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ORIGINAL ARTICLE

Combined transgenic expression of *Punica granatum* conjugase (*FADX*) and *FAD2* desaturase in high linoleic acid *Arabidopsis thaliana* mutant leads to increased accumulation of punicic acid

Elzbieta Mietkiewska · Robin Miles · Aruna Wickramarathna · Ariff Firman Sahibollah · Michael S. Greer · Guanqun Chen · Randall J. Weselake

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Abstract

Main conclusion ***Arabidopsis*** **was engineered to produce 21.2 % punicic acid in the seed oil. Possible molecular factors limiting further accumulation of the conjugated fatty acid were investigated.**

Punicic acid (18:3$\Delta^{9cis,11trans,13cis}$) is a conjugated linolenic acid isomer and is a main component of *Punica granatum* (pomegranate) seed oil. Medical studies have shown that punicic acid is a nutraceutical with anti-cancer and anti-obesity properties. It has been previously demonstrated that the conjugated double bonds in punicic acid are produced via the catalytic action of fatty acid conjugase (FADX), which is a homolog of the oleate desaturase. This enzyme catalyzes the conversion of the Δ^{12}-double bond of linoleic acid (18:2$\Delta^{9cis,12cis}$) into conjugated $\Delta^{11trans}$ and Δ^{13cis}-double bonds. Previous attempts to produce punicic acid in transgenic *Arabidopsis thaliana* seeds overexpressing *P. granatum FADX* resulted in a limited accumulation of punicic acid of up to 4.4 %, accompanied by increased accumulation of oleic acid (18:1Δ^{9cis}), suggesting that production of punicic acid in some way inhibits the activity of oleate desaturase (Iwabuchi et al. 2003). In the current study, we applied a new strategy to enhance the production of punicic acid in a high linoleic acid *A. thaliana fad3/fae1* mutant background using the combined expression of *P. granatum FADX* and *FAD2*. This approach led to the accumulation of punicic acid at the level of 21 % of total fatty acids and restored the natural proportion of oleic acid observed in the *A. thaliana fad3/fae1* mutant. In addition, we provide new insights into the high oleate phenotype and describe factors limiting the production of punicic acid in genetically engineered plants.

Keywords Conjugated fatty acids · Metabolic engineering · Triacylglycerol · Phosphatidylcholine

Abbreviations

CLNAs	Conjugated linolenic acids
FAD2	Δ^{12}-oleic acid desaturase
FAD3	Δ^{15}-linoleic acid desaturase
FADX	Fatty acid conjugase
ODP	Oleic acid desaturation proportion
PC	Phosphatidylcholine
3′ UTR	Three prime untranslated region
TAG	Triacylglycerol

Introduction

Most commercial oilseeds contain saturated and unsaturated fatty acids, such as palmitic (16:0), stearic (18:0), oleic (18:1Δ^{9cis}), linoleic (18:2$\Delta^{9cis,12cis}$) and α-linolenic acid (18:3$\Delta^{9cis,12cis,15cis}$) (Li et al. 2010). Typically, the double bonds of polyunsaturated fatty acids are separated by one or more methylene groups ($-CH_2-$). In contrast, seeds of a limited number of plant species contain oil enriched in fatty acids with conjugated non-methylene interrupted double bonds (Cahoon et al. 1999). Conjugated linolenic acids (CLNAs) are found in the seed oils of plant species from several families including Curcubitaceae,

E. Mietkiewska · R. Miles · A. Wickramarathna · A. F. Sahibollah · M. S. Greer · G. Chen · R. J. Weselake (✉)
Alberta Innovates Phytola Centre, Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-10 Agriculture/Forestry Centre, Edmonton, AB T6G 2P5, Canada
e-mail: randall.weselake@ualberta.ca

Punicaceae, Bignoniaceae, Rosaceae, Chrysobalanaceae, Lythraceae, Balasaminaceae and Euphorbiaceae (Smith 1971; Badami and Patil 1980; Rawat et al. 2012).

Oils enriched in CLNAs are valuable for nutraceutical and industrial applications (Cahoon et al. 1999; Rawat et al. 2012). CLNAs such as α-eleostearic acid ($18{:}3\Delta^{9cis,11trans,13trans}$), present in tung oil, are commonly used in formulations of inks, dyes, coatings and resins (Sonntag 1979; Hornung et al. 2004; Cahoon et al. 2006). There is also growing evidence showing that dietary supplementation with CLNAs has cytotoxic effects on tumor cells and alters lipid metabolism in animals (Igarashi and Miyazawa 2005; Vroegrijk et al. 2011). In particular, it has been shown that *Punica granatum* (pomegranate) seed oil, containing punicic acid ($18{:}3\Delta^{9cis,11trans,13cis}$) and polyphenols, retards oxidation and prostaglandin synthesis, inhibits breast and colon cancer cell proliferation and invasion, and promotes breast cancer cell apoptosis (Kim et al. 2002; Kohno et al. 2004). Also, in human clinical trials, it has been shown that consumption of pomegranate seed oil has a positive effect on cardiovascular health (Mirmiran et al. 2010). Therefore, there is a growing interest in transferring the genes responsible for the biosynthesis of punicic acid into crop plants to obtain significant amounts of it in the seed oil (Mietkiewska et al. 2014).

