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Plant *sn*-glycerol-3-phosphate acyltransferases: biocatalysts involved in the biosynthesis of intracellular and extracellular lipids

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Abstract

Acyl-lipids such as intracellular phospholipids, galactolipids, sphingolipids and surface lipids play a crucial role in plant cells by serving as major components of cellular membranes, seed storage oils and extracellular lipids such as cutin and suberin. Plant lipids are also widely used to make food, renewable biomaterials and fuels. As such, enormous efforts have been made to uncover the specific roles of different genes and enzymes involved in lipid biosynthetic pathways over the last few decades. *sn*-glycerol-3-phosphate acyltransferases (GPAT) are a group of important enzymes catalyzing the acylation of *sn*-glycerol-3-phosphate at the *sn*-1 or *sn*-2 position to produce lysophosphatidic acids. This reaction constitutes the first step of storage lipid assembly and is also important in polar and extracellular lipid biosynthesis. Ten GPAT have been identified in Arabidopsis, and many homologs have also been reported in other plant species. These enzymes differentially localize to plastids, mitochondria and the endoplasmic reticulum, where they have different biological functions, resulting in distinct metabolic fate(s) for lysophosphatidic acid. Although studies in recent years have led to new discoveries about plant GPAT, many gaps still exist in our understanding of this group of enzymes. In this article, we highlight current biochemical and molecular knowledge regarding plant GPAT, and also discuss deficiencies in our understanding of their functions in the context of plant acyl lipid biosynthesis.

Abbreviations

ACP, acyl-carrier protein

CoA, coenzyme A

Ptd_2Gro, cardiolipin

DAG, *sn*-1,2-diacylglycerol

DCA, alpha, omega dicarboxylic acid

DCA-CoA, dicarboxylic acyl-CoA

DGD, digalactosyldiacylglycerol

ER, endoplasmic reticulum

Gro3P, glycerol-3-phosphate

GPAT, *sn*-glycerol-3-phosphate acyltransferase

lysoPtdOH, lysophosphatidic acid

LPAAT, lysophosphatidic acid acyltransferase

MAG, monoacylglycerol

MGD, monogalactosyldiacylglycerol

PtdOH, phosphatidic acid

PtdCho, phosphatidylcholine

PtdEtn, phosphatidylethanolamine

PtdGro, phosphatidylglycerol

PtdIns, phosphatidylinositol

PtdSer, phosphatidylserine

SQD, sulfoguinovosyldiacylglycerol

TAG, triacylglycerol

ω-OH-CoA, ω-hydroxy acyl-CoA

Introduction

Plant lipids encompass a wide range of compounds, including fatty acids, oils/fats, steroids (sterols), waxes, cutin, suberin, glycerophospholipids (phospholipids), glyceroglycolipids (glycosylglycerides), terpenes and tocopherols (Ohlrogge and Browse, 1995), which are used by plants for an array of functions. In line with the complex nature of lipid constituents in plants, their biosynthesis is also extremely multifaceted, involving an enormous number of genes/enzymes. Although a great deal of effort has been dedicated in the last few decades towards the characterization of genes and enzymes implicated in lipid biosynthetic pathways, especially those with the potential to improve our ability to generate high-quality oilseed crops, many of their functions and interactions have yet to be revealed (Napier et al., 2014).

Plant glycerolipids are generated via three key biosynthetic pathways that take place within plastids, mitochondria or on the endoplasmic reticulum (ER), respectively (Roughan and Slack, 1982; Browse et al., 1986). The products resulting from each of these pathways are very distinct, with those synthesized in chloroplasts consisting of mainly galactolipids, which generally act as components of photosynthetic membranes (Dörmann and Benning, 2002), and those produced within mitochondria appearing to contribute to the production of membrane lipids and fatty acid composition of triacylglycerol (TAG) (Zheng et al., 2003). Glycerolipids produced on ER membranes, on the other hand, typically consist of cellular membrane phospholipids, and in developing seeds can be composed almost exclusively of TAG in certain species (Ohlrogge and Browse, 1995). While each of these lipid biosynthetic pathways makes use of a distinct set of enzymes, in every case they act to facilitate the stereospecific esterification of fatty acids to a glycerol backbone and provide a widespread coordinated

exchange of glycerolipid molecules between the three subcellular compartments (Browse et al., 1986; Kunst et al., 1988).

sn-glycerol-3-phosphate acyltransferases (GPAT) are one class of enzymes involved in plant lipid biosynthesis, catalyzing the first step of *de novo* membrane and storage lipid production, namely the transfer of an acyl group to the *sn*-1 or *sn*-2 position of *sn*-glycerol-3-phosphate (Gro3P) to produce lysophosphatidic acid (lysoPtdOH). In the model oilseed species *Arabidopsis thaliana* (hereafter Arabidopsis), ten GPAT, including ATS1 and GPAT1-9, have been identified and characterized to date (Table 1). The encoded proteins localize to three subcellular compartments, respectively, with one (ATS1) localizing to the plastid stroma, three (GPAT1-3) localizing to the mitochondrial membrane, and six (GPAT4-9) localizing to the ER membrane. Based on these differences in subcellular localization, plant GPAT are divided into three groups that are involved in the synthesis of distinct lipid classes, including extracellular lipids (for example suberin-associated waxes and the extracellular polyesters cutin and suberin), membrane lipids and storage TAG (Bertrams and Heinz, 1982; Chen et al., 2011; Gidda et al., 2009; Singer et al., 2016; Yang et al., 2012). Functional divergence of GPAT is also attained through substrate specificity, whereby their preference for acyl-coenzyme A (CoA) or acyl-carrier protein (ACP), as well as the particular species of acyl group, determines their output (Frentzen et al., 1983) (Fig. 1). In this review, we will discuss current knowledge, as well as gaps in our understanding, regarding each of the three classes of plant GPAT.

Plastidial GPAT play major roles in prokaryotic lipid biosynthesis and abiotic stress response in plants

Acyl-ACP, which is generated in the plastids, is the final product of fatty acid synthesis. Acyl-ACP can be used as an acyl donor in the synthesis of plastidial glycerolipids or the fatty acyl group can be cleaved from the fatty acid synthase complex and used in the downstream production of lipids on the ER and in mitochondria (Chen et al., 2015). In plant leaves, lipids are produced mainly through two parallel biosynthetic pathways that occur either in chloroplasts (prokaryotic pathway) or the ER (eukaryotic pathway). Since plastid and ER-localized acyltransferases have distinct fatty acyl substrate specificities, with acyltransferases derived from the prokaryotic pathway preferring 16-carbon acyl-ACP and those associated with the eukaryotic pathway preferring 18-carbon acyl-CoA, the lipids produced through the prokaryotic and eukaryotic pathways are differentiated by the preponderance of 16- or 18-acyl groups, respectively (Li-Beisson et al., 2013).

The majority of leaf lipids are in the form of thylakoid membrane galactolipids, which comprise mainly monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidylglycerol (PtdGro) and sulfoquinovosyldiacylglycerol (Chen et al., 2014), and provide functional and structural components of photosynthetic membranes. Other polar membrane lipids also make up a fraction of total leaf lipids, as well as a very small proportion of TAG, which is believed to play a role in carbon storage and/or membrane lipid remodeling (Slocombe et al., 2009). In most higher plants, leaf galactolipids are produced in large part through the eukaryotic pathway, and therefore contain a relatively high proportion of 18:3$\Delta^{cis9,cis12,cis15}$; hereafter 18:3), which are often referred to as '18:3 plants'. A smaller number of species, however, including Arabidopsis, contain substantial amounts of 16:3$\Delta^{cis6,cis9,cis13}$ (hereafter 16:3) in their galactolipid fraction, of which approximately half derives from the prokaryotic pathway (these species are often referred to as '16:3 plants') (Jamieson and Reid, 1972; Roughan and Slack, 1982; Kunst et al., 1988).

