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Sunflower *Ha*GPAT9-1 is the predominant GPAT during seed development

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ABSTRACT

In oil crops, triacylglycerol biosynthesis is an important metabolic pathway in which glycerol-3-phosphate acyltransferase (GPAT) performs the first acylation step. Mass spectrometry analysis of developing sunflower (*Helianthus annuus*) seed membrane fractions identified an abundant GPAT, *Ha*GPAT9 isoform 1, with a N-terminal peptide that possessed two phosphorylated residues with possible regulatory function. *Ha*GPAT9-1 belongs to a broad eukaryotic GPAT family, similar to mammalian GPAT3, and it represents one of the two sunflower GPAT9 isoforms, sharing 90% identity with *Ha*GPAT9-2. Both sunflower genes are expressed during seed development and in vegetative tissues, with *HaGPAT9-1* transcripts accumulating at relatively higher levels than those for *HaGPAT9-2*. Green fluorescent protein tagging of *Ha*GPAT9-1 confirmed its subcellular accumulation in the endoplasmic reticulum. Despite their overall sequence similarities, the two sunflower isoforms displayed significant differences in their enzymatic activities. For instance, *Ha*GPAT9-1 possesses *in vivo* GPAT activity that rescues the lethal phenotype of the cmy228 yeast strain, while *in vitro* assays revealed a preference of *Ha*GPAT9-1 for palmitoyl-, oleoyl- and linoleoyl-CoAs of one order of magnitude, with the highest increase in yield for oleoyl- and linoleoyl-CoAs. By contrast, no enzymatic activity could be detected for *Ha*GPAT9-2, even though its over-expression modified the TAG profile of yeast.

1. Introduction

Glycerol-3-phosphate acyltransferases (GPAT; E.C. 2.3.1.15) are enzymes that transfer the acyl moiety from an acyl-coenzyme A (CoA) donor (or acyl–acyl carrier protein [ACP] in plastids) to the *sn*-1 position of a glycerol-3-phosphate (G3P) molecule, yielding 1-acylglycerol-3-phosphate (or lysophosphatidic acid, LPA) [1]. This first acylation step occurs slower than the second [2], limiting the availability of LPA and producing a potential 'bottleneck' in the flow of carbon into glycerolipids, as originally defined by Eugene Kennedy [3].

It is now realized that GPAT-mediated regulation of glycerolipid synthesis is more complex than previously thought. Considerable effort has been dedicated to determine the major plant GPAT isoforms implicated in seed oil synthesis, leading to the identification of GPAT sequences from three protein families in different plant species [4–6]. The GPAT family homologue of GPAT9 (At5g60620)

Abbreviations: ACP, acyl carrier protein; ConA, Concanavalin A; DAF, days after flowering; ER, endoplasmic reticulum; FOA, fluoroorotic acid; G3P, glycerol-3-phosphate; GFP, green fluorescent protein; GPAT, glycerol-3-phosphate acyltransferase; LPA, 1-acylglycerol-3-phosphate or lysophosphatidic acid; LPAAT, 1-acylglycerol-3-phosphate acyltransferase; PDAT, phosphatidylcholine:diacylglycerol acyltransferase; TAG, triacylglycerol.

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Table 1
Strains and plasmids used in this study.

Yeast strains and plasmids	Description	Reference or source
S. cerevisiae		
S288C	*MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	[14]
W303-1A	*MATα* {*leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*}	[15]
gat1Δ	*MAT*a; *his3Δ1*; *leu2Δ0*; *lys2Δ0*;*ura3Δ0*; *YKR067w*:*kanMX4*	[16]
ale1Δ	*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YOR175c*:*kanMX4*	[17]
cmy228	W303-1A; *MATα*; *gat1Δ*:*TRP1 gat2Δ*:*HIS3* [p*GAL1*:*GAT1 URA3*]	[18]
Plasmids		
p416(*LEU2*)	Yeast vector for constitutive expression of proteins under the control of a GPD promoter	[19]
p*GAL1*(*URA3*):*GAT1*	Plasmid carried by cmy228 that drives galactose inducible expression of *GAT1* to confer viability	[18]
pUC18/NheI-mGFP	pUC18-based expression vector that harbors EmGFP gene driven by the CaMV35S promoter	[20]
pYES2(*URA3*)	Yeast expression vector for native expression of proteins regulated by galactose	Invitrogen

from *Arabidopsis thaliana* L. has been attributed a direct role in TAG biosynthesis [7,8]. This enzyme is located in the endoplasmic reticulum (ER) and its protein sequence shares certain similarity with mammalian GPAT3 and GPAT4, both of which are involved in lipid storage in white adipose tissue of the liver and mammary glands [9].

