Bioactivity and biotechnological production of punicic acid

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Abstract

Punicic acid (PuA; 18: 3$\Delta^{9cis,11trans,13cis}$) is an unusual 18-carbon fatty acid bearing three conjugated double bonds. It has been shown to exhibit a myriad of beneficial bioactivities including anti-cancer, anti-diabetes, anti-obesity, antioxidant, and anti-inflammatory properties. Pomegranate (*Punica granatum*) seed oil contains approximately 80% PuA, and is currently the major natural source of this remarkable fatty acid. While both PuA and pomegranate seed oil have been used as functional ingredients in foods and cosmetics for some time, their value in pharmaceutical/medical and industrial applications are presently under further exploration. Unfortunately, the availability of PuA is severely limited by the low yield and unstable supply of pomegranate seeds. In addition, efforts to produce PuA in transgenic crops have been limited by a relatively low content of PuA in the resulting seed oil. The production of PuA in engineered microorganisms with modern fermentation technology is therefore a promising and emerging method with the potential to resolve this predicament. In this paper, we provide a comprehensive review of this unusual fatty acid, covering topics ranging from its natural sources, biosynthesis, extraction and analysis, bioactivity, health benefits and industrial applications, to recent efforts and future perspectives on the production of PuA in engineered plants and microorganisms.

Keywords

Conjugated linolenic acid, Metabolic engineering, Yeast biotechnology, Functional food, Triacylglycerol biosynthesis, Anti-cancer

Introduction

Conjugated linolenic acids (CLNA) are polyunsaturated fatty acids bearing three conjugated double bonds (alternating single and double bonds). The most common positional and geometric CLNA isomers in seed oil include punicic acid (PuA; 18: 3$\Delta^{9cis,11trans,13cis}$), α-eleostearic acid (18: 3$\Delta^{9cis,11trans,13trans}$), calendic acid (18: 3$\Delta^{8trans,10trans,12cis}$), jacaric acid (18: 3$\Delta^{8cis,10trans,12cis}$), and catalpic acid (18:3$\Delta^{9trans,11trans,13cis}$) (Fig. 1a; Smith 1971). PuA has drawn considerable interest over the past two decades as researchers continuously unravel its extensive array of beneficial properties. Among others, it has been shown to exhibit anti-cancer, anti-diabetes, anti-obesity, hypolipidemic, and anti-inflammatory activities through various *in vitro* and *in vivo* animal studies (Suzuki et al. 2001; Arao et al. 2004; Kohno et al. 2004; Koba et al. 2007; Boussetta et al. 2009; Grossmann et al. 2010; Costantini et al. 2014; Wang et al. 2014; Yuan et al. 2014; Aruna et al. 2016). While the seeds of pomegranate (*Punica granatum*, Fig. 1b) are the major natural source of PuA, this plant is not suitable for large-scale agronomic production due to its low yield, low seed oil production and restricted cultivation to sub-tropical and tropical climates (Takagi and Itabashi 1981; Joh et al. 1995). Consequently, due to its beneficial bioactivities and limited availability, efforts are ongoing to generate a biotechnological platform for PuA production through the metabolic engineering of plants and microorganisms (Mietkiewska et al. 2014a; 2014b; Garaiova et al. 2017). Although there is increasing interest in PuA production and utilization, a comprehensive review about PuA-related research is

lacking. Here we describe recent advances in PuA research, focusing on its bioactivities, natural sources, extraction, and biotechnological production in plants and microorganisms.

Natural sources, biosynthesis, extraction and analysis of punicic acid

PuA is naturally present as a component of triacylglycerol (TAG), which is a storage lipid making up the major constituent of vegetable oil, in the seeds of some terrestrial plant species. The most abundant natural source of this fatty acid is by far pomegranate (*P. granatum*), which is a member of the *Punicaceae* family (recently re-classified within the *Lythraceae* family). Pomegranate contains up to 80% PuA and less than 4% other CLNAs in its seed oil (Takagi and Itabashi 1981), the content of which depends on genotype and ranges from 12–20% of the seed weight (Özgül-Yücel 2005; Khoddami et al. 2014). While pomegranate is certainly the major source of PuA, seed oils from several species of the *Cucurbitaceae* family also contain relatively high amounts of this fatty acid, and include *Ecballium elaterium* (22%), *Fevillea trilobata* (30%), *Trichosanthes anguina* (43%), *T. bracteata* (42%), *T. nervifolia* (52%), *T. kirilowii* (40%), and *Momordica balsamina* (50%) (Chisholm and Hopkins 1964; Tulloch and Bergter 1979; Gaydou et al. 1987; Lakshminarayana et al. 1988; Joh et al. 1995).

To accumulate PuA in seed oil, these plant species have evolved a unique mechanism for both synthesizing this fatty acid and channeling it from phospholipids into TAG. TAG biosynthesis begins with fatty acid biosynthesis inside the plastid. The *de novo* synthesized fatty acids, mostly in the form of palmitic (16:0), stearic (18:0) and oleic acid (18:1Δ^{9cis}), are then converted to acyl-Coenzyme A (CoA) through the action of acyl-CoA synthetase (ACS) before being exported out of the plastid for TAG assembly (Ohlrogge and Jaworski 2003; Harwood 2005; Chapman and Ohlrogge 2012). In plants producing oils enriched in conjugated fatty acids, the nascent fatty acids at the level of phosphatidylcholine (PC) undergo further modifications such as desaturation and conjugation on the ER (Cahoon et al. 1999). Oleic acid in the *sn*-2 position of PC is first desaturated to linoleic acid (18:2$\Delta^{9cis,12cis}$) and α-linolenic acid (18:3$\Delta^{9cis,12cis,15cis}$) via the sequential catalytic action of fatty acid desaturase (FAD) 2 and FAD3, respectively (Browse et al. 1993; Vrinten et al. 2005). The subsequent formation of conjugated fatty acids is then catalyzed by fatty acid conjugases (FADXs), which are divergent forms of FAD2 (Hornung et al. 2002; Iwabuchi et al. 2003; Mietkiewska et al. 2014a). In the developing seeds of *T. kirilowii* and *P. granatum*, FADXs catalyze the conversion of the Δ^{12cis} double bond of linoleic acid to $\Delta^{11trans}$ and Δ^{13cis} double bonds to form PuA (Hornung et al. 2002; Iwabuchi et al. 2003). Similarly, FADXs in tung tree (*Aleurites fordii*) and *Momordica charantia* catalyze the conversion of the Δ^{12cis} double bond of linoleic acid to $\Delta^{11trans}$ and $\Delta^{13trans}$ double bonds to produce α-eleostearic acid (Cahoon et al. 1999; Dyer et al. 2002). In the case of calendic acid, FADX from *Calendula officinalis* catalyzes the conversion of the Δ^{9cis} double bond of linoleic acid to Δ^{8trans} and $\Delta^{10trans}$ double bonds (Cahoon et al. 2001; Qiu et al. 2001). The formation of conjugated double bonds catalyzed by FADXs resulting in the production of PuA and other C18 conjugated fatty acids, such as α-eleostearic and calendic acid, are depicted in Fig. 2.