Plastidial GPAT contribute to the synthesis of these plastid-derived glycerolipids through the prokaryotic lipid biosynthetic pathway (Mackender and Leech, 1974), whereby they catalyze the transfer of an acyl group from acyl-ACP to the *sn-1* position of the Gro3P backbone (Fig. 1) (Chen et al., 2014). In the last few decades, plastidial *GPAT* have been identified, cloned and characterized from various plant species, including Arabidopsis, squash (*Cucurbita moschata*), pea (*Pisum sativum*), spinach (*Spinacia oleracea*), cucumber (*Cucumis sativus*), sunflower (*Helianthus annuus*), *Jatropha curca*, and western wallflower (*Erysimum asperum*). In almost every case, they tend to be nuclear in origin (Bertrams and Heinz, 1981; Chen et al., 2014; Johnson et al., 1992; Misra et al., 2017; Payá-Milans et al., 2015; Weber et al., 1991), encoding proteins with a putative transit peptide at their N-termini for translocation to the plastid (Chen et al., 2014). These proteins must compete for substrate with acyl-ACP hydrolase, which catalyzes the hydrolysis of acyl-ACP chains to produce free fatty acids (Chapman and Ohlrogge, 2012), and the relative activity of these two enzymes therefore directly affects the fate of fatty acids in the biosynthesis of acyl-lipids in plant cells (Nishida et al., 1993). Although plastidial acyltransferases, including GPAT and lysophosphatidic acid acyltransferase (LPAAT), use acyl-ACP as their natural acyl-donors, these enzymes can also utilize acyl-CoA (Frentzen, 1993). Plastidial LPAAT catalyzes the acyl-ACP-dependent acylation of the *sn*-2 position of Gro3P (Chen et al., 2015). Interestingly, increasing concentrations of acyl-CoA have been shown to exhibit a positive cooperative effect on certain plastidial GPAT implying that the enzyme was subject to allosteric regulation (Chen et al., 2014; Cronan and Roughan, 1987). Western wallflower plastidial GPAT was also shown to form multimers, which is a characteristic of many allosteric enzymes (Chen et al., 2014). Further investigation will be required, however, to

172 determine whether this is a general characteristic of plastidial GPAT, and if in fact acyl-ACP acts
173 as both acyl donor and positive effector.

174 In line with the fact that plastidial GPAT function in the prokaryotic lipid biosynthetic
175 pathway, Arabidopsis *ats1* mutant lines have been found to display significant alterations in leaf
176 lipid composition, with a substantial decrease in 16:3 and concomitant increases in unsaturated
177 C18 species. They exhibit virtually no change in total leaf lipid content, however, compared to
178 wild type. These results have been attributed to compensation by the eukaryotic lipid
179 biosynthetic pathway, which would explain both the lack of change in leaf lipid abundance and
180 the alterations in lipid composition, due to the distinct substrate specificities of the
181 acyltransferases associated with the two pathways (Kunst et al., 1988). In addition to the
182 abnormal leaf lipid composition, Arabidopsis *ats1* mutant and RNAi lines have also been found
183 to demonstrate reduced PtdGro levels in the plastids compared to wild type, and an
184 accompanying reduction in plant growth (Xu et al., 2006). These phenotypes were even more
185 severe in an Arabidopsis line with mutations in both *ATS1* and *ATS2*, which encodes the
186 plastidial LPAAT, suggesting that these two genes function in a coordinated manner in plastidial
187 lipid biosynthesis (Xu et al., 2006). In contrast to studies of Arabidopsis *ATS1* in leaf tissues,
188 down-regulation of *J. curcas GPAT1*, which is an ortholog of Arabidopsis *ATS1*, has recently
189 been found to result in a 12% relative reduction in seed oil content compared to wild type,
190 although the statistical significance of this finding was not provided (Misra et al., 2017). In line
191 with this, the heterologous expression of *J. curcas GPAT1* in Arabidopsis was found to increase
192 total seed oil content by 13-20% on a relative basis in transgenic lines, but again, the statistical
193 significance of these results is unknown (Misra et al., 2017). While further investigation will be
194 required to confirm this result, and also to establish whether this gene can boost seed oil content

in other plant species, it is not the first time the over-expression of a plastidial *GPAT* has led to enhanced seed oil content. Indeed, the heterologous expression of a plastidial GPAT from safflower (*Carthamus tinctorius*) in Arabidopsis has been found to increase seed oil content in the range of 10-21% on a relative basis (Jain et al., 2000). Although the mechanism behind this phenomenon is unknown at present, these findings hint at the possible applicability of plastidial GPAT in terms of the future engineering of oilseed species with increased levels of oil.

As a direct result of their role in lipid biosynthesis, plastidial GPAT have important physiological functions in plants, especially in terms of eliciting cold tolerance. Cold sensitivity in plants tends to correlate with the proportion of unsaturation in fatty acids within PtdGro in chloroplast membranes (Murata et al., 1992), with those species exhibiting high levels of unsaturation being relatively cold tolerant and those with low levels being sensitive. Indeed, the presence of even very low amounts (approximately 1% of total membrane phospholipids) of di-saturated molecular species can cause membrane phase transitions at low temperatures (Roughan, 1985). Intriguingly, at least certain cold-sensitive plants such as squash (*C. moschata*) and *Amaranthus lividus* possess plastidial GPAT that utilize both $18{:}1\Delta^{9cis}$ (hereafter 18:1)-ACP and 16:0-ACP at comparable rates (Kim and Huang, 2004; Cronan and Roughan, 1987), while cold-tolerant plants tend to possess plastidial GPAT that exhibit selectivity for 18:1-ACP. These distinctions in fatty acid specificities play a key role in determining the saturation level of the resulting PtdGro, and thus are central in terms of eliciting cold tolerance. In line with this, the heterologous expression of a plastidial *GPAT* from Arabidopsis (a plant with a high proportion of *cis*-unsaturated fatty acids in the PtdGro of chloroplast membranes) in *Nicotiana tabacum* (tobacco; a plant with lower levels of unsaturated fatty acids in PtdGro) has been found to result in enhanced tolerance to chilling (Murata et al., 1992).

ER-bound GPAT are involved in the biosynthesis of both intracellular and extracellular acyl lipids

The glycerolipids produced through the ER-localized eukaryotic pathway mainly constitute membrane phospholipids and in the case of seeds, TAG (Ohlrogge and Browse, 1995). As shown in Fig. 1, lipid biosynthesis on the ER begins with the acylation of a Gro3P backbone to produce lysoPtdOH (catalyzed by a GPAT), followed by a further acylation to produce phosphatidic acid (PtdOH) catalyzed by LPAAT. The PtdOH produced on the ER membrane can either act as substrate in the production of phospholipids, or it can be converted to *sn*-1,2-diacylglycerol by removing phosphate via the catalytic action of phosphatidic acid phosphatase. The resulting diacylglycerol can then be acylated to produce TAG via the catalytic action of diacylglycerol acyltransferase. The acyl-CoA-dependent pathway generating TAG from Gro3P is called the Kennedy pathway (Ohlrogge and Browse, 1995). In addition to the production of lysoPtdOH through the catalytic action of ER-bound GPAT, *sn*-2 regio-specific GPAT with phosphatase activity also contribute to the synthesis of monoacylglycerol (MAG), which acts as an intermediate of extracellular polyester biosynthesis (Pollard et al., 2008).

A number of studies have suggested that GPAT4-9 are localized to the ER membrane in plants, and that GPAT4-8 act in the biosynthesis of extracellular polyesters such as cutin (GPAT4, 6 and 8) and suberin (GPAT5 and 7) (Chen et al., 2011; Yang et al., 2012). Conversely, recent studies have shown that GPAT9 is very distinct both structurally and functionally from all other GPAT, and is responsible for catalyzing the first step of TAG biosynthesis on the ER, at least in certain plant species (Shockey et al., 2016; Singer et al., 2016).

Role of GPAT4, 6 and 8 in cutin biosynthesis

Cutin is a structural component of the cuticle and is synthesized within the epidermis of various aerial plant organs such as fruits, leaves, primary stems and floral organs (Pollard et al., 2008). This outermost lipid layer acts as a barrier to plant pathogen invasion, controls non-stomatal gas exchange and maintains plant morphology by separating organs during organogenesis (Pollard et al., 2008; Molina et al., 2006). Cutin is composed of a polyester of 16-carbon and 18-carbon oxygenated (i.e, hydroxyl and carboxy) fatty acids and glycerol (Fich et al., 2016). The oxygenation of the fatty acids at the terminal methyl group of the aliphatic molecules (ω-position) is catalyzed by the cytochrome-P450-dependent enzyme CYP86A, whereas midchain oxygenations (hydroxyl, epoxy, oxo, vicinal diol) are catalyzed by CYP77A (Molina et al., 2008). The ability of cutin to form a local branched structure is believed to be attributable to the presence of these mid-chain oxygen-containing functional groups in linear ω-hydroxy fatty acid chains. While the relevant fatty acid modifications and acyl lipid synthesis steps are believed to take place on the ER, the exact location of these fatty acid modifications and polyester assembly remains to be elucidated (Molina et al., 2006).

