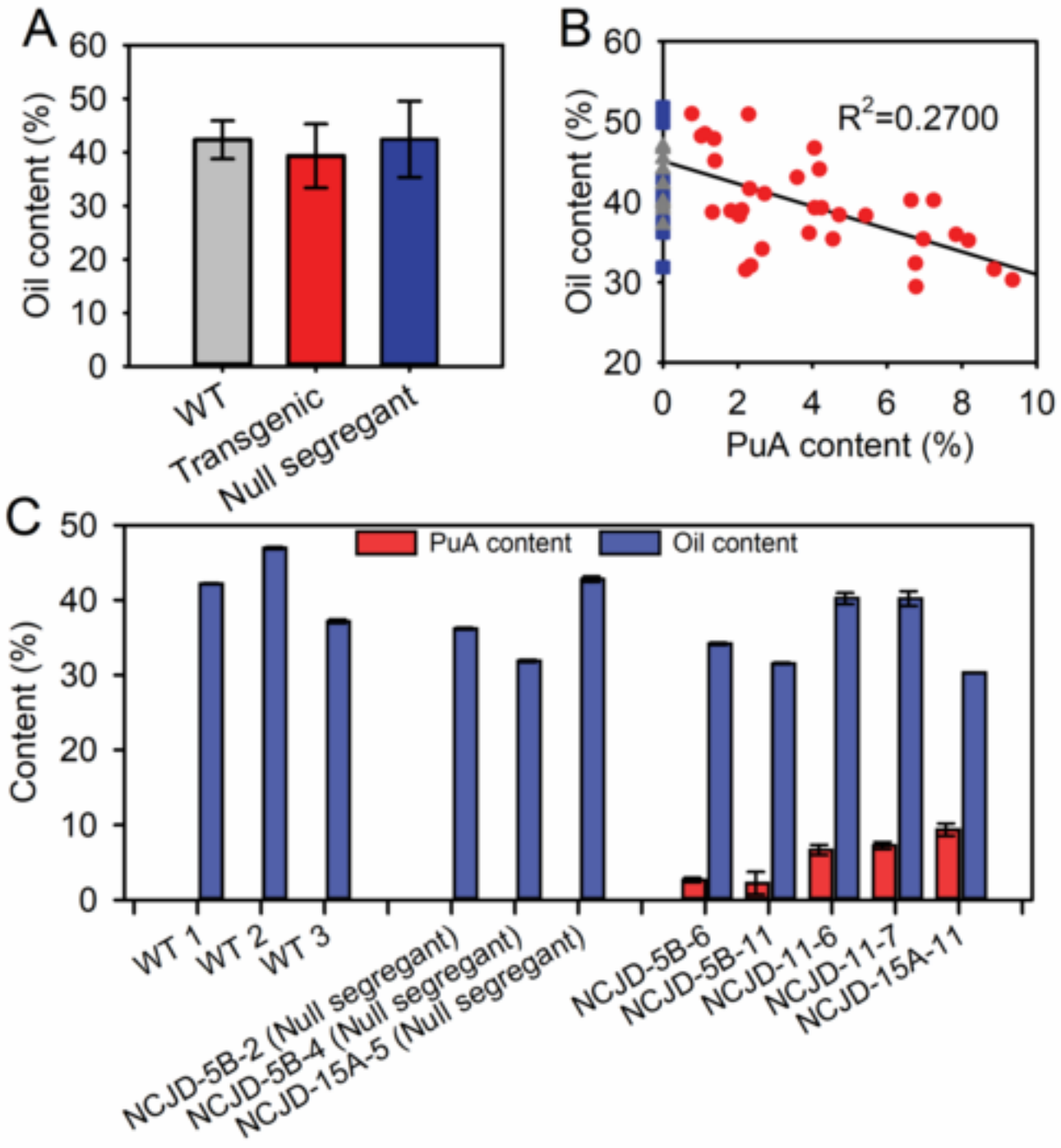

Figure 5

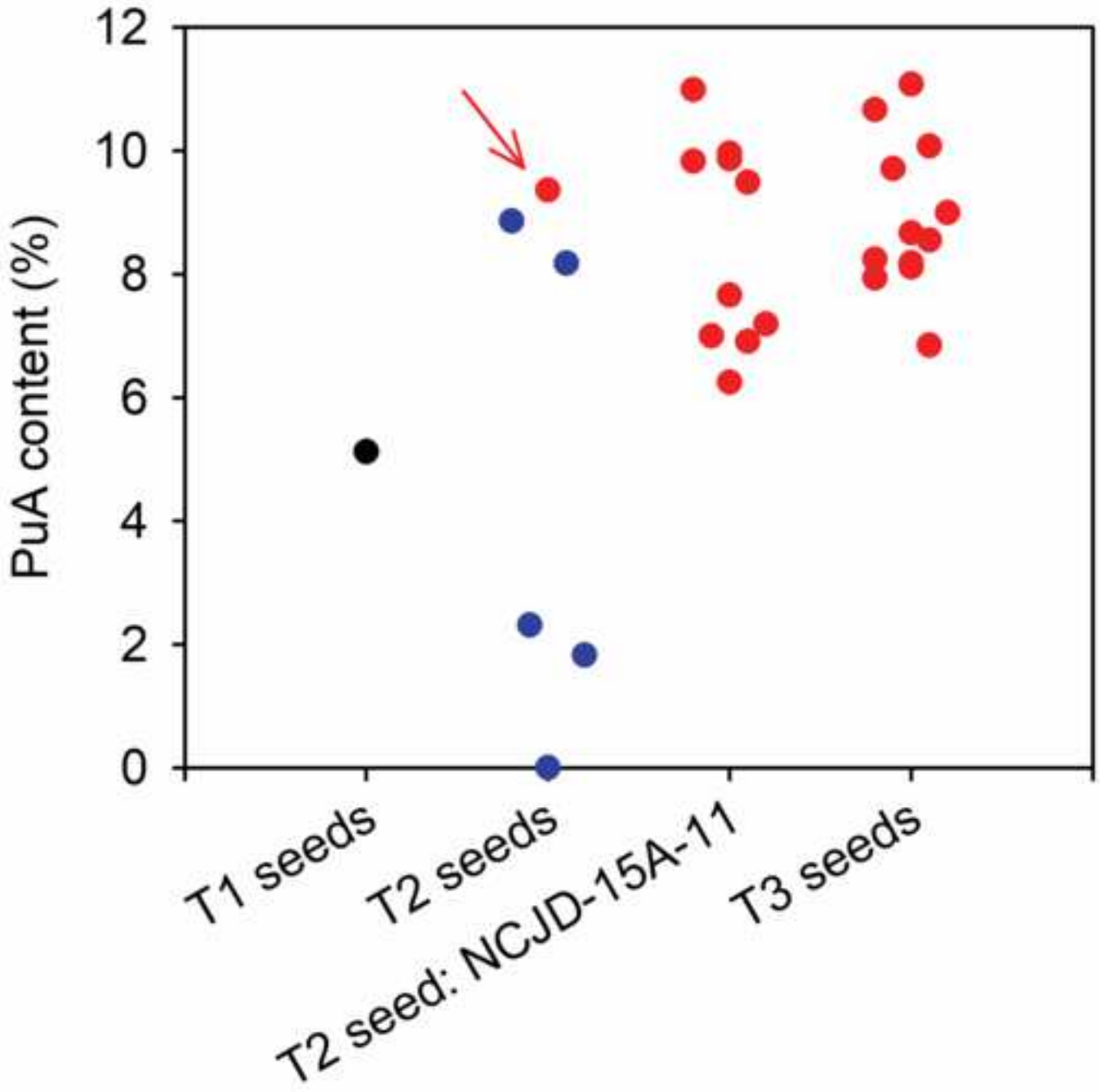