Following the synthesis of conjugated fatty acids on PC, they can then be incorporated into TAG via several distinct acyl-editing routes (Fig. 2) (Chen et al. 2015; Bates 2016). TAG assembly occurs on the ER and involves the sequential acylation of *sn*-glycerol-3-phosphate (G3P) to yield TAG. This process is known as the Kennedy

pathway and is catalyzed by three acyl-CoA dependent acyltransferases, including *sn*-glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT) (Snyder et al. 2009). Phosphatidic acid phosphatase (PAP) catalyzes the removal of the phosphate group from the glycerol backbone prior to the final acylation step. Fatty acids, including those that are modified, may also be channeled from PC to TAG directly through the catalytic action of phospholipid:diacylglycerol acyltransferase (PDAT; Kim et al. 2011; van Erp et al. 2011; Pan et al. 2013). In addition, fatty acids modified on the *sn*-2 position of PC can enter the acyl-CoA pool via a reverse reaction catalyzed by lysophosphatidylcholine acyltransferase (LPCAT) (Stymne and Stobart 1984; Lager et al. 2013; Pan et al., 2015) or combined action of phospholipase A_2 (PLA_2) and long chain acyl-CoA synthetase (LACS; Lands 1960). The subsequent acylation of the resulting lysophosphatidylcholine (LPC) with an unmodified acyl-CoA through the forward action of LPCAT regenerates PC for further modifications. Exchange of the acyl groups between the *sn*-1 and *sn*-2 positions of PC may also occur through the catalytic action of glycerophosphocholine acyltransferase (GPCAT) and lysophosphatidylcholine transacylase (LPCT) (Lager et al. 2015). Furthermore, PC-modified fatty acids can also be incorporated into TAG through a *sn*-1,2-diacylglycerol (DAG) intermediate. In this instance, *de novo* synthesized DAG can be converted into PC through the catalytic action of CDP-choline:1,2-diacyl-*sn*-gycerol cholinephosphotransferase (CPT) (Slack et al. 1983; Slack et al. 1985), and converted back to DAG and/or phosphatidic acid (PA) once the acyl chains on PC have been modified via the catalytic action of phospholipase C and/or D, respectively (Chapman and Ohlrogge 2012; Bates et al. 2013). Finally, phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT) also catalyzes the conversion between PC and DAG (Lu et al. 2009; Wickramarathna et al. 2015; see Fig. 2 for a schematic diagram of TAG biosynthesis in plants producing conjugated fatty acids).

The commercial production of PuA largely relies on the extraction of seed oils from producer plants. Various extraction procedures, including cold pressing (Khoddami et al. 2014), solvent extraction with stirring (Abbasi et al. 2008), Soxhlet extraction (Abbasi et al. 2008; Habibnia et al. 2012), microwave irradiation or ultrasonic irradiation solvent extraction (Abbasi et al. 2008), supercritical CO_2 extraction (Abbasi et al. 2008; Liu et al. 2009; Sargolzaei and Moghaddam 2013), and superheated solvent extraction (Eikani et al. 2012) have been used to extract pomegranate seed oil. In general, the oil yield largely depends on the efficiencies of the different extraction methods. The lowest yields of 1~4% (dry weight, extraction efficiency < 22%) and 6.9% (dry weight, extraction efficiency 54%) are obtained from supercritical CO_2 extraction and cold pressing, respectively, whereas the highest yield of 22.18% (dry weight, extraction efficiency 124%) is obtained using superheated solvent extraction (Eikani et al. 2012). Although cold pressing results in low yield, this method provides an environmentally friendly process for pomegranate seed oil extraction and the resulting oils display enhanced physico-chemical properties including lower atherogenicity and higher thrombogenicity compared to oils extracted using organic solvents (Khoddami et al. 2014). Superheated solvent extraction provides a higher extraction efficiency and yields oil with a similar fatty acid profile to that obtained using the cold pressing approach (Eikani et al. 2012). Supercritical CO_2 extraction, on the other hand, yields oils with a similar fatty acid profile to those extracted using solvents, but results in an extracted oil with a higher tocopherol content (Liu et al. 2009).