In typical 16-rich cutin, the dominant fatty acid components include 16-hydroxy- and 10,16-dihydroxy-palmitic acids, whereas 18-rich cutin mainly consists of 9,10,18-trihydroxystearic acid and 9,10-epoxy-18-hydroxy-stearic acid (Pollard et al., 2008). Infrequently, in certain species such as Arabidopsis and *Brassica napus*, leaf and stem cutin also contains a high proportion of dicarboxylic acid (DCA; containing 2 carboxyl groups), which is mainly derived from linoleic acid ($18{:}2\Delta^{9cis,12cis}$; hereafter 18:2) (Molina et al., 2006; Pollard et al., 2008; Li et al., 2007a). Generally, however, DCA is not characteristic of plant cutin and is typically associated with suberin instead.

263 The formation of mature monoacylglycerol cutin monomers begins with the transfer of an
264 acyl group from acyl-CoA to a glycerol backbone by a GPAT. Arabidopsis GPAT4, 6 and 8 all
265 function in the biosynthesis of cutin and correspondingly display a strong preference for
266 terminal-hydroxy or carboxy fatty acids compared to unsubstituted fatty acids (Yang et al.,
267 2012), suggesting that they act after oxidation reactions have occurred (Fich et al., 2016). *In vitro*
268 enzymatic assays of these three enzymes using yeast or wheat germ cell-free translation systems
269 revealed that they possess both *sn*-2 acyltransferase and phosphatase activities, the latter of
270 which allows the removal of the phosphate from the Gro3P acyl acceptor (Fich et al., 2016). This
271 leads to the conversion of a substantial proportion of lysoPtdOH to *sn*-2 MAG, which acts as a
272 precursor for surface lipid biosynthesis, and is in contrast to *sn*-1 lysoPtdOHs, which are the
273 precursors of storage lipid biosynthesis (Yang et al., 2010, 2012).

274 Both *sn*-2 lysoPtdOH and MAG products are thermodynamically less stable than their *sn-*
275 *1* counterparts, and although they are the major products of cutin-biosynthesizing GPAT, the
276 purpose for this *sn*-2 specificity has yet to be determined (Yang et al., 2012). It is possible,
277 however, that the biosynthesis of these extracellular polyesters with *sn*-2 regio-specificity
278 provide unique advantages in terms of polymer properties and/or deliver a recognition signal for
279 targeting polymer precursors to specific transport and assembly processes, allowing
280 differentiation between lysoPtdOHs destined for extracellular polyester biosynthesis and other
281 lipid biosynthetic pathways (Yang et al., 2012).

282 *AtGPAT4* and *AtGPAT8* are functionally redundant (Li et al., 2007a) and are essential for
283 cutin biosynthesis in both leaves and stems. The expression of these genes has been noted in a
284 variety of tissue types, including roots, leaves, stem, flowers and seeds (Molina et al., 2008;
285 Yang et al., 2010; Yang et al., 2012; Li et al., 2007a), which suggests that they may have

286 additional functions and/or play a similar role in other plant parts. Arabidopsis *gpat4/gpat8*
287 double mutants exhibit a strong reduction in cutin content, leading to increased water loss rates
288 and susceptibility to pathogens, as well as altered stomatal structure. Correspondingly, over-
289 expression of either of these two genes in Arabidopsis leads to enhanced accumulation of16 and
290 18 ω-hydroxy fatty acids, as well as DCA, which are known monomers of stem and leaf cutin (Li
291 et al., 2007a). Similarly, a GPAT (EpGPAT1) from *Echium pitardii* (Boraginaceae) with high
292 homology to AtGPAT4 (75% identity, 88% similarity) and AtGPAT8 (75% identity, 86%
293 similarity) has also been found to play a role in cutin biosynthesis (Mañas-Fernández et al.
294 2010).

295 *AtGPAT6*, on the other hand, plays a role in cutin biosynthesis in floral tissues, with
296 Arabidopsis *gpat6* mutant lines exhibiting significant reductions in 16-carbon cutin monomer
297 content (DCA, 16-hydroxy- and 10,16-dihydroxypalmitates) in sepals and petals (Li-Beisson et
298 al., 2009). These mutants also display defective tapetum cell development with reduced ER stack
299 assembly which results in the abortion of pollen grains and defective pollen wall formation in
300 Arabidopsis (Li et al., 2012). This suggests that *AtGPAT6* also plays a very important role in
301 anther/pollen development, thereby affecting fertility and seed yield (Li et al., 2012). Functional
302 analyses of *GPAT6* have been also reported in other plants with similar results. For instance,
303 mutation of the tomato (*Solanum lycopersicum*) *GPAT6* gene results in perturbed pollen
304 formation, altered cuticle thickness, composition and function, and reduced fruit brightness,
305 which implies that in certain species, *GPAT6* may also be involved in cutin biosynthesis in fruit
306 (Petit et al., 2014, 2016).

307 Role of *GPAT5* and *7* in suberin biosynthesis

Suberin is an aliphatic polyester with a glycerol backbone associated with cross-linked polyaromatics that is located between the primary cell wall and the plasma membrane, and is commonly found in outer bark, as well as the epidermis and endodermis of roots (Molina et al., 2006). In addition, suberinized cell walls can be found in the bundle sheaths of grasses and the chalazal region of seed coats (Pollard et al., 2008). Suberin provides strength to the cell wall and controls solute and water diffusion, and increases in its production are often observed in response to stress and wounding (Beisson et al., 2007; Pollard et al., 2008). In contrast to cutin, suberin constitutes a large proportion of 16-28 ω -hydroxy fatty acids and C16-C26 DCAs, and higher levels of primary and aromatic alcohols, as well as hydroxycinnamic acids (predominantly ferulate) (Pollard et al., 2008; Molina et al., 2006). Waxes are also often closely associated with suberin, and unlike cuticular waxes, these show a similar chemical composition to suberin itself, being composed mainly of alkanes, primary alcohols, fatty acids, and alkyl ferulates (Li et al., 2007b). The composition of Arabidopsis root suberin-associated waxes, however, is distinct from that of suberin-associated waxes on aerial portions of the plant. Indeed, esters of $_{p}$-coumaric, caffeic, and ferulic acids with 18-22 saturated fatty alcohols are the major components of Arabidopsis root waxes (Li et al., 2007b), and MAGs (both *sn*-1 and *sn*-2) can also be present (Li et al., 2007b). Due to the similarities in their composition, the biosynthesis of suberin and suberin-associated waxes appear to be linked, at least in certain species (Pollard et al., 2008; Li et al., 2007b).

Like cutin, suberin monomer biosynthesis occurs on the ER. AtGPAT5 and 7 are the main GPAT involved in suberin biosynthesis in Arabidopsis (Yang et al., 2012). Similarly to GPAT4, 6 and 8, suberin biosynthesizing GPAT show a preference for *sn-2* acylation, although this partiality is somewhat weaker than with the cutin-associated GPAT (Fich et al., 2016; Yang

et al., 2012). Indeed, in regio-specificity experiments, GPAT5 produced *sn*-2:*sn*-1 products in a ratio of ~2:1, whereas with cutin-associated GPAT this ratio was ~ 5:1 (Yang et al., 2010). Unlike the ER-bound cutin-synthesizing GPAT, AtGPAT5 and 7 lack phosphatase activity, and therefore produce a mixture of *sn*-1 and *sn*-2 acyl-lysoPtdOHs (predominantly *sn*-2) as their major products, rather than *sn*-2 MAG (Yang et al., 2012, 2010; Beisson et al., 2007; Li et al., 2007b). The production of MAG for the downstream synthesis of suberin-associated waxes likely results instead from the activities of other enzyme/enzymes such as lysophospholipases (Li et al., 2007b). The substrate preference of AtGPAT5 and 7 also differs from that of AtGPAT4, 6 and 8, with a preference for very-long-chain fatty acids (especially 22:0 and 24:0 ω-hydroxy acyl-CoA and DCA-CoA), which are characteristic of suberin (Beisson et al., 2007; Yang et al., 2010, 2012).