It has been previously demonstrated that the conjugated double bonds in punicic acid are synthesized by a divergent form of the Δ^{12}-oleic acid desaturase (FAD2). This enzyme, which has been designated 'fatty acid conjugase, or FADX', catalyzes the conversion of the Δ^{12}-double bond of linoleic acid into two conjugated double bonds at positions $\Delta^{11trans}$ and Δ^{13cis} from the carboxyl end of the fatty acid (Hornung et al. 2002; Iwabuchi et al. 2003). Fatty acid conjugase involved in the biosynthesis of punicic acid in *P. granatum* (*PgFADX,* GenBank# AY178446) and *Trichosanthes kirilowii (TkFADX,* GenBank# AY178444) was previously isolated by PCR-based cloning. Earlier attempts to produce punicic acid in transgenic plants resulted in the limited accumulation of the conjugated fatty acid. *Arabidopsis thaliana* seeds overexpressing *PgFADX* or *TkFADX* showed accumulation of punicic acid up to 4.4 % or 10 %, respectively (Iwabuchi et al. 2003). In addition, *TkFADX Brassica napus* was developed showing production of punicic acid up to 2.5 % of total fatty acids in transgenic seeds (Koba et al. 2007). These levels of punicic acid were considerably lower compared to those observed in *P. granatum* (up to 80 % w/w) or *T. kirilowii* (40 % w/w).

Similar problems with low accumulation of other conjugated fatty acids were reported earlier in transgenic plants overexpressing cDNAs encoding other divergent FAD2 isoforms including the conjugase from *Momordica charantia*, *Vernica fordii*, *Impatients balsamina* and *Calendula officinalis* (Cahoon et al. 1999, 2001; Cahoon and Kinney 2004; Cahoon et al. 2006). This indicates that although the biosynthesis of conjugated fatty acids might be supported by a single gene trait (*FADX*), the accumulation of these unusual fatty acids in the storage lipids requires a network of additional genes/enzymes. In all of these cases, transgenic expression of *FAD2* divergent genes resulted in low accumulation of modified fatty acids and was also accompanied by a significant increase in the content of oleic acid (Napier 2007; Carlsson et al. 2011).

Here, we describe a new strategy developed to increase the accumulation of punicic acid to higher than previously reported levels (Iwabuchi et al. 2003). First, overexpression of *PgFADX* in high oleic *A. thaliana* double mutant *fad3/fae1* led to 2.6-fold higher amounts of punicic acid compared to that previously reported in the wild-type *A. thaliana* background. Secondly, combined overexpression of *PgFADX* with *PgFAD2* in a *fad3/fae1* mutant background brought the production of punicic acid to 21.2 % of total fatty acids. These investigations reveal metabolic constraints limiting the accumulation of punicic acid in developing transgenic seeds. Furthermore, the current data also provide new insights into the phenomenon of reduced endogenous FAD2 desaturase activity observed in plants engineered to produce punicic acid.

Materials and methods

RNA isolation and cDNA synthesis from *P. granatum* seeds

Punica granatum L. seeds were obtained from fruits purchased at the local market. Total RNA was isolated using Spectrum Plant Total RNA Kit (Sigma-Aldrich, Oakville, ON, Canada) followed by additional cleaning and concentration with RNeasy MinElute™ Cleanup Kit (Qiagen, Mississauga, ON, Canada). Single-stranded cDNA was synthesized at 42 °C from 1 μg of seed total RNA with Superscript II (Invitrogen, Burlington, ON, Canada) and used as a template for PCR amplification.

Preparation of plant expression constructs and transformation

The napin promoter (Josefsson et al. 1987) was amplified by PCR with the primers:

F1: 5′-ATAGAATTCAAGCTTTCTTCATCGGTGAT-3′ (*Eco*RI site is underlined) and R1: 5′-ATACCCGGGGTCCGTGTATGTTTTTAATC-3′ (*Sma*I site is underlined). A *P. granatum FADX* ORF (GenBank# AY178446) was amplified with the primers: F2: 5′-TATCCCGGGATGGGAGCTGATGGAACA-3′ (*Sma*I site is

underlined) and R2: 5′-CGCGCGGCCGCTCAGA ACTTGCTCTTGAAC-3′ (*Not*I site is underlined).

The NOS terminator (Bevan 1983) was generated by PCR with the primers:

F3: 5′-CGCCGGCGGCCGCGATCGTTCAAACATTT GGCA-3′ (*Not*I site is underlined) and R3: 5′-TATGGTAC CCGATCTAGTAACATAGATGAC-3′ (*Kpn*I site is underlined).

Subsequently, napin, *PgFADX* and NOS PCR fragments digested with the appropriate endonucleases were ligated into *Eco*RI and *Kpn*I sites of pRD400 (Datla et al. 1992) and that resulting in the NCJ construct.

Then, the napin promoter was amplified with the primers:

F4: 5′-ATAGGTACCAAGCTTTCTTCATCGGTGAT (*Kpn*I site is underlined) and R4: 5′-ATACTCGAGGTCCG TGTATGTTTTTAATCT-3′ (*Xho*I site is underlined). A *P. granatum FAD2* ORF (GenBank# AY178447) was generated by PCR with the primers: F5: 5′-TAACTCGAGATG GGAGCCGGTGGAAG-3′ (*Xho*I site is underlined) and R5: 5′-TATTCTAGATCAGAGGTTCTTCTTGTAC-3′ (*Xba*I site is underlined).

