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Research article

# *Arabidopsis thaliana* GPAT8 and GPAT9 are localized to the ER and possess distinct ER retrieval signals: Functional divergence of the dilysine ER retrieval motif in plant cells

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ABSTRACT

Glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15) catalyzes the committed step in the production of glycerolipids, which are major components of cellular membranes, seed storage oils, and epicuticular wax coatings. While the biochemical activities of GPATs have been characterized in detail, the cellular features of these enzymes are only beginning to emerge. Here we characterized the phylogenetic relationships and cellular properties of two GPAT enzymes from the relatively large *Arabidopsis thaliana* GPAT family, including GPAT8, which is involved in cutin biosynthesis, and GPAT9, which is a new putative GPAT that has extensive homology with a GPAT from mammalian cells involved in storage oil formation and, thus, may have a similar role in plants. Immunofluorescence microscopy of transiently-expressed myc-epitope-tagged GPAT8 and GPAT9 revealed that both proteins were localized to the endoplasmic reticulum (ER), and differential permeabilization experiments indicated that their N- and C-termini were oriented towards the cytosol. However, these two proteins contained distinct types of ER retrieval signals, with GPAT8 possessing a divergent type of dilysine motif (–KK–COOH rather than the prototypic –KKXX–COOH or –KXKXX–COOH motif) and GPAT9 possessing a hydrophobic pentapeptide motif (–ϕ–X–X–K/R/D/E–ϕ–; where ϕ are large hydrophobic amino acid residues). Notably, the divergent dilysine motif in GPAT8 only functioned effectively when additional upstream residues were included to provide the proper protein context. Extensive mutational analyses of the divergent dilysine motif, based upon sequences present in the C-termini of other GPAT8s from various plant species, further expanded the functional definition of this molecular targeting signal, thereby providing insight to the targeting signals in other GPAT family members as well as other ER-resident membrane proteins within plant cells.



## 1. Introduction

Glycerolipids play an essential role in plant biology by serving as major components of cellular membranes, storage oils in developing seeds, and the protective, hydrophobic barrier on the cuticular surface of plant organs [41]. Glycerolipids and their associated metabolites also serve as part of the dynamic signaling processes associated with many aspects of plant growth and development and resistance to both biotic and abiotic stresses.

The production of glycerolipids in plant cells begins with the activity of a glycerol-3-phosphate acyltransferase (GPAT) enzyme, which catalyzes the transfer of a fatty acid from the acyl-CoA pool (or acyl-ACP pool in plastids) to the *sn*-1 position of glycerol-3-phosphate, resulting in formation of lysophosphatidic acid [38]. Subsequent acylation of lysophosphatidic acid at the *sn*-2 position by lysophosphatidic acid acyltransferase (LPAT) results in

*Abbreviations:* ACP, acyl-carrier protein; BY-2, Bright Yellow-2; Cf9, *Cladosporium fulvum*-9; ConA, concanavalin A; DIC, differential interference contrast; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FAD, fatty acid desaturase; GPAT, glycerol-3-phosphate acyltransferase; GFP, green fluorescent protein; LPAT, lysophosphatidic acid acyltransferase; MCS, multiple cloning site; ORF, open reading frame; RFP, red fluorescent protein; SNARE, soluble N-ethyl-maleimide sensitive factor attachment protein receptor; SRP, signal-recognition particle; TAG, triacylglycerol; TMD, transmembrane domain.

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production of phosphatidic acid, which is a key intermediate in the metabolic pathways responsible for both membrane polar lipid and neutral storage lipid biosynthesis [41]. Overall, the metabolic fate(s) of these glycerolipid metabolites is determined in part by the subcellular localization of the enzymatic reactions responsible for their production. For example, glycerolipids synthesized in chloroplasts are converted primarily to galactolipids, which serve as major structural and functional components of photosynthetic membranes [12]. Glycerolipids produced in the endoplasmic reticulum (ER), however, are converted primarily to cellular membrane phospholipids and, in developing seeds, to storage oil triacylglycerols (TAGs) [41]. Glycerolipid synthesis is known to occur also in mitochondria, and evidence from both mammals and plants suggests that this pathway also contributes to the synthesis of membrane lipids and several important aspects of TAG formation, such as the relative composition of TAGs in plant cells [62] and the formation of serum lipids in mammals [18].

Genes encoding membrane-bound GPAT activity were first identified in bacteria [59], and subsequently in mammals for mitochondrial-localized GPATs [18]. In plants, the first GPAT genes identified were those that encoded enzymes localized to plastids, which are soluble enzymes and, thus, facilitated their purification and gene cloning [38]. Comparative analysis of these soluble GPATs and other GPATs from evolutionarily diverse organisms has since revealed that all members of the GPAT superfamily contain at least four highly conserved amino acid sequence motifs that are essential for both acyltransferase activity and the binding of glycerol-3-phosphate substrate [27].