AtGPAT5 is expressed mainly in roots, hypocotyls, seed coats, open flowers and anthers (but not in stems or rosette leaves) (Table 1; Beisson et al., 2007; Yang et al., 2012). Arabidopsis *gpat5* mutant plants have been shown to display a 50% reduction in aliphatic suberin content in their roots, as well as a several-fold reduction in very-long-chain DCAs and ω-hydroxy fatty acids in seed coats (Beisson et al., 2007). In line with this, the permeability of *Atgpat5* mutant seed coats to tetrazolium salts was increased due to a reduction in suberin levels, and the mutants were also less tolerant to salt stress than wild type plants (Beisson et al., 2007). No changes in the composition or content of membrane/storage glycerolipids or surface cuticular waxes were observed in *Atgpat5* lines, which indicates that *AtGPAT5* is not involved in their biosynthesis. Arabidopsis overexpressing *AtGPAT5* did not result in any significant alterations in the suberin content of roots, but did enhance the proportion of saturated very-long-chain fatty acids (Li et al., 2007b).

In addition to its apparent function in suberin biosynthesis, AtGPAT5 also plays a role in the production of suberin-associated root waxes, at least in Arabidopsis (Li et al., 2007b). Indeed, *AtGPAT5* over-expression was found to increase MAG content in root waxes while its ectopic expression led to the accumulation of a substantial amount of *sn*-2 MAG in cuticular waxes as a novel component in aerial parts of the plants where it is typically not present (Li et al., 2007b). Although the precise mechanism by which GPAT5 functions in suberin-associated wax biosynthesis remains to be determined, it is likely that its contribution is indirect, with GPAT5 being responsible for the production of lysoPtdOH, which can either be utilized for suberin production or converted to MAG and free fatty acids for the generation of suberin-associated waxes through the activity of additional enzymes (Li et al., 2007b). It is also possible that extra lysoPtdOH present once suberin levels have reached a threshold channels instead to wax biosynthesis, which is supported by the fact that *AtGPAT5* over-expression lines did not exhibit alterations in root suberin content in later developmental stages, but instead increased MAG and free fatty acid levels in root waxes (Li et al., 2007b).

AtGPAT7 is very closely related to AtGPAT5, exhibiting 81% amino acid identity (Beisson et al., 2007; Yang et al., 2012) and also functional similarities, although the expression patterns of the two encoding genes suggest that they may exert their functions in different tissue types, since *AtGPAT7* is expressed in stems, rosette leaves and flowers (but not in roots) (Table 1; Beisson et al., 2007). Similar to *GPAT5*, over-expression of *GPAT7* in Arabidopsis has been found to result in increased production of very-long-chain MAGs along with C22:0 and C24:0 free fatty acids, but in seed and stem waxes rather than those of roots. *AtGPAT7* expression has also been found to be strongly induced by wounding in aerial tissues, suggesting an additional involvement of this gene in suberin biosynthesis in response to mechanical damage (Yang et al.,

2012; Li et al., 2007b). Indeed, suberin deposition in aerial tissues is a common feature in response to wounding (Kolattukudy, 2001). Interestingly, Arabidopsis *gpat7* lines did not display any alterations in fatty acid composition in leaf tissues, which insinuates further that AtGPAT7 only contributes to suberin biosynthesis under specific conditions, such as wounding, in leaves (Yang et al., 2012).

GPAT9 plays a key role in storage lipid biosynthesis

In addition to their role in the biosynthesis of extracellular lipid polyesters, ER-bound GPAT(s) also contribute to TAG biosynthesis by catalyzing the first acyl-CoA-dependent acylation reaction in the Kennedy pathway. GPAT9 is very closely related phylogenetically to the mammalian ER-bound GPAT3, which is known to play a crucial role in storage lipid biosynthesis in humans (Gidda et al., 2011), Furthermore various algal species possess homologs of AtGPAT9 that are responsible for producing an abundance of TAG (Iskandarov et al., 2016; Niu et al., 2016). Hence, studies have been focused on unraveling the function of GPAT9 in plants for some time (Singer et al., 2016).

GPAT9 is expressed rather ubiquitously in plants and encodes a protein that exhibits unique *sn*-1 acyltransferase activity with a high specificity for acyl-CoA, which is essential for TAG biosynthesis (Singer et al., 2016). This corresponds with its proposed role in TAG and membrane biosynthesis in seeds, leaves and pollen, rather than having a function in the production of extracellular lipids. Indeed, down-regulation of *AtGPAT9* has been found to cause a reduction in seed oil content, while its over-expression leads to modest increases in seed TAG content and the number of pollen lipid droplets, as well as more substantial increases in leaf TAG content, but no obvious alterations in total cutin or cuticular wax content (Singer et al., 2016). Interestingly, *Atgpat9* knockout mutants have also been found to exhibit both male and

female gametophytic lethality (Shockey et al., 2016), which is reminiscent of Arabidopsis *gpat1* mutants, whereby lipid accumulation in pollen grains is also disturbed (Zheng et al., 2003).

Increased levels of 18:3 and reduced levels of 16:3 were observed in the polar lipid fractions of leaves from *GPAT9* over-expressing plants (Singer et al., 2016), which implies a shift from the prokaryotic pathway (16:3) to the eukaryotic pathway (18:3), and is essentially the converse of what occurs in leaves when the plastidial *ATS1* is knocked out in Arabidopsis (Kunst et al., 1988). In terms of substrate specificity, Arabidopsis GPAT9 was shown to demonstrate a preference for 18:1-CoA in an *in vitro* study, a finding that corresponded with increased levels of 18:1 in *GPAT9* over-expressing Arabidopsis seeds (Singer et al., 2016). Conversely, sunflower GPAT9 appears to exhibit a high specificity for 16:0-CoA and 18:2-CoA, and a lower preference for 18:1-CoA and 18:0-CoA, which corresponds well with the normal TAG composition of sunflower seeds (high in 16:0) (Payá-Milans et al., 2016). This indicates that GPAT9 enzymes from different oilseed plants may possess distinct substrate preferences for certain fatty acyl chains, which could be of immense importance in terms of improving our ability to produce high-value unusual fatty acids in agronomically amenable crop species in the future.

Intriguingly, a *GPAT9*-like transcript from castor bean (*Ricinus communis*) has been identified as the most abundant *GPAT* transcript in developing seed endosperm (Brown et al., 2012), and two closely related forms of *GPAT9* from sunflower (GPAT9-1 and GPAT9-2) were found to increase TAG content in a GPAT-deficient yeast strain when expressed heterologously (Payá-Milans et al., 2016). Both sunflower genes are normally expressed during seed development and in vegetative tissues where TAG and polar lipids tend to accumulate, but *HaGPAT9-1* in particular exhibits high levels of expression particularly during early embryo development and during the late stages of seed maturation. Taken together, these findings imply

423 that the role of *GPAT9* in TAG biosynthesis is conserved across plant species. Along the same
424 lines, the heterologous over-expression of *J. curcas GPAT2,* which is a homolog of Arabidopsis
425 *GPAT9*, in Arabidopsis has recently been found to result in a 43-60% increase in seed oil content
426 compared to wild type, although the statistical significance of these results was not provided
427 (Misra et al., 2017). This latter result is somewhat surprising due to the fact that the over-
428 expression of Arabidopsis *GPAT9* genes did not yield anywhere near such substantial
429 enhancements in seed TAG, with constitutive and seed-specific over-expression of Arabidopsis
430 *GPAT9* resulting in only 2.8% and 3% relative increases in seed TAG content, respectively, on a
431 per weight basis compared to wild type (Singer et al., 2016; Payá-Milans et al., 2016). Given the
432 fact that unlike other plastidial *GPAT*, the heterologous over-expression of *JcGPAT1* also led to
433 a relatively large accumulation of TAG in seeds (Misra et al., 2017), it may be possible that
434 GPAT from this species are functionally divergent from those in other plants. As such, a
435 comparison of JcGPAT with other plant GPAT should be of high priority in order to shed light
436 on the mechanism behind their purported ability to increase TAG accumulation.