The NOS terminator was amplified with the primers:

F6: 5′-TATTCTAGAGATCGTTCAAACATTTGGCA A-3′ (*Xba*I site is underlined) and R6: 5′-ATAGTCGACC GATCTAGTAACATAGATGAC-3′ (*Sal*I site is underlined). Subsequently, napin, *PgFAD2* and NOS PCR fragments digested with the appropriate endonucleases were cloned into *Kpn*I and *Sal*I sites of NCJ resulting in the NCJD construct.

The binary vectors: NCJ or NCJD were electroporated into *Agrobacterium tumefaciens* cell strain GV3101 (Koncz and Schell 1986) and transformed into *A. thaliana fad3/fae1* (Smith et al. 2003) mutant background using the floral dip method as described earlier by Clough and Bent (1998). Transgenic plants were selected and analyzed as described by Mietkiewska et al. (2007). *Arabidopsis thaliana* plants were grown in a growth chamber at 22 °C with a photoperiod of 18 h light and 250 μmol m^{-2} s^{-1} light intensity.

Gene expression analysis

Total RNA was extracted from T_4 transgenic *A. thaliana* seed lines and wild type at the beginning of the late developmental stage (14 days after flowering) using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). First-strand cDNA synthesis was performed using 1 μg of total RNA as template and the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Developing seeds were harvested at 14 DAF for gene expression analysis, since it has been shown earlier that the accumulation of polyunsaturated fatty acids starts to increase significantly from 14 to 18 DAF (Baud and Lepiniec 2009).

Quantification of *AtFAD2* transcripts in various transgenic lines was performed using 6 μL of a 1/10 dilution of cDNA as template. The reactions were performed as described by Chen et al. (2011) with minor modifications. In brief, the 25 μL reaction contained 12.5 μL of 2x SYBR-Green Master Mix (Molecular Biology Facility, University of Alberta, Canada), 1.2 μL of forward primer (10 μM; final concentration 48 nM) and 1.2 μL of reverse primer (10 μM; final concentration 48 nM). PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Gene expression analyses were carried out using three biological replicates. Three technical replicate reactions were performed with each cDNA sample and individual primer pairs. The *Arabidopsis* 18S small subunit nuclear ribosomal RNA gene was used as the internal reference gene (GenBank# NC001284) and the relative transcript abundance of the target gene in the individual seed sample was calculated using the comparative Ct method (Livak and Schmittgen 2001). The primers for the three prime untranslated regions (3′ UTR) of *AtFAD2* (GenBank# L26296) were: F7: 5′-AT GATGGTGAAGAAATTGTCG-3′ and R7: 5′-GTCATAA CACAACAAAATGGAC-3′. The primers for 18S rRNA were F8: 5′-CAAAACGGCTCCGAAACAA-3′ and R8: 5′-ACTGGCAGTCCCTCGTGAGT-3′.

Lipid analysis

Analysis of fatty composition of plant material was performed by homogenizing 4−5 mg of *A. thaliana* seeds in 1 mL of a mixture of chloroform and isopropanol (2:1, v/v). The homogenate was then dried under N_2 and transmethylated with 1 mL of 5 % sodium methoxide ($NaOCH_3$) in methanol at room temperature for 30 min. The FAMEs were extracted twice with hexanes and after drying under N_2 resuspended in 1 mL of iso-octane with internal standard (21:0, methyl heneicosanoin, 0.1 mg mL^{-1}).

For separation of individual lipid classes by thin-layer chromatography (TLC), lipids were extracted from 100 mg of *A. thaliana* seeds as described earlier (Bligh and Dyer 1959). Lipid extracts were separated by one-dimensional TLC on silica gel plates (SIL G25, 0.25 mm, Macherey-Nagel, Düren, Germany) using the two solvent systems. The TLC plates were placed in the chamber with chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, by vol.) for running until the first solvent reached halfway up the plate. Then, the plate was moved into the second solvent, hexane/diethyl ether/glacial acetic acid (70:30:1, by vol) and developed until the solvent was 1 cm from the top. Lipid classes were visualized under UV after spraying with 0.05 % primuline solution. Spots corresponding to

TAG and PC were scraped out and transmethylated with 5 % sodium methoxide in methanol at RT for 30 min. The FAMEs were extracted with hexanes and dried under N_2. Finally, FAMEs were resuspended in 1 mL of iso-octane with an internal standard (21:0, methyl heneicosanoin, 0.1 mg mL^{-1}). The FAMEs were analyzed as described by Mietkiewska et al. (2011). ODP values were calculated as described earlier (Singh et al. 2001).