While it has long been recognized that GPAT enzyme activity is present also in ER microsomal preparations from yeast, plants and mammalian cells and that these activities contributed to both membrane lipid and storage lipid biosynthesis, the genes encoding these enzymes have only recently been identified. For instance, two microsomal GPAT enzymes termed GAT1 and GAT2 were identified in yeast [2,63], and more recently an enzyme termed GPAT3 was identified in mammalian cells [9]. Mammalian GPAT3 is particularly notable among the GPAT family because it is associated with storage oil formation in mammalian cells and is strongly induced during adipocyte differentiation [18]. In plants, a bioinformatics-based search of the *Arabidopsis* genome with the yeast GAT1 and GAT2 sequences revealed a family of eight related genes (termed GPAT1 through GPAT8) that encode proteins of similar size and amino acid sequence identity, including the four amino acid motifs that are characteristic of all members of the GPAT family [5,62]. Indeed, functional analysis of 7 of these 8 GPAT enzymes through heterologous expression in yeast cells demonstrated that at least 5 possess GPAT activity [62]. Moreover, characterization of GPAT1 revealed that it is localized to mitochondria in vitro and plays an essential role in pollen fertility [62]. GPAT4, 5 and 8, on the other hand, are involved in the production of suberin or cutin [5,28]. The subcellular localization of these latter three GPAT enzymes, however, has not been clearly defined. Furthermore, no gene(s) encoding GPAT activity involved in ER-localized storage oil production in plants has been identified.

We are interested in the cellular and organizational properties of lipid-metabolic enzymes in plant cells, with an emphasis on those enzymes involved in storage oil production in the ER. Towards this end, we previously characterized the fatty acid desaturase and diacylglycerol acyltransferase enzymes from *Arabidopsis* and/or tung (*Vernicia fordii*), the latter of which catalyze the committed step in storage oil formation [13,14,32,54]. Here, we describe the cellular properties of several GPAT enzymes, using the *Arabidopsis* GPAT enzyme family as a model system, including the subcellular localization and topological orientation of the GPAT8 protein. We also describe the identification of a new putative GPAT gene from *Arabidopsis* (At5g60620), referred to here as GPAT9, which was identified through homology-based searches using the recently identified mammalian GPAT3 gene [9] as a probe.

Overall, we show that *Arabidopsis* GPAT8 and GPAT9 are both localized to the ER and exhibit a similar topological orientation in ER membranes, but that they contain distinct types of ER retrieval sequences. Further characterization of the ER retrieval signals in other members of the plant GPAT8 gene family allowed us to significantly expand the functional definition of dilysine-type targeting signals in plants. The implications of these results in terms of our understanding of ER protein trafficking in plant cells and the regulation of glycerolipid biosynthesis by GPATs located in different subcellular organelles in plants, compared to other evolutionarily diverse organisms, are discussed.

## 2. Results and discussion

### 2.1. Sequence analysis of the Arabidopsis GPAT family – identification of GPAT9

As mentioned in the Introduction, GPAT enzyme activity in *Arabidopsis* is encoded by a relatively large gene family containing at least eight members [5,62]. As shown in Fig. 1, alignment of the deduced polypeptide sequences of these GPAT proteins (GPAT1–8) demonstrates that they are similar in length and share several features that are characteristic of other membrane-bound GPATs from evolutionarily diverse organisms, including the presence of one or more predicted TMDs and four conserved amino acid motifs (Blocks I–IV) known to be important for acyltransferase activity [27]. The GPAT protein sequences also show a modest amount of sequence similarity (18% identical, 40% similar) with the highest degree of conservation detected in their C-terminal halves near the putative active sites (Fig. 1). The GPAT1 protein also possesses an extended N-terminal region that exhibits characteristics of a mitochondrial targeting peptide (albeit weakly detected by web-based protein targeting signal algorithms; data not shown), which is consistent with its reported localization to mitochondria in vitro [62]. All of the other GPAT proteins (GPAT2–8) lack any recognizable N-terminal intracellular targeting signal motifs, but do contain putative C-terminal ER retrieval signals (see below, Section 2.4).

Recent identification of an ER-localized GPAT protein in mammals (GPAT3) [9] provided an opportunity to identify a similar gene(s) in plants. Towards this end, we utilized a bioinformatics approach to identify a potential homolog of mammalian GPAT3 in *Arabidopsis* (see Materials and Methods for details). Referred to here as GPAT9, this putative mammalian GPAT3 homolog corresponds to locus number At5g60620 in the *Arabidopsis* genome database. Analysis of the encoded polypeptide sequence of GPAT9 reveals the presence of three predicted TMDs and all four conserved GPAT-type domains, but notably, the overall length of the protein (376 amino acids) is significantly shorter than that of other *Arabidopsis* GPAT family members (Fig. 1). Furthermore, the GPAT9 polypeptide sequence shares just 3% identity and 17% similarity with the GPAT1–8 consensus sequence, suggesting that it is distantly related to these GPATs.

To determine the evolutionary relationships of this newly-identified GPAT9 gene relative to other members of the *Arabidopsis* GPAT family, as well as to other GPAT genes from other organisms and that are well characterized in the literature, we performed a phylogenetic analysis. As shown in Fig. 2A, *Arabidopsis* GPAT9 is more closely related to the mammalian ER-localized GPAT3 and GPAT4 enzymes than it is to other members of the *Arabidopsis* GPAT family (GPATs 1–8), suggesting that the divergence of the GPAT9 gene from the GPAT1–8 family of *Arabidopsis* occurred prior to the evolutionary split between plants and mammals. The involvement of mammalian GPAT3 and GPAT4 in the production of storage TAG