437

438 **The role of mitochondrial GPAT in pollen lipid biosynthesis**

439 Glycerolipids produced within mitochondria may also be involved in the biosynthesis of
440 cell membrane lipids and storage TAG in a number of plant organs (Zheng et al., 2003), and
441 mitochondria are known to utilize a unique set of enzymes to synthesize a certain set of lipids
442 that are used for their structural and functional needs (Michaud et al., 2017). The capacity of
443 mitochondria to generate their own lipids, however, is somewhat limited, and they therefore need
444 to import certain lipid species from other parts of the cell (Horvath and Daum, 2013; Michaud et
445 al., 2017). While mitochondria have the ability to synthesize phosphatidylethanolamine, PtdOH,

PtdGro and cardiolipin, phosphatidylinositol, phosphatidylserine and phosphatidylcholine are imported. Phosphatidylethanolamine synthesized through the decarboxylation of phosphatidylserine is also exported to other organelles (Michaud et al., 2017).

The acylation of Gro3P via the catalytic action of GPAT has been noted in plant mitochondria in several studies (Horvath and Daum, 2013). Although AtGPAT1-3 are predicted to localize to this organelle, this has only been confirmed for AtGPAT1, which is the only gene/enzyme of the three to be characterized in detail thus far (Zheng et al., 2003; Yang et al., 2012). AtGPAT1 exhibits *sn*-2 acyltransferase activity and has the ability to use either acyl-CoAs or DCA-CoAs as substrates, but lacks phosphatase activity (Yang et al., 2012). The highest level of activity is observed with 20:0-CoA as substrate, followed by 18:0-CoA. Its ability to use 22:0 DCA-CoA is lower than with the aforementioned acyl-CoA species, but equal to 22:0-CoA. This ability of AtGPAT1 to utilize DCA-CoA as a substrate is reminiscent of the ER-bound GPAT4, 5, 6, 7 and 8, which are mainly involved in the biosynthesis of the extracellular polymers, cutin and suberin. No alterations in polymeric lipids, however, have been noted in the leaves, seeds or flowers of the *Atgpat1* mutant (Yang et al., 2012), and as such, the biological significance of the ability of GPAT1 to use DCA-CoA as substrate remains uncertain.

Interestingly, *AtGPAT1* exhibits relatively high levels of expression in developing siliques and flower buds compared to other plant tissues that were tested (i.e., roots, seedlings and leaves) (Zheng et al., 2003). In addition, Arabidopsis *gpat1* mutant lines have been found to exhibit impaired male fertility via a gametophytic effect on pollen performance accompanied by reduced seed yield. Since mature pollen grains contain several classes of lipids, including galactolipids, neutral esters located in pollen coat cells (tapetal cells), as well as polar and neutral lipids (TAG) in vegetative cells (Ischebeck, 2016), it is possible that this reduction in pollen

performance resulted from altered lipid biosynthesis in this tissue type. These lines also displayed several fatty acid compositional changes in floral tissues and seeds compared to wild type Arabidopsis plants (Zheng et al., 2003). In addition, subsequent studies have demonstrated that AtGPAT1 acts together with the ER-bound AtGPAT6 in the release of microspores from tetrads, as well as in stamen filament elongation (Li et al., 2012).

Functional and biochemical characterizations of AtGPAT2 and AtGPAT3 have yet to be carried out, although structural modeling and sequence analysis of the active sites suggest that these enzymes should also display *sn*-2 regio-specificity (Yang et al., 2012). Unlike *AtGPAT1*, however, both *AtGPAT2* and *AtGPAT3* have been found to be expressed at low levels in a rather broad range of plant tissues (including roots, seedlings, leaves stem, flower and siliques) (Zheng et al., 2003; Suh et al., 2005; Beisson et al., 2007; Yang et al., 2012). Moreover, GPAT2 activity may be induced in some plant tissues through external stimuli such as salt stress (Zheng et al., 2003; Sui et al., 2017). Intriguingly, a recent study of the rice *OsGPAT3*, which is a closely related homolog of *AtGPAT3*, demonstrated that in a similar manner to *AtGPAT1*, this gene plays a crucial role in male fertility through its function in anther development and pollen formation (Men et al., 2017). Somewhat surprisingly, however, OsGPAT3 has been shown to localize to the ER rather than mitochondria (Men et al., 2017). Due to this distinction in its localization and since *Atgpat3* mutants have not been shown to exhibit any obvious macroscopic or chemical phenotypic changes, it is possible that monocot *GPAT3* genes provide divergent functions in male reproduction from their dicot counterparts (Men et al., 2017; Yang et al., 2012).

Other plant and algal GPAT

Although plant GPAT tend to be classified based on their subcellular locations and functions, several plant and algal GPAT are not so easily categorized. For instance, over-expression of a *GPAT* from the halophyte *Suaeda salsa* (*SsGPAT*) in Arabidopsis resulted in an enhancement of salt tolerance, possibly through the alleviation of the photoinhibition of PSII and PSI under salt stress by elevating unsaturated fatty acid content (Sui et al., 2017). Unfortunately, sequence information regarding the *SsGPAT* gene is not currently available, and therefore, it is unknown where this gene falls phylogenetically. Reduced level of salt tolerance was also reported for *Atgpat2* and *Atgpat6* mutants in the same study but the mechanism of these two GPAT's contribution to salt tolerance has yet to be fully characterized (Sui et al., 2017). Moreover, though a role in stress response (chilling tolerance) has been well documented for plastidial GPAT, as previously discussed, very little is currently known regarding the function of other GPAT in this context. Given the fact that the over-expression of *JcGPAT1* (plastidial) and *JcGPAT2* (homolog of *GPAT9*) in Arabidopsis resulted in elevated unsaturation levels in seed lipids (Misra et al., 2017), it is highly possible that additional GPAT may provide a role in imparting resiliency to particular forms of abiotic stress.

Unlike plant GPAT, the majority of GPAT from green algae species are predicted to localize within plastids (Misra and Panda, 2013), and many appear to be involved in TAG biosynthesis (Iskandarov et al., 2016). Sequence similarity between algal and plant GPAT has been found to be low in general, however there is a highly conserved topology arrangement in these two GPAT types (Misra and Panda, 2013). Interestingly, the site-specific mutation of the Gro3P binding site from the single-celled microalga *Lobosphaera incisa* has been found to result in increased phospholipid levels when expressed in GPAT-deficient yeast (Ouyang et al., 2016). Furthermore, the heterologous over-expression of another *LiGPAT* gene, which is a close

ortholog of Arabidopsis *GPAT9* and predicted to localize to the ER, in the green microalga *Chlamydomonas reinhardtii* resulted in up to 50% increases in TAG content on a dry weight basis as compared to the control in stationary phase cultures (Iskandarov et al., 2016).

Conclusions and future perspectives

Our understanding of plant lipid metabolism is increasing steadily due to recent progress in reverse genetic studies and detailed metabolite analyses. Indeed, the adoption of such multidisciplinary approaches has extended our understanding of the functions of GPAT in plant lipid metabolism considerably. Nevertheless, a plethora of opportunities still exist for further functional characterization of GPAT and their interactions, as well as the role that regio-specificity plays on the separation of fatty acyl chains destined for the extracellular lipid polyester, membrane or storage lipids. For example, further analyses of plastidial GPAT with selectivity for unsaturated fatty acids may direct the future development of plants with improved stress tolerance. In addition, although it appears that GPAT9 is the only GPAT directly involved in TAG biosynthesis in Arabidopsis, it is possible that other GPAT (such as JcGPAT1 or JcGPAT2) or GPAT-like enzymes also contribute to the first step of the Kennedy pathway.

There is considerable interest in boosting oil content in both plants and microalgae for the generation of renewable oil sources that can be readily converted into biodiesel. Since GPAT9 and its homologs are clearly involved in TAG biosynthesis in oilseeds and microalgae, it provides a promising candidate for improving oil quantity through metabolic engineering.

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Conflict of Interest

The authors declare no conflicts of interest.