Results

Overexpression of *PgFADX* in *A. thaliana*

As described before, seed-specific overexpression of *P. granatum* conjugase (*PgFADX)* in the wild-type *A. thaliana* background led to the limited accumulation of punicic acid of up to 4.4 % (Iwabuchi et al. 2003). The level of linoleic acid which serves as a precursor for punicic acid synthesis is limited in the wild-type seeds of *A. thaliana* background to 27 % (Zhou et al. 2006). Therefore, to increase the production of punicic acid, we decided to overexpress *PgFADX* in a *A. thaliana fad3/fae1* mutant background which has an increased content of linoleic acid in the seed oil of up to 51 %, due to inhibition of Δ^{15}-desaturase (FAD3) and fatty acid elongase (FAE1) activity (Smith et al. 2003). We developed a binary vector NCJ carrying *PgFADX* under the control of a seed-specific napin promoter and expressed in *A. thaliana fad3/fae1*. Twenty eight kanamycin resistant plants were grown to seed maturity and seeds were collected individually from each plant. The fatty acid composition of T_2 segregating seeds collected from all plants is shown in Table 1. Significant changes in fatty acid composition in comparison to the control lines (nt-*fad3/fae1*) were found. Seed-specific expression of *PgFADX* resulted in an increased proportion of punicic acid from 0 % in the control lines up to an average of 6.43 % of total fatty acids. In the highest transgenic line NCJ-11, punicic acid accounted for as much as 11.26 % of total fatty acids in T_2 segregating seeds. The increased proportion of punicic acid was correlated with a concomitant reduction in the proportion of its corresponding precursor 18:2 (reduced on average by 33 %). The production of punicic acid in *A. thaliana* seeds was also accompanied on average by a 34 % increase in oleic acid content. This indicates that overexpression of *PgFADX* led to the inhibition of the native FAD2 desaturase activity. Decreases in the proportion of palmitic acid were also found in *A. thaliana* seeds engineered to produce punicic acid. Seeds from the T_2 lines with the highest proportion of punicic acid were grown to obtain the T_3 seed generation. As shown in Fig. 1, a consistent level of punicic acid production was observed in T_3 seeds. The proportion of punicic acid increased from 0 % in wild-type and null segregant controls to as high as 11.2 and 11.5 % in the highest homozygous transgenic lines NCJ: 19-2 and 11-4, respectively. As in T_2 segregating seeds, the production of punicic acid in the T_3 generation was accompanied by a significant increase in oleic acid to levels of 50 % of the total fatty acids. Furthermore, seedlings of NCJ transgenic lines germinated as well as the corresponding controls (data not shown). The 100-seed weight averaged across eight T_3 lines was 2.169 ± 0.049 mg (average $\pm$ standard error), compared to 2.226 ± 0.098 mg for plants of the non-transformed control. The average total fatty acid content of eight transgenic lines was 0.224 ± 0.004 mg/mg of seeds, compared with 0.211 ± 0.006 mg/mg for non-transformed control lines. Statistical analysis (*t* test) showed that the difference of 100-seed weight as well as total fatty acid content was not significant ($P < 0.05$) between the controls and the NCJ transgenic lines. Taken together, these data indicate that the accumulation of punicic acid has no detrimental effect on the size or total fatty acid accumulation in comparison with non-transformed controls.

Overexpression of *PgFADX* and *PgFAD2* in *A. thaliana*

Since overexpression of *PgFADX* affected the activity of the native *A. thaliana* FAD2 desaturase, we decided to study if combined overexpression of *PgFAD2* with *PgFADX* could reduce the negative effect on AtFAD2 desaturase activity and increase the accumulation of punicic acid. We developed a tandem construct (NCJD) carrying both genes under the control of napin promoters and introduced into the *A. thaliana fad3/fae1* mutant. As shown in Table 1, the level of punicic acid found in lines overexpressing both *PgFADX* and *PgFAD2* was higher compared to the lines overexpressing only *PgFADX*. On average the level of punicic acid was 9.2 % of total fatty acid in the seed oil, which was 43 % higher compared to the average level found in NCJ transgenic lines. The average level of oleic acid (33.5 %) found in the NCJD lines was lower compared with the level observed in lines overexpressing only *PgFADX* (NCJ) and was equivalent to the proportion observed in non-transformed *A. thaliana fad3/fae1* seeds. These results indicate that overexpression of *PgFAD2* could compensate for the negative effect of *PgFADX* on AtFAD2 desaturase activity observed in NCJ lines. Seeds from the best T_2 transgenic lines with punicic acid content over 12 % were selected and grown to the T_3 seed generation. As shown in Fig. 2, punicic acid accounted for as much as 21.2 % of total fatty acids in seeds of NCJD-30-2 and 34-3 transgenic lines representing a 61 % and 43 % increase, respectively, compared to the T_2 parental lines. Increased production of punicic acid in

Table 1 Fatty acid composition of transgenic *A. thaliana* T_2 seed oils

Construct	Fatty acid composition					
	16:0	18:0	18:1Δ^9	18:2$\Delta^{9,12}$	18:3$\Delta^{9,12,15}$	Punicic acid
	Percentage (w/w) of total fatty acids {range} [% increase [(a)] or decrease [(b)]][A]					
NCJ	6.84 ± 0.05 {6.07−7.60}	3.12 ± 0.03 {2.69−3.45}	45.03 ± 0.37 {37.62−48.16} [34.06[a]]	34.82 ± 0.52 {25.76−48.94} [33.0[b]]	0.62 ± 0.01 {0.50−1.02}	6.43 ± 0.39 {0.3−11.26}
NCJD	7.04 ± 0.07 {6.5−7.87}	3.46 ± 0.04 {3.09−3.86}	33.53 ± 0.44 {30.74−44.65}	42.12 ± 0.81 {32.74−49.85} [18.95[b]]	0.58 ± 0.02 {0.51−0.66}	9.21 ± 0.39 {1.54−15.24}
nt-*fad3/fae1*	8.15 ± 0.09 {8.04−8.39}	3.57 ± 0.05 {3.51−3.67}	33.59 ± 0.45 {32.70−34.06}	51.97 ± 0.31 {51.96−52.59}	0.96 ± 0.02 {0.93−1.01}	0.0