Since the conjugated fatty acids derived from plant seed oils are usually composed of different positional and geometric isomers (Özgül-Yücel 2005), a reliable method for the separation and characterization of each conjugated fatty acid isomer is necessary. Gas chromatography (GC)-based methods are the most commonly used for the separation, quantification and identification of PuA and other conjugated fatty acids from plant seed oils (Joh et al. 1995; Cahoon et al. 1999; Cahoon et al. 2001; Hornung et al. 2002; Cahoon et al. 2006; Mietkiewska et al. 2014b; Garaiova et al. 2017). These methods, however, only provide information regarding the C=C double bond location rather than the bond configuration (i.e., *cis* versus *trans*) (Cao et al. 2007). Thus, they cannot be used to separate PuA from its CLNA isomers, which display very minor positional and geometrical differences in their structures. For example, GC in conjunction with acetonitrile chemical ionisation tandem MS was successfully used to determine both the position and configuration of the double bonds of conjugated linoleic acid (CLA) isomers (Michaud et al. 2003), However, when the same technique was applied to PuA and other CLNAs, only the double bond position, but not configuration, could be obtained (Lawrence and Brenna 2006). To fully characterize the double bond position and configuration of CLNA isomers, additional separation or characterization methods are required. These methods include thin layer chromatography (TLC) (Sita Devi 2003), capillary electrophoresis (Bohlin et al. 2003), gas liquid chromatography (Takagi and Itabashi 1981), silver ion impregnated high-performance liquid chromatography (Ag^+-HPLC) (Cao et al. 2006; Chen et al. 2007), and NMR spectroscopy (Cao et al. 2006; Cao et al. 2007; Sassano et al. 2009), all of which have been successfully applied to separate PuA from other CLNA isomers and thus provide alternative approaches for geometrical identification.

Bioactivity, health benefits and potential industrial uses of punicic acid

PuA has been reported to exhibit a host of beneficial therapeutic benefits (Fig. 3; reviewed by Shabbir et al. 2017, Yuan et al. 2014; AlMatar et al. 2017). As cancer remains to be the leading cause of death in developed countries, there is a need for a safe and acceptable bioactive oil that could be used in prevention and treatment. In the case of prostate cancer, pomegranate seed oil has been shown to suppress the proliferation of a number of different prostate cancer cell lines, including LNCaP, PC-3 and DU-145 (Albrecht et al. 2004). Although the other components of the pomegranate fruit (namely ellagic acid, caffeic acid and luteolin) also have anti-cancer activity against human prostate cancer cells (Lansky et al. 2005a), PuA has been demonstrated to have anti-cancer activity on its own and act synergistically with the other bioactives in pomegranate (Lansky et al. 2005a). Indeed, combining PuA, caffeic acid and luteolin in equal amounts (3 μg/mL) was reported to synergistically inhibit the invasive properties of PC-3 prostate cancer cells (Lansky et al. 2005b). PuA has also been shown to reduce the growth of LNCaP cells through effects on antiandrogenic and proapototic signals (Gasmi and Sanderson 2010). In another study involving a mouse (*Mus musculus*) model injected with human prostate cancer cells, PuA in combination with other pomegranate phytochemicals (luteolin and ellagic acid) inhibited the progression of tumor growth, migration and chemotaxis towards CXCL12, a chemokine involved in metastasis (Wang et al. 2014).

PuA (Grossmann et al. 2010) and a PuA-enriched pomegranate seed oil fraction (Costantini et al. 2014) were also found to inhibit the proliferation of triple negative (MDA-MB-231) and estrogen receptor positive (MCF-7)

breast cancer cells. These studies suggest that PuA induced apoptosis and mitochondrial membrane potential disruption, possibly through mechanisms related to lipid peroxidation and protein kinase C pathways (Grossmann et al. 2010) or through a reduction of inflammatory mediators (Costantini et al. 2014). There is also evidence for a beneficial effect of PuA or pomegranate seed oil and PuA against other forms of cancer, including bladder carcinoma (Wang et al. 2013), colon adenocarcinoma (Kohno et al. 2004, Constantini et al. 2014), skin cancer (Hora et al. 2003), liver cancer (Costantini et al. 2014) and leukemia (Suzuki et al. 2001).

PuA has also been found to have potentially beneficial effects on diabetes/insulin intolerance in various animal models (reviewed by Shabbir et al. 2017). For example, an obese rat strain with type II diabetes (Otsuka Long Evans Tokushima Fatty rats) fed with PuA exhibited reduced hepatic TAG compared to the control group (Arao et al. 2004). In this study, the mechanism of TAG reduction was partly attributed to the inhibition of a Δ9 desaturase. Similarly, in rats with streptozotocin-induced type II diabetes, the addition of pomegranate seed extract to their diet lowered their fasting blood glucose levels, thus reducing the incidence of obesity and insulin resistance (Das et al. 2001). However, in the same model, feeding PuA increased insulin secretion but did not change blood glucose levels (Nekooeian et al. 2014). It has been suggested that PuA may serve as an agonist of peroxisome proliferator-activated receptors (PPAR), which are present in adipose tissue and are common drug targets of anti-diabetic agents (Anusree et al. 2015). Pomegranate seed oil has also been shown to prevent obesity induced by a high-fat diet and enhance insulin sensitivity in mice (Vroegrijk et al. 2011), consequently reducing the tendency to acquire type II diabetes (McFarlin et al. 2009). Supplementation with PuA has also been shown to reduce the effects of diabetes in mouse models through its antioxidant and anti-inflammatory activities (Saha and Ghosh 2012). *In vitro* studies have suggested some other mechanisms behind PuA activity. For example, incubation with PuA stimulated adiponectin secretion and upregulated GLUT4 expression and translocation in adipocytes, which is possibly mediated by the high binding affinity of PuA to PPARγ (Anusree et al. 2014). Furthermore, mitochondrial dysfunction is observed in insulin resistant states such as diabetes, and PuA treatment improved glucose uptake and prevented changes in mitochondrial proteins associated with dysfunction in 3T3-L1 adipocytes (Anusree et al. 2015). More recent data from this group found that in this *in vitro* model, PuA prevented the deleterious effects of TNF-α on leptin and insulin receptor substrate production (Anusree et al. 2017). Despite these promising results, not all animal studies have found beneficial effects of feeding PuA/pomegranate seed oil (reviewed by Banihani et al. 2013) and further research is needed.