References

Beisson, F., Li, Y., Bonaventure, G., Pollard, M., and Ohlrogge, J.B. (2007) The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of Arabidopsis. The Plant Cell, **19**: 351-368.

Bertrams, M. and Heinz, E. (1981) Positional specificity and fatty acid selectivity of purified *sn*-glycerol 3-phosphate acyltransferases from chloroplasts. Plant Physiology, **68**: 653-657.

Brown, A.P., Kroon, J.T.M., Swarbreck, D., Febrer, M., Larson, T.R., Graham, I.A., Caccamo, M., and Slabas, A.R. (2012) Tissue-specific whole transcriptome sequencing in castor, directed at understanding triacylglycerol lipid biosynthetic pathways. PLoS One, **7**.

Browse, J., Warwick, N., Somerville, C.R., and Slack, C.R. (1986) Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the “16:3” plant *Arabidopsis thaliana*. Biochemical Journal, **235**: 25-31.

Chapman, K.D. and Ohlrogge, J.B. (2012) Compartmentation of triacylglycerol accumulation in plants. Journal of Biological Chemistry, **287**: 2288-2294.

558 Chen, G., Woodfield, H.K., Pan, X., Harwood, J.L., and Weselake, R.J. (2015) Acyl-
559 trafficking during plant oil accumulation. Lipids, **50**: 1057-1068.

560 Chen, X., Miles, R., Snyder, C., Truksa, M., Zhang, J., Shah, S., and Weselake, R.J. (2014)
561 Possible allostery and oligomerization of recombinant plastidial *sn*-glycerol-3-phosphate
562 acyltransferase. Archives of Biochemistry and Biophysics, **554**: 55-64.

563 Chen, X., Snyder, C.L., Truksa, M., Shah, S., and Weselake, R.J. (2011) *sn*-glycerol-3-
564 phosphate acyltransferases in plants. Plant Signaling Behavior, **6**: 1695-9.

565 Cronan, J.E. and Roughan, P.G. (1987) Fatty acid specificity and selectivity of the
566 chloroplast *sn*-glycerol 3-phosphate acyltransferase of the chilling sensitive plant,
567 *Amaranthus lividus*. Plant Physiology, **83**: 676-680.

568 Dörmann, P. and Benning, C. (2002) Galactolipids rule in seed plants. Trends in Plant
569 Science, **7**: 112-118.

570 Fich, E.A., Segerson, N.A., and Rose, J.K.C. (2016) The plant polyester cutin: biosynthesis,
571 structure, and biological roles. Annual Review of Plant Biology, **67**: 207-233.

572 Frentzen, M. (1993) Acyltransferases and triacylglycerols. In Lipid Metabolism in Plants,
573 T.. Moore, ed (CRC Press. Ann Arbor, Massachusetts), pp. 195-230.

574 Frentzen, M., Heinz, E., Mckeon, T.A., and Stumpf, P.K. (1983) Specificities and
575 selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate
576 acyltransferase from pea and spinach chloroplasts. European Journal of Biochemistry, **129**:
577 629-636.

578 Gidda, S.K., Shockey, J.M., Falcone, M., Kim, P.K., Rothstein, S.J., Andrews, D.W., Dyer,
579 J.M., and Mullen, R.T. (2011) Hydrophobic-domain-dependent protein-protein interactions

580 mediate the localization of GPAT enzymes to ER subdomains. Traffic, **12**: 452-472.

581 Gidda, S.K., Shockey, J.M., Rothstein, S.J., Dyer, J.M., and Mullen, R.T. (2009)
582 *Arabidopsis thaliana* GPAT8 and GPAT9 are localized to the ER and possess distinct ER
583 retrieval signals: Functional divergence of the dilysine ER retrieval motif in plant cells.
584 Plant Physiology and Biochemistry, **47**: 867-879.

585 Horvath, S.E. and Daum, G. (2013) Lipids of mitochondria. Progress in Lipid Research, **52**:
586 590-614.

587 Ischebeck, T. (2016). Lipids in pollen-They are different. Biochimica et Biophysica Acta -
588 **1861**: 1315-1328.

589 Iskandarov, U., Sitnik, S., Shtaida, N., Didi-Cohen, S., Leu, S., Khozin-Goldberg, I., Cohen,
590 Z., and Boussiba, S. (2016) Cloning and characterization of a GPAT-like gene from the
591 microalga *Lobosphaera incisa* (Trebouxiophyceae): overexpression in *Chlamydomonas*
592 *reinhardtii* enhances TAG production. Journal of Applied Phycology, **28**: 907-919.

593 Jain, R.K., Coffey, M., Lai, K., Kumar, A., and MacKenzie, S.L. (2000) Enhancement of
594 seed oil content by expression of glycerol-3-phosphate acyltransferase genes. Biochemical
595 Society Transactions, **6**: 958-961.

596 Jamieson, G.R. and Reid, E.H. (1972) The component fatty acids of some marine algal
597 lipids. Phytochemistry, **11**: 1423-1432.

598 Johnson, T.C., Schneider, J.C., and Somerville, C. (1992) Nucleotide sequence of acyl-acyl
599 carrier protein: glycerol-3-phosphate acyltransferase from cucumber. Plant Physiology, **99**:
600 771-772.

601 Kim, H.U. and Huang, A.H.C. (2004) Plastid lysophosphatidyl acyltransferase is essential

602 for embryo development in Arabidopsis. Plant Physiology, **134**: 1206-1216.

603 Kolattukudy, P.E. (2001) Polyesters in higher plants. Advances in Biochemical Engineering
604 / Biotechnology, **71**: 1-49.

605 Kunst, L., Browse, J., and Somerville, C. (1988) Altered regulation of lipid biosynthesis in a
606 mutant of Arabidopsis deficient in chloroplast glycerol-3-phosphate acyltransferase activity.
607 Proceedings of the National Academy of Sciences of the United States of America, **85**:
608 4143-7.

609 Li, X.C., Zhu, J., Yang, J., Zhang, G.R., Xing, W.F., Zhang, S., and Yang, Z.N. (2012)
610 Glycerol-3-Phosphate acyltransferase 6 (GPAT6) is important for tapetum development in
611 Arabidopsis and plays multiple roles in plant fertility. Molecular Plant, **5**: 131-142.

612 Li, Y., Beisson, F., Koo, A.J., Molina, I., Pollard, M., and Ohlrogge, J. (2007a)
613 Identification of acyltransferases required for cutin biosynthesis and production of cutin
614 with suberin-like monomers. Proceedings of the National Academy of Sciences of the
615 United States of America, **104**: 18339-18344.

616 Li, Y., Beisson, F., Ohlrogge, J., and Pollard, M. (2007b) Monoacylglycerols are
617 components of root waxes and can be produced in the aerial cuticle by ectopic expression of
618 a suberin-associated acyltransferase. Plant Physiology, **144**: 1267-1277.

619 Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., Arondel, V., Bates, P.D.,
620 Baud, S., Bird, D., DeBono, A., Durrett, T.P., Franke, R.B., Graham, I.A., Katayama, K.,
621 Kelly, A.A., Larson, T., Markham, J.E., Miquel, M., Molina, I., Nishida, I., Rowland, O.,
622 Samuels, L., Schmid, K.M., Wada, H., Welti, R., Xu, C., Zallot, R., and Ohlrogge, J.
623 (2013) Acyl-lipid metabolism. The Arabidopsis Book, **11**: e0161.

Li-Beisson, Y., Pollard, M., Sauveplane, V., Pinot, F., Ohlrogge, J., and Beisson, F. (2009) Nanoridges that characterize the surface morphology of flowers require the synthesis of cutin polyester. Proceedings of the National Academy of Sciences of the United States of America, **106**: 22008-22013.

Mackender, R.O. and Leech, R.M. (1974) The galactolipid, phospholipid, and fatty acid composition of the chloroplast envelope membranes of *Vicia faba* L. Plant Physiology, **53**: 496-502.

Mañas-Fernández, A., Li-Beisson, Y., Alonso, D.L., García-Maroto, F. (2010) Cloning and molecular characterization of a glycerol-3-phosphate O-acyltransferase (GPAT) gene from *Echium* (Boraginaceae) involved in the biosynthesis of cutin polyesters. Planta, **232**:987–997.