Results represent the average ± SE from 28 NCJ and 45 NCJD independent transgenic lines in *A. thaliana fad3/fae1* mutant background and 5 non-transformed controls (nt-*fad3/fae1*). Construct: NCJ: napin:*PgFADX*; NCJD: napin:*PgFADX*+napin:*PgFAD2*

[A] Relative to value for seeds from non-transformed *A. thaliana* (nt-*fad3/fae1*) controls, set at 100 %

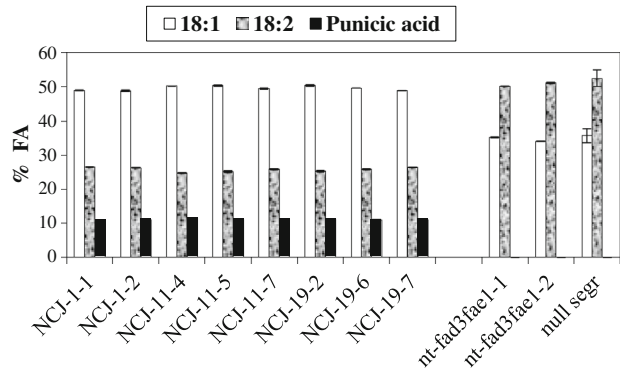

Fig. 1 Fatty acid composition of mature seed of non-transformed *A. thaliana fad3/fae1* mutant (nt-fad3fae1), null segregant (null segr) and T_3 seeds of transgenic *A. thaliana* lines (NCJ) overexpressing the *PgFADX* gene under the control of the napin promoter. The values are the average of three determinations ± SD

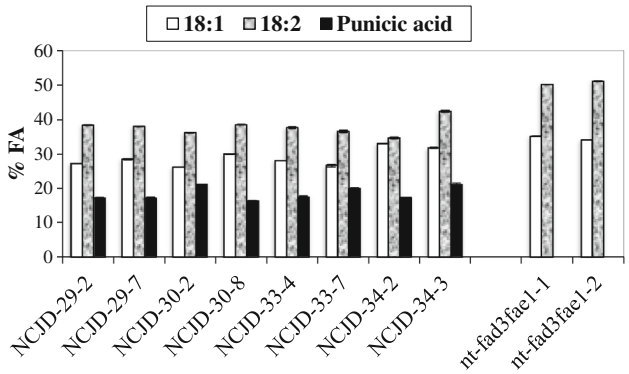

Fig. 2 Fatty acid composition of mature seed of non-transformed *A. thaliana fad3/fae1* mutant (nt-fad3fae1) and T_3 seeds of transgenic *A. thaliana* lines (NCJD) overexpressing *PgFADX* and *PgFAD2* genes under the control of the napin promoter. The values are the average of three determinations ± SD

these two NCJD lines correlated with decreased levels of oleic acid compared to non-transformed seed controls. This may indicate that T_3 NCJD seed lines overexpressing *PgFAD2* exhibited increased Δ^{12}-desaturase activity (Fig. 2).

Effect of *PgFADX* on oleate desaturation levels in transgenic seeds

As shown in Table 1, production of punicic acid in the *A. thaliana fad3/fae1* mutant was accompanied by increased accumulation of oleic acid. Therefore, to assess the cumulative effects of Δ^{12}-desaturase activity, we applied an indirect method developed by Singh et al. (2001). Oleic acid desaturation proportion (ODP) values were calculated for all T_2 NCJ and NCJD lines and controls (Fig. 3). Oleate desaturase is highly active in the developing seeds of wild-type *A. thaliana*, with 73 % of 18:1 being converted to 18:2 and 18:3, for an ODP value of 0.73 (Stoutjesdijk et al. 2002). In the *A. thaliana fad3/fae1* mutant background with affected Δ^{15}-desaturase activity, only 60 % of 18:1 is being further desaturated (ODP of 0.6, Fig. 3). NCJ transgenic lines overexpressing *PgFADX* exhibited a reduced level of Δ^{12}-desaturase activity (ODP of 0.44−0.57). Regression analysis revealed a significant inverse correlation ($R^2 = 0.761$) between the amount of punicic acid and ODP value; higher accumulation of punicic acid was associated with lower ODP values (Fig. 3a). For instance, in NCJ-11 line with the highest content of punicic acid (11.26 %), ODP was only 0.44 indicating that the amount of Δ^{12}-desaturated fatty acids was reduced by 27 % due to the overexpression of *PgFADX* compared to the