PuA may also have beneficial effects on a number of cardiometabolic risk factors. In several mice models, feeding PuA reduced adipose tissue accumulation and suppressed adipogenesis (reviewed by Shabbir et al. 2017). For example, mice supplemented with PuA have been shown to display decreased body fat mass, possibly through the stimulation of carnitine-palmitoyl transferase in adipose tissues (Koba et al. 2007), while mice supplemented with PuA exhibited reduced perirenal and epididymal adipose tissues and decreased hepatic TAG accumulation (Yuan et al. 2009). Consistent with this, supplementation with pomegranate seed oil has been shown to lower TAG in the plasma lipids of hypercholesterolemic rats (Elbandy and Ashoush 2012). PuA has also been shown to display anti-inflammatory activity in mice and sheep (reviewed by Shabbir et al. 2017, Yuan et al. 2015). In a rat model with 2, 4, 6-trinitrobenzenesulfonic acid-induced colitis, feeding PuA relieved colon inflammation by inhibiting

TNFα-induced priming of NADPH oxidase, an enzyme associated with the intestinal inflammatory response (Boussetta et al. 2009). In other studies, PuA has been shown to relieve intestinal inflammation and activate PPARγ, a key regulator of inflammatory and immune responses (Bassaganya-Riera et al. 2011; Yuan et al. 2015). In neonatal rats, oral administration of 1.5% pomegranate seed oil decreased the incidence and severity of necrotizing enterocolitis, a life threatening intestinal inflammatory condition observed in preterm infants (Coursodon Boyiddle et al. 2012). In this study, improved outcome was associated with improvements in intestinal integrity and decreased mRNA encoding inflammatory cytokines (Coursodon Boyiddle et al. 2012). Another mechanism for the anti-inflammatory effects of PuA may be its antioxidant properties (Saha and Ghosh 2009; Saha and Ghosh 2012), which likely contribute to the anti-nephrotoxic effects reported in rats (Boroushaki et al. 2014).

In summary, there is a growing body of literature that ingesting PuA may have beneficial effects on a variety of chronic health conditions. Although most of this work has been done in cell culture and animal models, PuA and other pomegranate-derived phytochemicals have been available on the market for a number of years as a nutraceutical, primarily in the form of powdered capsules (Newman et al. 2007). Carefully conducted clinical trials are needed to determine the potential benefits of this bioactive lipid for potential use in the prevention and treatment of chronic diseases.

Although the use of PuA as a functional food product has been well-established, the possible industrial application of this fatty acid has yet to be explored in depth even though other CLNAs have been widely used in a number of industries. For example, α-eleostearic acid, which is found at high levels in tung tree oil, has been used for many years as an industrial drying oil for coating wood and as a component of different inks, coatings, and resin formulations (He et al. 2014). CLAs have also been used in the poultry industry as a feed supplement to improve meat quality (Suksombat et al. 2007; Cho et al. 2013; Jiang et al. 2014). The fact that PuA has limited availability as it is exclusively extracted from seeds that are not readily available almost certainly contributes to this lack of industrial interest, and it is therefore likely that the development of sustainable alternative sources of PuA would enable its full exploitation.

Production of punicic acid in plants via genetic engineering

Although a handful of plant species are known to naturally produce seed oils enriched in conjugated fatty acids (Smith 1971; Badami and Patil 1980; Takagi and Itabashi 1981; Joh et al. 1995), these plants (including pomegranate) usually possess challenging agronomic characteristics and are therefore not suitable for large-scale or widespread production. As a result, the price of pomegranate seed oil is generally very high, with the cost of oil of unknown quality varying from $2,000 - $100,000 USD per metric tonne (based on prices from 50 suppliers on www.alibaba.com, Accessed 15 November 2017). Therefore, one promising strategy to address our need for conjugated fatty acids is to produce them via the metabolic engineering of established oilseed crops. Varying degrees of success have been achieved thus far in the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*) and oilseed crops [e.g., canola (*Brassica napus*)] in terms of their genetic manipulation to produce conjugated fatty acids in the seed oil. However, even in the highest accumulators only exhibited modest PuA production at best (Table 1).

Both TkFADX (from *T. kirilowii*) and PgFADX (from *P. granatum*) have been found to recruit linoleic acid as substrate and convert its Δ12-double bond into conjugated $\Delta^{11trans}$ and $\Delta^{13trans}$ double bonds to form PuA (Hornung et al. 2002; Iwabuchi et al. 2003). These enzymes are bifunctional as they also exhibit Δ12-oleate desaturase activity (Iwabuchi et al. 2003). As expected, the expression of *PgFADX* and *TkFADX* in *Arabidopsis* led to the accumulation of PuA, but only at levels up to 4.4% (w/w) and 10.2% (w/w) of the total fatty acids in seeds, respectively (Iwabuchi et al. 2003). Similarly, over-expression of *TkFADX* in canola-type *B. napus* resulted in the production of transgenic lines that accumulated PuA up to only 2.5% of the seed oil (Koba et al. 2007). This limited accumulation of PuA in the seed oils of these transgenic plants may be due to the poor availability of the linoleic acid substrate for FADX, with less than 27% and 20% linoleic acid present in wild-type *Arabidopsis* and *B. napus* seeds, respectively. In addition, the low accumulation of PuA in transgenic *Arabidopsis* expressing *FADX* cDNAs was also accompanied by elevated levels of oleic acid, suggesting that the activity of FAD2 was somehow inhibited in these lines (Iwabuchi et al. 2003). Similar effects have also been observed in transgenic plants expressing cDNAs encoding other FAD2-like enzymes (Napier 2007). It is therefore possible that the conjugated fatty acid product may trigger the transcriptional repression of genes encoding other relevant enzymes in its biosynthetic pathway (Song et al. 2017). Additionally, post-transcriptional gene silencing may occur in *PgFADX* transgenic lines considering the high sequence identity (>65%) between *PgFADX* and *AtFAD2*, and the fact that reduced *AtFAD2* expression levels were observed in *Arabidopsis* plants expressing *PgFADX* (Mietkiewska et al. 2014b). To address these issues, *PgFADX* was expressed either alone or in combination with *P. granatum FAD2* in an *Arabidopsis fad3fae1* mutant background, leading to the accumulation of PuA in seed oil up to 11.5% in *PgFADX* lines and up to 21.0% in *PgFAD2* + *PgFADX* over-expression lines (Mietkiewska et al. 2014b). *Arabidopsis fad3fae1* mutant lines lack the activities of FAD3 and the fatty acid elongase 1 (FAE1) condensing enzyme, and thus provide a suitable fatty acid background with more than 50% linoleic acid available for conjugated fatty acid production (Smith et al. 2003). Along these same lines, when *PgDGAT2* was expressed in conjunction with *PgFADX* and *PgFAD2*, the resulting PuA content in seeds increased up to 24.8% in *Arabidopsis fad3fae1* transgenic lines. The efficiency with which the promoter contained within the transgenic cassette drives the expression of the *PgFADX* cDNA may also affect the yield of PuA in engineered plants. While the napin promoter was used in the aforementioned studies, the linin promoter has been found to be the most efficient for this purpose, leading to the accumulation of PuA in *Arabidopsis* seeds up to 13.2% of the total fatty acid content, which is 30% higher than that obtained using the napin promoter (Song et al. 2017). Considerable effort is also being devoted to the production of PuA in established oilseed crops, including canola-type *B. napus* and flax (*Linum usitatissimum*), and the results are promising (Weselake and Mietkiewska, 2014).