Men, X., Shi, J., Liang, W., Zhang, Q., Lian, G., Quan, S., Zhu, L., Luo, Z., Chen, M., and Zhang, D. (2017) Glycerol-3-phosphate acyltransferase 3 (OsGPAT3) is required for anther development and male fertility in rice. Journal of Experimental Botany, **68**: 513-526.

Michaud, M., Prinz, W.A., and Jouhet, J. (2017) Glycerolipid synthesis and lipid trafficking in plant mitochondria. FEBS Journal, **284**: 376-390.

Misra, A., Khan, K., Niranjan, A., Kumar, V., and Sane, V.A. (2017) Heterologous expression of two GPATs from Jatropha curcas alters seed oil levels in transgenic *Arabidopsis thaliana*. Plant Science, **263**: 79-88.

Misra, N. and Panda, P.K. (2013) In search of actionable targets for agrigenomics and microalgal biofuel production: sequence-structural diversity studies on algal and higher

646 plants with a focus on GPAT protein. OMICS, **17**: 173-186.

647 Molina, I., Bonaventure, G., Ohlrogge, J., and Pollard, M. (2006) The lipid polyester
648 composition of *Arabidopsis thaliana* and *Brassica napus* seeds. Phytochemistry, **67**: 2597-
649 2610.

650 Molina, I., Ohlrogge, J.B., and Pollard, M. (2008) Deposition and localization of lipid
651 polyester in developing seeds of *Brassica napus* and *Arabidopsis thaliana*. Plant Journal,
652 53: 437-449.

653 Murata, N., Ishizakinishizawa, Q., Higashi, S., Hayashi, H., Tasaka, Y., and Nishida, I.
654 (1992) Genetically engineered alteration in the chilling sensitivity of plants. Nature, **356**:
655 710-713.

656 Napier, J.A., Haslam, R.P., Beaudoin, F., and Cahoon, E.B. (2014) Understanding and
657 manipulating plant lipid composition: Metabolic engineering leads the way. Current
658 Opinion in Plant Biology, **19**: 68-75.

659 Nishida, I., Tasaka, Y., Shiraishi, H., and Murata, N. (1993) The gene and the RNA for the
660 precursor to the plastid-located glycerol-3-phosphate acyltransferase of *Arabidopsis*
661 *thaliana*. Plant Molecular Biology, **21**: 267-277.

662 Niu, Y.F., Wang, X., Hu, D.X., Balamurugan, S., Li, D.W., Yang, W.D., Liu, J.S., and Li,
663 H.Y. (2016) Molecular characterization of a glycerol-3-phosphate acyltransferase reveals
664 key features essential for triacylglycerol production in *Phaeodactylum tricornutum*.
665 Biotechnology for Biofuels, **9**: 60.

666 Ohlrogge, J. and Browse, J. (1995) Lipid biosynthesis. Plant Cell, **7**: 957-70.

667 Ouyang, L.L., Li, H., Yan, X.J., Xu, J.L., and Zhou, Z.G. (2016) Site-directed mutagenesis

668 from Arg195 to His of a microalgal putatively chloroplastidial glycerol-3-phosphate
669 acyltransferase causes an increase in phospholipid levels in yeast. Frontiers in Plant
670 Science, **7**: 1-14.

671 Payá-Milans, M., Aznar-Moreno, J.A., Balbuena, T.S., Haslam, R.P., Gidda, S.K., Pérez-
672 Hormaeche, J., Mullen, R.T., Thelen, J.J., Napier, J.A., Salas, J.J., Garcés, R., Martínez-
673 Force, E., and Venegas-Calerón, M. (2016) Sunflower HaGPAT9-1 is the predominant
674 GPAT during seed development. Plant Science, **252**: 42-52.

675 Payá-Milans, M., Venegas-Calerón, M., Salas, J.J., Garcés, R., and Martínez-Force, E.
676 (2015) Cloning, heterologous expression and biochemical characterization of plastidial *sn*-
677 glycerol-3-phosphate acyltransferase from *Helianthus annuus*. Phytochemistry, **111**: 27-36.

678 Petit, J., Bres, C., Just, D., Garcia, V., Mauxion, J.-P., Marion, D., Bakan, B., Joubes, J.,
679 Domergue, F., and Rothan, C. (2014) Analyses of tomato fruit brightness mutants uncover
680 both cutin-deficient and cutin-abundant mutants and a new hypomorphic allele of GDSL
681 Lipase. Plant Physiology, **164**: 888-906.

682 Petit, J., Bres, C., Mauxion, J.-P., Tai, F.W.J., Martin, L.B.B., Fich, E.A., Joubès, J., Rose,
683 J.K.C., Domergue, F., and Rothan, C. (2016) The glycerol-3-phosphate acyltransferase
684 GPAT6 from tomato plays a central role in fruit cutin biosynthesis. Plant Physiology, **171**:
685 894-913.

686 Pollard, M., Beisson, F., Li, Y., and Ohlrogge, J.B. (2008) Building lipid barriers:
687 biosynthesis of cutin and suberin. Trends in Plant Science, **13**: 236-246.

688 Roughan, P.G. (1985) Phosphatidylglycerol and chilling sensitivity in plants. Plant
689 Physiology, **77**: 740-746.

690 Roughan, P.G. and Slack, C.R. (1982) Cellular organization of glycerol metabolism. Annual
691 Review of Plant Physiology, **33**: 97-132.

692 Shockey, J., Regmi, A., Cotton, K., Adhikari, N., Browse, J., Bates, P.D. (2016)
693 Identification of Arabidopsis *GPAT9* (At5g60620) as an essential gene involved in
694 triacyglycerol biosynthesis. Plant Physiology, **170**: 163-179.

695 Singer, S.D., Chen, G., Mietkiewska, E., Tomasi, P., Jayawardhane, K., Dyer, J.M., and
696 Weselake, R.J. (2016) Arabidopsis GPAT9 contributes to synthesis of intracellular
697 glycerolipids but not surface lipids. Journal of Experimental Botany, **67**: 4627-4638.

698 Slocombe, S.P., Cornah, J., Pinfield-Wells, H., Soady, K., Zhang, Q., Gilday, A., Dyer,
699 J.M., and Graham, I.A. (2009) Oil accumulation in leaves directed by modification of fatty
700 acid breakdown and lipid synthesis pathways. Plant Biotechnology Journal, **7**: 694-703.

701 Suh, M., Samuels, A., and Jetter, R. (2005) Cuticular lipid composition, surface structure,
702 and gene expression in Arabidopsis stem epidermis. Plant Physiology, **139**: 1649-1665.

703 Sui, N., Tian, S., Wang, W., Wang, M., and Fan, H. (2017) Overexpression of glycerol-3-
704 phosphate acyltransferase from *Suaeda salsa* improves salt tolerance in Arabidopsis.
705 Frontiers in Plant Science, **8**: 1-14.

706 Weber, S., Wolter, F.P., Buck, F., Frentzen, M., and Heinz, E. (1991) Purification and
707 cDNA sequencing of an oleate-selective acyl-ACP:*sn*-glycerol-3-phosphate acyltransferase
708 from pea chloroplasts. Plant Molecular Biology, **17**: 1067-1076.

709 Xu, C., Yu, B., Cornish, A.J., Froehlich, J.E., and Benning, C. (2006) Phosphatidylglycerol
710 biosynthesis in chloroplasts of Arabidopsis mutants deficient in acyl-ACP glycerol-3-
711 phosphate acyltransferase. The Plant Journal, **47**: 296-309.