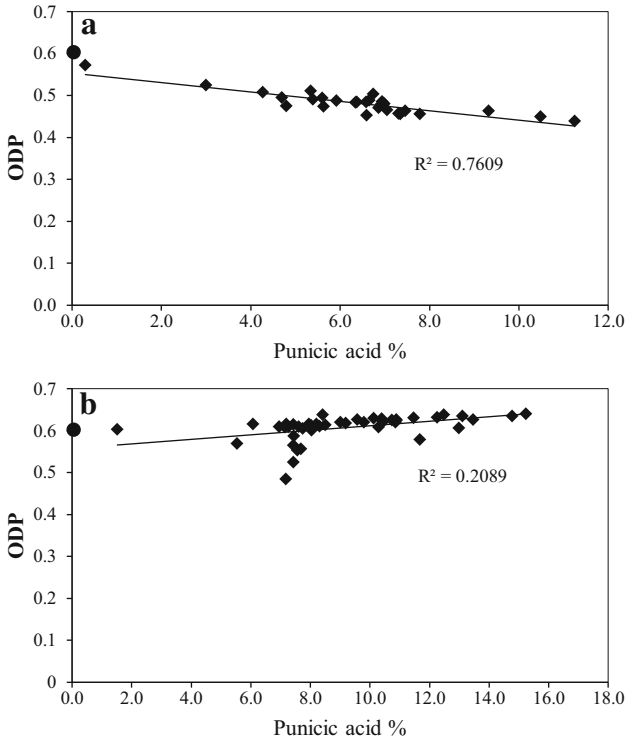

Fig. 3 Relationship between oleate desaturation proportion (ODP) value and level of punicic acid in transgenic *A. thaliana fad3/fae1* mutant in T_2 segregating seeds. **a** Data for transgenic NCJ lines overexpressing *PgFADX* under the control of the napin seed-specific promoter. **b** Data for the transgenic NCJD lines overexpressing *PgFADX* and *PgFAD2*. In both panels, *filled circle* shows non-transformed *A. thaliana fad3fae1* mutant

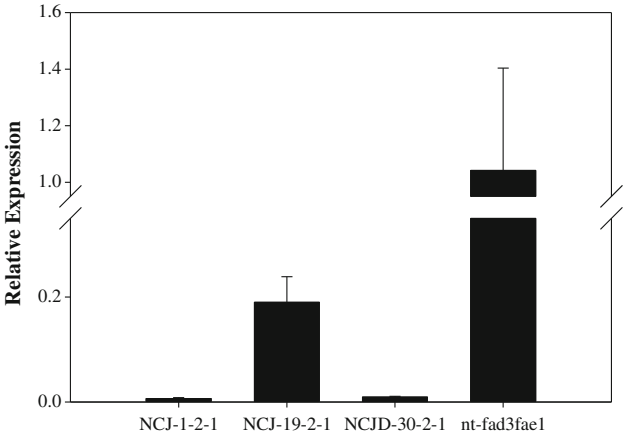

Fig. 4 Relative expression of *A. thaliana FAD2* gene in transgenic lines overexpressing *PgFADX* alone (NCJ lines) or in combination with *PgFAD2* (NCJD line) and non-transformed control (nt-fad3fae1). Total RNA was obtained from T_4 *A. thaliana* at 14 DAF. Equal amounts of total RNA were used for cDNA synthesis and serial dilutions of the resulting reaction were used for quantitative RT-PCR. Each bar represents the mean from three determinations ± SD with the *Arabidopsis* 18S small subunit nuclear ribosomal RNA gene as the internal reference gene

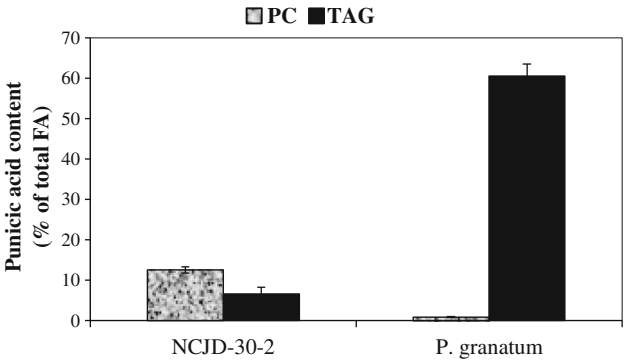

Fig. 5 Relative content of punicic acid in phosphatidylcholine (PC) and triacylglycerol (TAG) from *P. granatum* and *A. thaliana* T_3 seed line NCJD-30-2. The values represented by the *bars* are the average from analyses of three independent seed samples ± SD

non-transformed control. The linear regression analysis for NCJD lines exhibited a lower R^2 value (0.209), and there was also a change in the slope of the fitted line (Fig. 3b). The inverse relationship observed in lines overexpressing only *PgFADX* was found to be changed dramatically in the lines overexpressing both *PgFADX* and *PgFAD2*. The ODP levels for NCJD lines varied regardless of the level of punicic acid accumulation.