Considering that up to 40% and 80% PuA accumulates in the oil of *T. kirilowii* (Joh et al. 1995) and *P. granatum* (Takagi and Itabashi 1981) seeds, respectively, the level of PuA that accumulates in transgenic plants has been modest at best. A major challenge that hinders the production of conjugated fatty acids in these plants involves the inefficient trafficking of conjugated fatty acids from PC into TAG (Cahoon et al. 2006; Mietkiewska et al. 2014a; 2014b; Napier et al. 2014). Indeed, in contrast to *P. granatum* seeds in which PuA is predominantly present in TAG (60%) rather than PC (0.8%), transgenic *Arabidopsis* co-expressing *PgFADX* and *PgFAD2* accumulated more PuA

in PC (12.5%) than TAG (6.6%) (Mietkiewska et al. 2014b). Therefore, it appears that native plants that naturally accumulate conjugated fatty acids have evolved unique mechanisms for efficiently channeling these fatty acids into TAG following their synthesis on PC (Mietkiewska et al. 2014a). To further increase conjugated fatty acid production in non-native species, it will therefore be necessary to first identify native acyl-trafficking enzymes from plants accumulating conjugated fatty acids and introduce them along with other necessary enzymes. Such an approach has shown great promise in terms of improving the accumulation of other unusual fatty acids. For instance, hydroxy fatty acid production was attained via the co-expression of cassettes encoding specialized acyltransferases and acyl-editing enzymes, including DGAT, PDAT, phospholipase A and PDCT (Burgal et al. 2008; van Erp et al. 2011; Pan et al. 2013; Bayon et al. 2015; Wickramarathna et al. 2015). It has also been suggested that the introduction of exogenous lipid biosynthetic machinery from other plant sources into oilseed crops may lead to competition with the endogenous enzyme network, which could impose a limitation on accumulation of the desired target fatty acid (Vanhercke et al. 2013; van Erp et al. 2015). This is supported by recent research on producing unusual fatty acids in transgenic plants in which the accumulation of unusual fatty acids was limited by the competition between endogenous and transgenic isozymes (van Erp et al. 2015). Therefore, it may be possible to further enhance the accumulation of conjugated fatty acids in transgenic plants by reducing this competition through silencing the expression of endogenous genes encoding the enzymes which compete with those that are introduced.

Moreover, since TAG is exclusively stored in lipid droplets, it has been suggested that plant seeds accumulating unusual fatty acids may have developed a mechanism allowing them to possess two or more pools of lipid droplets, each exclusively enriched in different TAG species. For instance, one pool of lipid droplets containing TAG enriched in common fatty acids might serve to provide precursors for the generation of cell membranes and signaling, whereas lipid droplets enriched in TAG species containing PuA might play a different role in seeds (e.g., germination, protection from predators, attraction of animals for its nutritional effects). The process by which various types of lipid droplets may coexist in a single cell is currently being investigated (Wolins et al. 2005; Fujimoto and Parton 2011; Hsieh et al. 2012; Ohsaki et al. 2014). Such studies might shed additional insight into PuA production in both engineered plants and microorganisms (as described in the section below) in the future.

Biotechnological production of PuA in microorganisms

Although plants naturally accumulating PuA have great industrial potential, many factors such as plant over-utilization, climate-dependency, large space requirements and sensitivity to the environment are limiting in terms of the ever increasing demand of the growing market. In contrast, microorganisms could provide a less challenging alternative for PuA production due to their capacity to recycle industrial waste, minimal space requirements for controlled cultivation, rapid growth and wide availability of genetic resources and tools (Ledesma-Amaro 2015; Liu et al. 2017). For example, oleaginous microorganisms are considered a suitable source for renewable fuel production since these organisms accumulate more than 20% lipids per dry cell weight. Among them, the oleaginous yeast *Yarrowia lipolytica*, which is recognized as a safe microorganism for humans, has been successfully employed to produce a variety of fatty acids, including CLAs (reviewed in Ledesma-Amaro and Nicaud 2016). As an example, in

the case of 18:2$\Delta^{10trans,12cis}$ CLA production, a strategy employing soybean-based growth media combined with multi-copy integration and co-expression of heterologous genes was used to greatly enhance its accumulation (Zhang et al. 2013; Ledesma-Amaro and Nicaud 2016). The lack of efficient and established genetic manipulation methods in oleaginous microorganisms, however, has restricted their widespread use until very recently.