There have been several attempts to purify plant GPATs from ER fractions. For instance, partial purification of a solubilized GPAT from avocado mesocarp (*Persea americana* Mill.) was achieved by Eccleston and Harwood [10] using affinity chromatography, although the purified enzyme was very unstable. A GPAT from palm callus (*Elaeis guineensis* Jacq.) was also partially purified using ion exchange and molecular exclusion chromatography by Manaf and Harwood [11]. Based on this latter approach, Ruiz-Lopez et al. [12] obtained fractions from sunflower (*Helianthus annuus* L.) enriched in microsomal GPAT activity, although the enzyme responsible for that activity was not successfully sequenced.

Common sunflower seeds accumulate high levels of TAGs that are rich in oleic and linoleic acids [13]. Moreover, several mutant sunflower lines have been selected and bred to produce oils with diverse properties and fatty acid content [13]. The study of the genetics and biochemistry of oil biosynthesis in sunflower seeds not only provides a basic understanding of the underlying processes but also, it yields potential targets to customize the lipid composition of sunflower oil in order to improve both oil quality and production. As such, the GPAT activity in developing seed embryos was characterized, whereby the highest activity was measured in seeds between 15 and 20 days after flowering (DAF), showing specificity towards palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA derivatives [12]. The results of this study reflected the fatty acid composition at the *sn*-1 position of sunflower TAGs, supporting the involvement of this activity in oil assembly. To complement this earlier study, we report here the successful identification of the major GPAT isoform present in developing sunflower seed membranes, as well as its molecular and biochemical characterization. The role of this enzyme in sunflower oil synthesis is discussed in view of these findings.

2. Materials and methods

2.1. Biological material and growth conditions

The wild-type CAS-6 sunflower (*H. annuus*) line (Sunflower Collection of Instituto de la Grasa, CSIC, Spain) was grown as described in Ruiz-Lopez et al. [12]. Root, hypocotyl, leaf tissues and seeds at different days after flowering (DAF) from at least three plants were harvested for downstream analyses. *S. cerevisiae* strains were grown at 22 °C in restricted SC medium supplemented with glucose, galactose or raffinose (2%, w/v). Tobacco (*Nicotiana tabacum* L.) Bright Yellow-2 (BY-2) cells were cultured in suspension and prepared as described elsewhere [7]. The strains and plasmids used in this study are described in Table 1.

For heterologous expression studies and complementation assays, the PLATE transformation method [21] was used to separately introduce the plasmid constructs into the yeast strains. Yeast transformants were grown at 30 °C overnight in liquid selective SC medium (SC-U for pYES2 and SC-L for p416) supplemented with 2% galactose. Complementation assays were performed on 5 μL aliquots of serial dilutions plated on various selective media and incubated at 22 °C. Yeast transformed with the empty plasmids were used as controls.

2.2. Cloning and expression of Ha*GPAT9s*

Sunflower *HaGPAT9* isoforms 1 and 2 were identified based on a BLAST search of *Helianthus* sp. EST sequences using human GPAT3 as the query. Alignments from these ESTs revealed two complete GPAT sequences in sunflower, which were amplified from 15 DAF sunflower seed cDNA using specific primer pairs (HaGPAT9_[1,2]-F/-R: Electronic Material Table S1). The sequences of these cDNAs were confirmed (Secugen, Madrid, Spain) and complete sequences for *HaGPAT9-1* and *2* were deposited in GenBank under accession numbers EF552845 and EF552846, respectively.

Full-length sequences were amplified using the GPAT9_[1,2]pYES2-F/-R primer pairs (Table S1) and they were cloned into the pYES2(*URA3*) galactose inducible vector (Invitrogen, Carlsbad, CA, USA). Alternatively, the GPAT9_[1,2]p416-F/-R primer pairs were used for constitutive expression in the yeast p416(*LEU2*) vector [19] (kindly provided by Dr Ana Rincón). Quantitative PCR was performed using cDNA from seeds and tissues obtained at various DAF, as described in Sánchez-García et al. [22], and using the qGPAT9_[1,2]-F/-R primers (Table S1) and the corresponding constructs in the pYES2 plasmid.

2.3. Mass spectrometry analysis of sunflower seed proteins

Solubilized proteins from sunflower seed microsomal fractions were analyzed on the LTQ-Orbitrap XL mass spectrometer (Methods S1). The resulting spectra were searched against a custom sunflower database containing data from NCBI, TIGR and UniGene, as well as from the sunflower acyltransferases cloned to date that were added manually.

2.4. Confocal microscopy of GFP-tagged Ha*GPAT9-1 in tobacco cell suspensions*

The full-length open reading frame (ORF, minus the stop codon) of *Ha*GPAT9-1 was amplified by PCR using GPAT9_1pUC18-F/-R primers, designed with *Nhe*I restriction sites (Table S1). Cloned products were inserted into the unique *Nhe*I restriction site of pUC18/*Nhe*I-mGFP [20]. The resulting construct encoded