To further investigate the nature of FAD2 inhibition observed in *PgFADX A. thaliana* transgenic lines, we checked *AtFAD2* transcript levels in three independent transgenic T_4 homozygous seed lines at 14 DAF. For real-time PCR analysis, primers targeted to the 3′ UTR region of *AtFAD2* were used since *PgFADX* and *AtFAD2* exhibit high sequence identify (>65 %) in the coding region. As shown in Fig. 4, the relative *AtFAD2* expression level was significantly reduced (up to 99 %) in the *A. thaliana* seeds overexpressing *PgFADX* only (NCJ lines) or in combination with *PgFAD2* (NCJD line) compared to the non-transformed *fad3/fae1* mutant line background. These data may indicate that punicic acid that accumulated in *A. thaliana* seeds overexpressing *PgFADX* may act as a transcriptional repressor causing significant reduction of *AtFAD2* transcript level. Considering the high sequence identity (>65 %) of *PgFADX* and *AtFAD2*, the occurrence of post-transcriptional genes silencing in *PgFADX* transgenic lines cannot be completely excluded.

Relative content of fatty acid in phosphatidylcholine (PC) and triacylglycerol (TAG) from *A. thaliana* engineered to produce punicic acid

To investigate factors limiting the production of punicic acid in genetically engineered *A. thaliana* plants, we

decided to check fatty acid composition of the selected lipid classes. Examination of the fatty acid content of the selected lipid classes was performed for the *A. thaliana* line overexpressing *PgFADX*+*PgFAD2* (NCJD-30-2) with the highest content of punicic acid (21.2 %) in the T_3 seeds (Fig. 2) and *P. granatum* seeds. In transgenic NCJD-30-2 *A. thaliana* seeds, the punicic acid content of PC was 12.5 %, which was higher than that observed in TAG (6.6 %). On the contrary, in *P. granatum* seeds, punicic acid accounted for 60 % of the fatty acids in TAG and only 0.8 % of fatty acids in PC (Fig. 5). Taken together, the above results indicate that in *P. granatum* an efficient mechanism of trafficking of punicic acid from PC to TAG has evolved and that mechanism is missing in developing *A. thaliana* seeds.

Discussion

Metabolic engineering of oilseed crops to produce large amounts of conjugated and other industrial fatty acids has proven to be very difficult (Mietkiewska et al. 2014). Over the years of intensive studies, enzymes involved in the biosynthesis of many unusual fatty acids have been identified and corresponding genes have been cloned. Conjugated fatty acids were shown to be synthesized by a divergent form of Δ^{12}-desaturase (designated as FADX), either from oleic or linoleic acid precursors esterified to *sn-2* position of PC (Cahoon et al. 2007; Vanhercke et al. 2013). Transgenic expression of *FADX* genes in *A. thaliana* or soybean (*Glycine max*) resulted in limited accumulation of the desired fatty acid product compared with the levels in seeds from plants of the source species.

As described earlier, punicic acid was synthesized from linoleic substrate on *sn-2* position of PC by fatty acid conjugase (PgFADX) in *P. granatum* seeds. As with other unusual fatty acids, overexpression of *P. granatum FADX* led to the limited accumulation of punicic acid in *A. thaliana* seeds (Iwabuchi et al. 2003). Since the level of linoleic acid substrate is limited to <27 % in wild-type *A. thaliana* seeds, we overexpressed *PgFADX* in a high 18:2 *A. thaliana fad3/fae1* mutant background. This resulted in increased accumulation of punicic acid up to 11.3 % in T_2 segregating *A. thaliana* seeds (Table 1, Fig. 1) compared with the 4.4 % reported earlier by Iwabuchi et al. (2003). Our results have shown that a high 18:2 *A. thaliana* mutant background is a better host for transgenic production of punicic acid compared with the wild type. A similar observation was reported by Cahoon et al. (2006) when producing α-eleostearic and calendic acid ($18{:}3\Delta^{8trans,10trans,12cis}$) in *A. thaliana* seeds. The amounts of these fatty acids accumulated in seeds of wild-type background were below 5 % compared with more than 10 % found in the *A. thaliana fad3/fae1* mutant background overexpressing either the *M. charantia* or *C. officinalis* conjugase. Similar effects of increased vernolic acid (12,13-epoxy-$18{:}1\Delta^{9cis}$) production in response to the increased linoleic acid availability in the *A. thaliana fad3/fae1* double mutant were reported by Zhou et al. (2006). In contrast to the above, lack of such a direct relationship between substrate level (18:1) and ricinoleic acid (12-OH $18{:}1\Delta^{9cis}$) synthesis catalyzed by FAH12 (another type of FAD2 divergent enzyme) was reported earlier (Smith et al. 2003; Napier 2007).