To date, only a small number of research groups have investigated the recombinant production of enzymes required for the synthesis of PuA in microorganisms. For example, the activities of native FADX from *P. granatum* (PgFADX) and *T. kirilowii* (TkFADX) have been characterized in the yeast *Saccharomyces cerevisiae* (Hornung et al. 2002; Iwabuchi et al. 2003). In these studies, the formation of PuA in strains heterologously expressing the corresponding cDNAs was not detected. Instead, linoleic acid and hexadecadienoic acid (16:2 $\Delta^{9cis,12cis}$) accumulated up to 1.2% (w/w), confirming that these FADX enzymes possessed FAD2 activity (Hornung et al. 2002; Iwabuchi et al. 2003). Further experiments have shown that PuA is only detected in strains expressing *FADX* after supplementation of the culture media with linoleic acid and that the accumulation of PuA was reduced at lower cultivation temperatures, which is in contrast to linoleic acid and hexadecadienoic acid formation derived from FAD2 desaturase activities (Hornung et al. 2002). In both studies, however, the heterologous production of PuA in *S. cerevisiae* reached less than 2 % (w/w) of total fatty acids, suggesting that as is the case for plants, additional modifications will be necessary to further improve PuA accumulation.

Recently, we metabolically engineered the fission yeast *Schizosaccharomyces pombe*, which naturally has a high oleic acid content, to produce PuA by heterologously co-expressing codon optimized *PgFAD2* and *PgFADX* coding sequences under the control of the strong, inducible, *nmt1* promoter (Garaiova et al. 2017). In contrast to previous studies carried out in *S. cerevisiae*, expression of *PgFADX* on its own resulted in the production of PuA at levels up to 19.6% (w/w) of total fatty acids without any fatty acid supplementation. In addition to PuA accumulation, a limited production of linoleic acid up to 2.2% of total fatty acids was also observed in these strains. Co-expression of codon optimized *PgFADX* with *PgFAD2* resulted in a further increase in PuA content up to 25.1% of total fatty acids (corresponding to 38.7 µg PuA/mL culture). In addition, differences were also noted in PuA accumulation dynamics between single and double expression strains. In cells expressing *PgFADX* alone, the level of PuA was steadily high from day 3 to day 6, with the maximal content occurring on day 4. In the case of cells co-expressing *PgFAD2* and *PgFADX*, PuA content only peaked at days 2 and 3. Interestingly, the accumulated PuA in *S. pombe* expressing *PgFADX* is mainly found at a single position of the glycerol backbone of TAG (Fig. 4), which is in contrast with pomegranate seed oil, where the majority of PuA incorporated into TAG occupies all three positions of the glycerol backbone (Fig. 4; Kaufman and Wiesman 2007). This indicates that *S. pombe* may lack the enzyme specificities that are needed to maximize PuA accumulation in TAG.

The results obtained from our studies with *S. pombe* imply that metabolically engineered microorganisms can potentially represent an alternative source of PuA, and even higher yields of PuA could be expected in the event that oleaginous microorganisms were to be similarly engineered. Recently, CRISPR-Cas9 technology for multigene editing of the *Y. lipolytica* genome was established (Gao et al. 2016), thus providing an efficient and precise tool that might pave the way for designing industrial microbial strains that rapidly generate PuA. Other cutting edge

approaches such as metabolome (Pomraning et al. 2015), transcriptome and proteome analyses (Horn et al. 2016), cDNA library screening (Yazawa et al. 2013), lipid body proteome analysis (Zhu et al. 2015) and *in silico* metabolic engineering (Zhang and Hua 2015) may also help to identify key players required for the efficient heterologous production of this unusual fatty acid in microorganisms. As seems to be the case in plants, high levels of microbial-based PuA production may require the heterologous co-overexpression of acyltransferases (e.g., DGAT and PDAT) from plants naturally producing PuA along with modifications of enzymes involved in lipid remodeling processes in order to redirect the flow of PuA from PC to TAG. Furthermore, blocking PuA degradation and decreasing any microorganism-specific toxicity might also enhance accumulation in this system. Indeed, it is anticipated that by combining a variety of these strategies, we will begin to reach, and potentially surpass, PuA contents of 60-80% total fatty acids within microbial cells as is observed in the seed oils of plants that naturally produce this bioactive fatty acid.

Conclusions and future perspectives

PuA is being studied extensively for its beneficial effects in terms of alleviating cancer, diabetes, obesity, and inflammation, among others. As researchers continue to expand our knowledge regarding its wide range of bioactivities, interest in the use of this fatty acid as a functional food product and nutraceutical will continue to grow. However, the full exploitation of PuA for food, medical, and possibly industrial applications will require the establishment of a viable alternative source due to the fact that natural sources of PuA are not amenable to widespread agronomic production. As the biosynthetic genes for PuA production are already well-characterized, and those likely to be required for high levels of expression are in the process of being deciphered, a genetic toolkit is well on its way for biotechnological production efforts. Recently, *Arabidopsis* and *S. pombe* have been successfully engineered to produce this compound at moderate levels using genes derived from pomegranate, and as our synthetic biology tools become more advanced and readily available, future research involving the optimization of plant and microbial pathways will almost certainly result in further increases in PuA accumulation to reach its maximum potential in the future.

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Compliance with ethical standards

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

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

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Figure Legends

Fig 1. (a) Structures of conjugated linolenic acids commonly found in seed oil. Fatty acid structures were drawn using ChemDraw Prime (PerkinElmer Informatics); (b) pomegranate (*Punica granatum*) (Photograph by Roman Holic).