Figure 6

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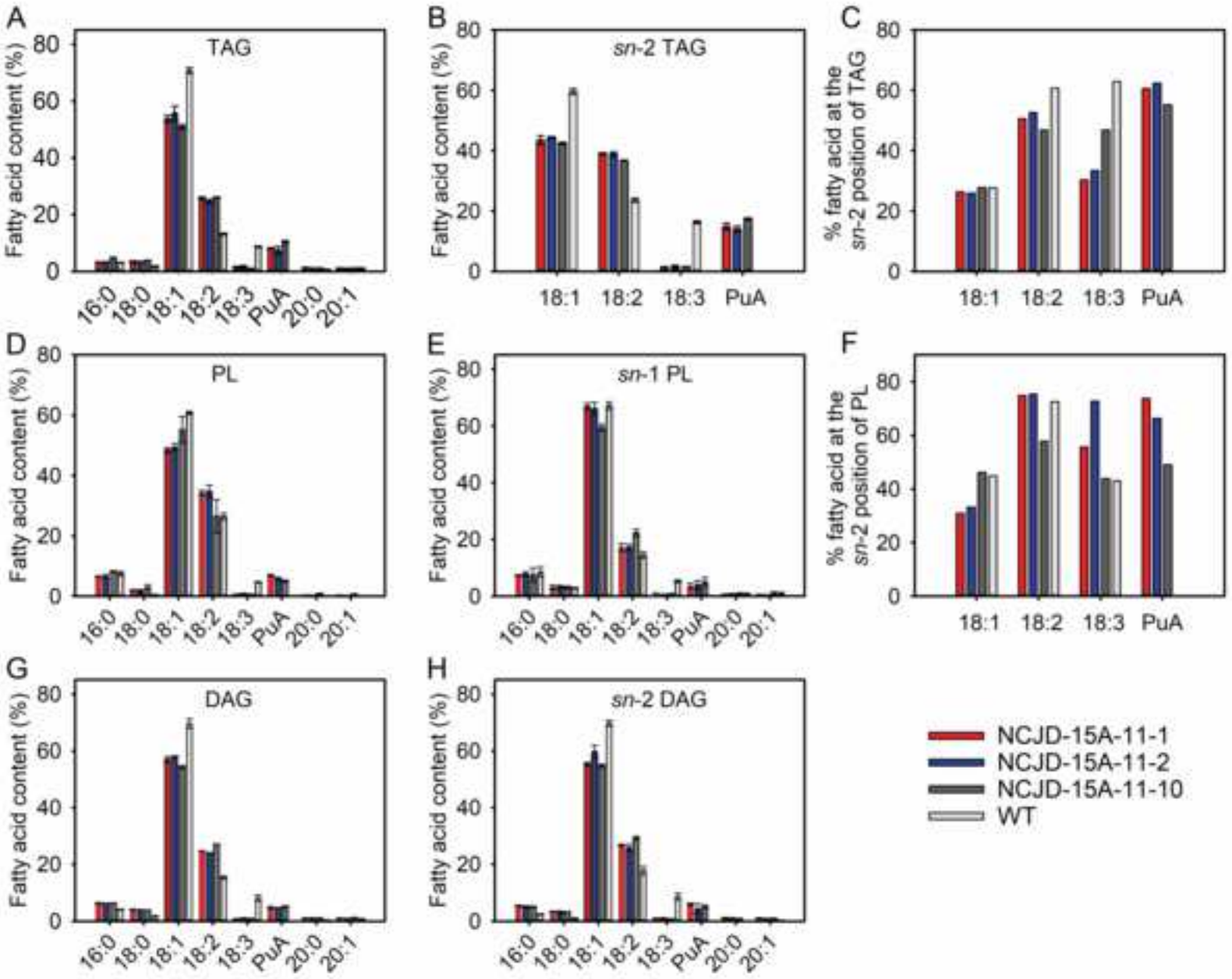

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