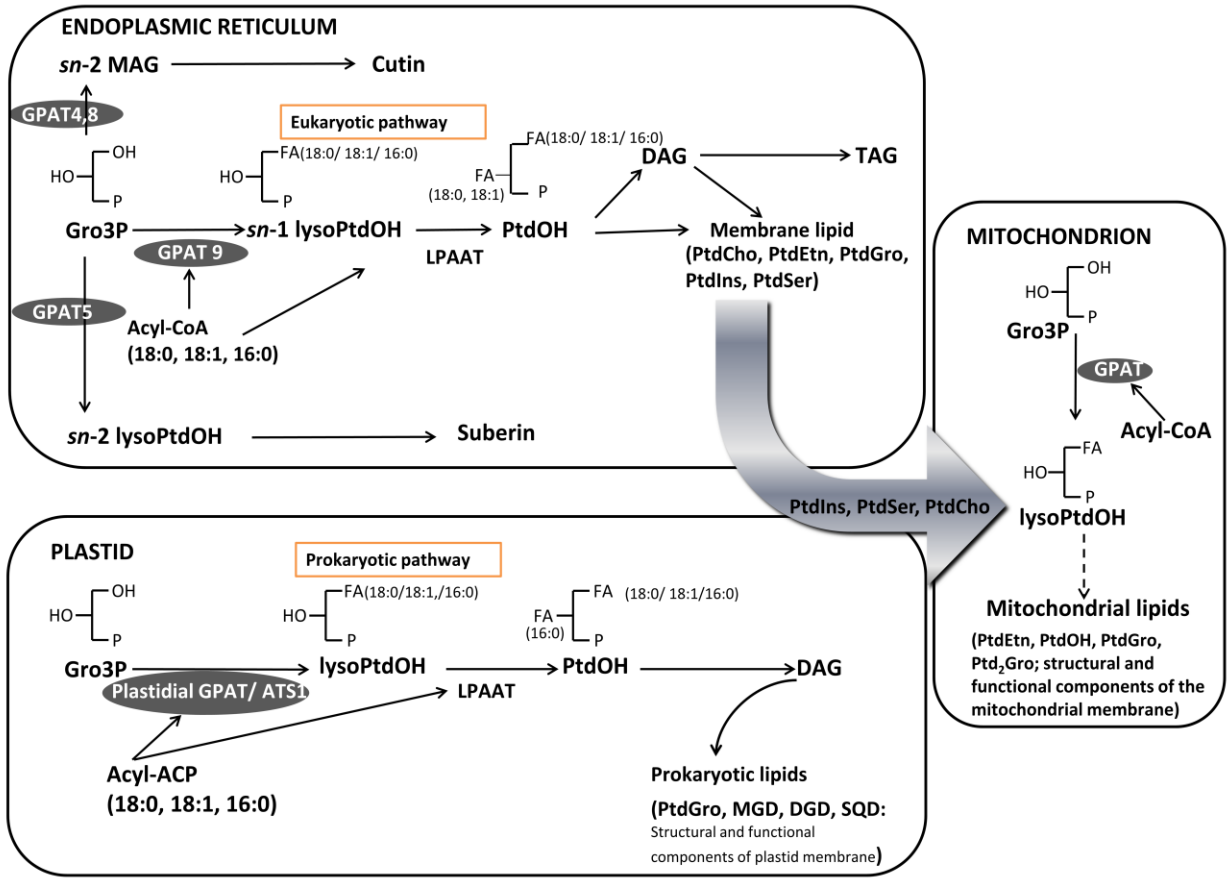
Yang, W., Pollard, M., Li-Beisson, Y., Beisson, F., Feig, M., and Ohlrogge, J. (2010) A distinct type of glycerol-3-phosphate acyltransferase with *sn*-2 preference and phosphatase activity producing 2-monoacylglycerol. Proceedings of the National Academy of Sciences of the United States of America, **107**: 12040-12045.

Yang, W., Simpson, J.P., Li-Beisson, Y., Beisson, F., Pollard, M., and Ohlrogge, J.B. (2012) A land-plant-specific glycerol-3-phosphate acyltransferase family in Arabidopsis: Substrate specificity, *sn*-2 preference, and evolution. Plant Physiology, **160**: 638-652.

Zheng, Z., Xia, Q., Dauk, M., Shen, W., Selvaraj, G., and Zou, J. (2003) Arabidopsis *AtGPAT1*, a member of the membrane-bound glycerol-3-phosphate acyltransferase gene family, is essential for tapetum differentiation and male fertility. The Plant Cell, **15**: 1872-87.

Figure Legends

Fig. 1 Role of *sn*-glycerol-3-phosphate acyltransferases (GPAT) in plastidial, mitochondrial and endoplasmic reticulum-based lipid biosynthesis. Abbreviations: Ptd_2Gro, cardiolipin; DAG, *sn*-1,2-diacylglycerol; DGD, digalactosyldiacylglycerol; Gro3P, *sn*-glycerol-3-phosphate; LPAAT, lysophosphatidic acid acyltransferase; lysoPtdOH, lysophosphatidic acid; MGD, monogalactosyldiacylglycerol; PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; SQD, sulfoguinovosyldiacylglycerol; TAG, triacylglycerol.



744 Table 1. Arabidopsis glycerol-3-phosphate acyltransferases (AtGPAT)

745

Gene name	Gene ID (Name)	Gene expression [a]	Subcellular protein localization	Substrate preference [b]	Regio-specificity	Physiological role	References
ATS1	AT1G32200	F, S, Sh, Po, Rt, Em, St	Plastid, Chloroplast	Acyl-ACP> Acyl-CoA	*sn-1*	Membrane lipid biosynthesis	(Frentzen et al., 1983; Kim and Huang, 2004; Xu et al., 2006)
AtGPAT1	AT1G06520	F, S, Po, Rt, E, L St	Mitochondria	Acyl-CoA>DCA-CoA	*sn-2 > sn-1*	cutin biosynthesis, dephosphorylation, pollen sperm cell differentiation	(Zheng et al., 2003; Yang et al., 2012)
AtGPAT2	AT1G02390	P, Rt, L, St	Mitochondria	DCA-CoA	*sn-1, sn-2*	cutin biosynthesis, dephosphorylation	(Yang et al., 2012; Beisson et al., 2007)
AtGPAT3	AT4G01950	F, Rt, Sh, L	Mitochondria	DCA-CoA	*sn-1, sn-2*	Cutin biosynthesis, dephosphorylation, metabolic processes	(Yang et al., 2012; Beisson et al., 2007)
AtGPAT4	AT1G01610	L, St, Fr ,F, Sh, Rt, Em	ER	DCA-CoA> ω-OH-CoA, Acyl-CoA	*sn-2 > sn-1*	Leaf and stem cutin biosynthetic process, phospholipid biosynthetic process (functionally redundant with *GPAT8*)	(Li et al., 2007a, 2007b; Li-Beisson et al., 2009; Yang et al., 2012)
AtGPAT5	AT3G11430	S, Rt, Po, F, Em	ER	Very long chain (C22) Acyl-CoA, DCA-CoA, ω-OH-CoA	*sn-2 > sn-1*	Root and seed coat suberin synthesis	(Beisson et al., 2007; Yang et al., 2010, 2012)
AtGPAT6	AT2G38110	F, Po, L, Em, Sh, S, Fr	ER	ω-OH-CoA > DCA-CoA	*sn-2 > sn-1*	Flower cutin biosynthetic process, flower development, phospholipid biosynthetic process	(Li et al., 2012; Yang et al., 2012)

AtGPAT7	AT5G06090	L, F, St	ER	Very long chain (C22) Acyl-CoA, DCA-CoA, ω-OH-CoA	*sn-2 > sn-1*	Suberin biosynthesis as a response to wounding in aerial tissues	(Yang et al., 2012; Beisson et al., 2007)
AtGPAT8	AT4G00400	L, St, F, Sh, Em, S	ER	ω-OH-CoA > DCA-CoA	*sn-2 > sn-1*	Cutin biosynthesis (functionally redundant with *GPAT4*)	(Yang et al., 2012)
AtGPAT9	AT5G60620	F, S, L, Po, Em, Sh, St	ER	Acyl-CoA>DCA-CoA	*sn-1 > sn-2*	Intracellular glycerolipid biosynthesis (seeds, pollen, and leaves)	(Singer et al., 2016; Shockey et al., 2016)

[a] F, flower; L, leaf; Fr, Fruit; Rt, root; S, seed; Po, Pollen; Sh, shoot; Em, Embryo; St, stem.

[b] Substrate specificity may vary in different reactions conditions with different substrate fatty acid compositions. Please check the references for the detailed reaction conditions. ACP, acyl-carrier protein; CoA, acyl-coenzyme A; DCA-CoA, dicarboxylic acyl-CoA; ω-OH-CoA, ω-hydroxy acyl-CoA

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