Fig 2. Schematic representation of triacylglycerol (TAG) biosynthesis and acyl-editing in plants producing oils containing conjugated fatty acids. Fatty acid modification, such as desaturation and conjugation, occurs on the *sn*-2 position of phosphatidylcholine (PC). In major oil crops, linoleic acid ($18{:}2\Delta^{9cis,12cis}$) and α-linolenic acid ($18{:}3\Delta^{9cis,12cis,15cis}$) are synthesized from oleic acid ($18{:}1\Delta^{9cis}$) via the sequential catalytic action of fatty acid

desaturase (FAD) 2 and FAD3. In plant species producing conjugated fatty acids, the formation of conjugated fatty acids is catalyzed by fatty acid conjugases (FADXs), which are a divergent form of FAD2, using linoleic acid or α-linolenic acid as substrates. Other abbreviations: ACS, acyl-CoA synthetase; CPT, choline phosphotransferase; DAG, *sn*-1,2-diacylglycerol; DGAT, diacylglycerol acyltransferase; FA, fatty acid; FAE, fatty acid elongase; GPAT, *sn*-glycerol-3-phosphate acyltransferase; GPC, glycerophosphocholine; GPCAT, glycerophosphocholine acyltransferase; G3P, *sn*-glycerol 3- phosphate; LPA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcoline; LPCAT, lysophosphatidylcholine acyltransferase; LPCT, lysophosphatidylcholine transacylase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PDAT, phospholipid:diacyglycerol acyltransferase; PDCT, phosphatidylcholine: diacylglycerol cholinephosphotransferase; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; TAG, triacylglycerol. Fatty acid structures were drawn using ChemDraw Prime (PerkinElmer Informatics).

Fig 3. Beneficial bioactivities of punicic acid found through studies involving *in vitro* and *in vivo* animal models (see section *Bioactivity, health benefits and potential industrial uses of punicic acid* for details).

Fig 4. Thin layer chromatography of pomegranate (*Punica granatum*) seed oil and neutral lipids of fission yeast *Schizosaccharomyces pombe* strains expressing empty vector (EV) and *PgFADX*, respectively. Detection of fatty acid composition of various triacylglycerol (TAG) species was performed by gas chromatography analysis. Abbreviations: *Erg,* ergosterol; *Lan,* lanosterol; *OA,* oleic acid; *PgFADX*, *Punica granatum* fatty acid conjugase gene; *SE,* steryl ester (cholesteryl oleate); *SQ,* squalene; *St,* standards; *TAG* (triolein); *TAG-A*, TAG containing one punicic acid (PuA) moiety; *TAG-B*, TAG containing two PuA moieties; *TAG-C*, TAG containing three PuA moieties; *TAG-N*, TAG containing no PuA; *TAG-T*, TAG containing traces of PuA.

739 Table 1. Examples of the production of PuA in transgenic plants.

Target gene(s)	Native species	Promoter	Transgenic plants/ engineered microorganism	PuA content (%, w/w)	Total lipid content (%, w/w)	References
FADX	*Punica granatum*	Napin	*Arabidopsis*	4.4	Not reported	(Iwabuchi et al. 2003)
FADX	*Trichosanthes kirilowiiI*	Napin	*Arabidopsis*	10.2	Not reported	(Iwabuchi et al. 2003)
FADX	*Trichosanthes kirilowiiI*	Napin	*Brassica napus*	2.5	Not reported	(Koba et al. 2007)
FADX	*Punica granatum*	Napin	*Arabidopsis fad3fae1* mutant	11.5	22.4%	(Mietkiewska et al. 2014b)
FAD2 +FADX	*Punica granatum*	Napin	*Arabidopsis fad3fae1* mutant	21	Not reported	(Mietkiewska et al. 2014b)
FAD2+FADX+ DGAT2	*Punica granatum*	Napin	*Arabidopsis fad3fae1* mutant	24.8	Not reported	(Weselake and Mietkiewska 2014)
FADX	*Punica granatum*	Linin	*Arabidopsis fad3fae1* mutant	13.2	Not reported	(Song et al. 2017)

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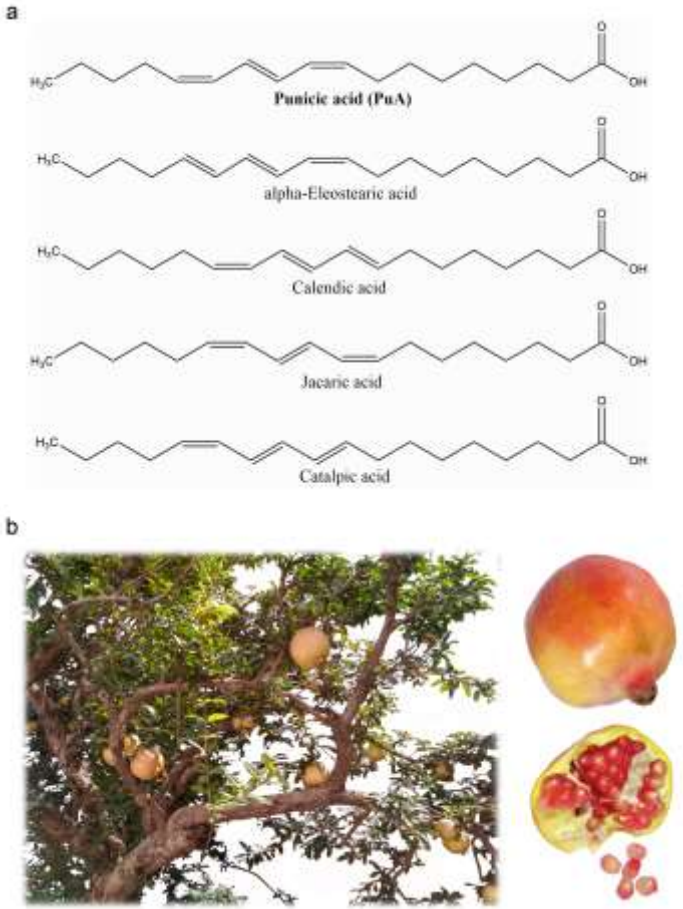
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Punicic acid (PuA)
alpha-Eleostearic acid
Calendic acid
Jacaric acid
Catalpic acid
b