The production of punicic acid obtained by overexpression of *PgFADX* was accompanied by changes in the relative proportion of other fatty acids in the seed oil. The most striking change was found for oleic acid which increased by up to 43 % compared to the seeds from non-transformed plants (Table 1, Fig. 1). This phenotype of reduced endogenous FAD2-mediated desaturase activity has also been observed in soybean somatic embryos producing α-eleostearic acid and *A. thaliana* seeds (Cahoon et al. 2006). An increased accumulation of oleic acid was also documented in transgenic plants overexpressing cDNAs encoding hydroxylases (Broun and Somerville 1997; Smith et al. 2003), epoxygenases (Singh et al. 2001; Li et al. 2010) and acetylenases (Thomaeus et al. 2001). In the current study, the suppression of AtFAD2 desaturase activity as indicated by lower ODP values correlated with higher punicic acid levels (Fig. 3a). To investigate the nature of this high oleic acid phenotype, we checked the *AtFAD2* transcript levels using a quantitative RT-PCR approach with primers targeted to the 3′ UTR of *AtFAD2*. The results clearly demonstrate a reduced level of *AtFAD2* mRNA in transgenic seeds at 14 DAF, which may suggest that accumulation of punicic acid may have repressed *AtFAD2* mRNA expression (Fig. 4). Similar to the current study, jacaric acid ($18{:}3\Delta^{8cis,10trans,12cis}$) and another isomer of CLNA ($18{:}4\Delta^{9cis,11trans,10trans,12cis}$) were shown to decrease the expression level of *stearoyl-CoA desaturase* mRNA in mouse (*Mus musculus*) liver (Lee et al. 1998; Shinohara et al. 2012). Also, the occurrence of post-transcriptional genes silencing in *PgFADX* transgenic lines cannot be completely excluded, since *PgFADX* and *AtFAD2* exhibit high sequence identity (>65 %). However, the possibility of decreased *AtFAD2* mRNA level was excluded before for *A. thaliana* overexpressing *Ricinus communis* (*RcFAH12*) or *Crepis palestina* epoxygenase (*Cpal2*) based on Northern blot analysis which showed unaltered *AtFAD2* transcript levels (Broun and Somerville 1997; Singh et al. 2001). This discrepancy could be possibly explained by the two different approaches used to check *AtFAD2* transcript level (i.e., Northern blot versus q-RT-PCR in our study). Secondly, there was a difference in the plant material used. The current study used late

developing seeds as a source of transcript, whereas earlier studies used entire siliques.

There are also other factors that could explain high oleate levels and low levels of punicic acid accumulation in transgenic plants. Given that AtFAD2 and PgFADX utilize membrane-linked substrates, impaired synthesis of 18:2 might result from inhibition of AtFAD2 desaturase activity by punicic acid. As we have shown (Fig. 5), punicic acid accounted for 12.5 % of total fatty acids of PC in transgenic *A. thaliana* compared to only 0.8 % in *P. granatum* seeds and that may have resulted in either the inhibition of AtFAD2 desaturase activity or in the reduction of oleic acid flux available for Δ^{12}-desaturation. This scenario has been discussed before by Napier (2007) in relation to other unusual fatty acids synthesized by divergent FAD2. As in the current study, higher relative amounts of unusual fatty acids in PC compared to TAG were previously reported for the transgenic production of acetylenic, epoxy, hydroxy and other conjugated fatty acids (Thomaeus et al. 2001; Cahoon et al. 2006).

We have shown here that combined overexpression of *PgFADX* with *PgFAD2* resulted in a 1.9-fold further increase in accumulation of punicic acid compared to the lines overexpressing only *PgFADX* along with restoration of oleic acid to the level found in non-transformed *A. thaliana fad3/fae1* (Figs. 2, 3b). Based on the data presented here, it should be noted that overexpression of an extra *PgFAD2* could restore Δ^{12}-desaturation to the level found in non-transformed plants even in the presence of punicic acid. As described previously for enhanced accumulation of vernolic acid in *Cpal2* + *Cpdes A. thaliana* (Zhou et al. 2006), the effect of an extra *PgFAD2* expression on the increased punicic acid production and restoration of Δ^{12}-desaturation in plants carrying *PgFADX*+*PgFAD2* is more likely related to the higher levels of FAD2 enzyme availability.

Comparison of fatty acid composition of PC and TAG revealed that in transgenic *A. thaliana* seeds, an efficient mechanism of trafficking punicic acid from its site of synthesis to the storage lipids is missing. By contrast, such a mechanism evolved in *P. granatum* seeds since punicic acid accounted for less than 0.8 % of total fatty acids in PC (Fig. 5). Similar to the studies described here, increased accumulation of other unusual fatty acids in PC was reported in *A. thaliana* engineered to produce hydroxy, epoxy, or conjugated fatty acids (Thomaeus et al. 2001; Cahoon et al. 2006; Napier 2007). As has also been shown for transgenic production of hydroxy and epoxy fatty acids (Burgal et al. 2008; Li et al. 2010), further increase in the transgenic production of punicic acid could be possibly achieved by combined overexpression of *PgFADX* and *PgFAD2* with cDNAs encoding specialized diacylglycerol acyltransferases and/or phospholipid:diacylglycerol acyltransferase from *P. granatum*. In addition, enzymes such as phospholipases C or D, lysophosphatidylcholine acyltransferase, or phospholipid:diacylglycerol cholinephosphotransferase sourced from *P. granatum* and introduced into transgenic plants might be useful for efficient removal of punicic acid from PC via acyl editing and exchange mechanisms (Vanhercke et al. 2013).

In conclusion, by using an *A. thaliana fad3/fae1* mutant host combined with seed-specific overexpression of *P. granatum conjugase* (*FADX*) with *FAD2*, the transgenic production of punicic acid was increased to 21.2 % of total fatty acids, the highest level reported to date. These results indicate that the transgenic production of very high levels of punicic acid will require identification and characterization of other specialized genes/enzymes involved in the metabolic network in plants naturally accumulating punicic acid.

Author contribution EM and RJW designed the research project, analyzed the data and wrote the manuscript. EM, RM, AW, AFS, MSG and GC conducted experiments and helped with data analysis. All authors read and approved the manuscript.

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