Fig. 1

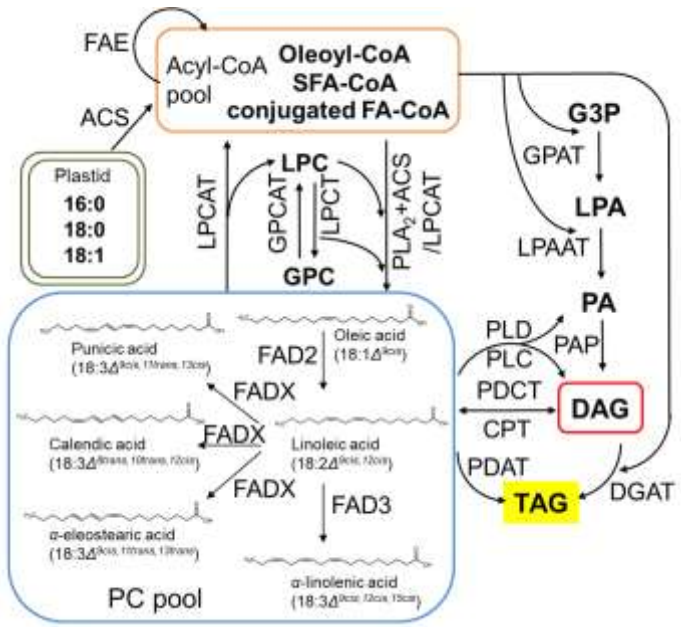
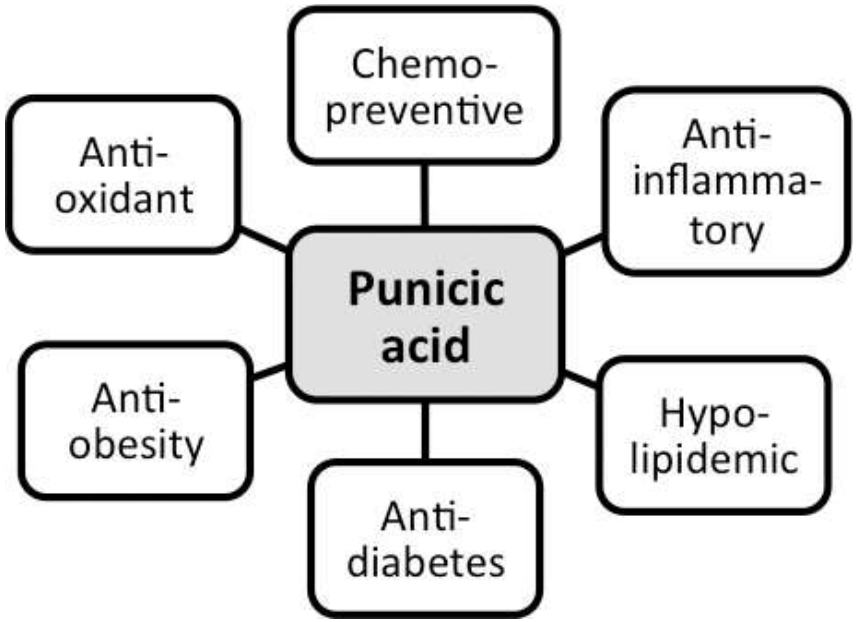

FAE
Acyl-CoA pool
Oleoyl-CoA
SFA-CoA
conjugated FA-CoA
ACS
Plastid
16:0
18:0
18:1
LPCAT
LPC
GPCAT
LPCT
GPC
PLA2+ACS /LPCAT
G3P
GPAT
LPA
LPAAT
PA
PLD
PLC
PAP
PDCT
CPT
DAG
PDAT
TAG
DGAT
Punicic acid
FAD2
Oleic acid
FADX
Calendic acid
Linoleic acid
FADX
FADX
FAD3
α-eleostearic acid
α-linolenic acid
PC pool

Fig. 2


Chemo-
preventive
Anti-
oxidant
Anti-
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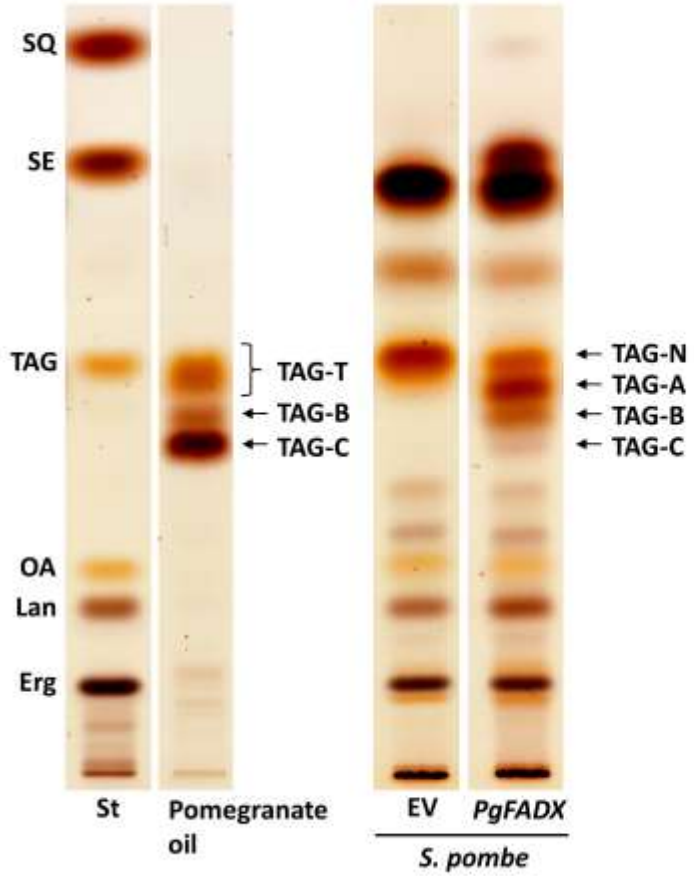
tory
Punicic
acid
Anti-
obesity
Hypo-
lipidemic
Anti-
diabetes

Fig. 3



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763 Fig. 4