

# Acyl carrier proteins from sunflower (*Helianthus annuus* L.) seeds and their influence on FatA and FatB acyl-ACP thioesterase activities

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## Abstract

**Main conclusion** The kinetics of acyl-ACP thioesterases from sunflower importantly changed when endogenous ACPs were used. Sunflower FatB was much more specific towards saturated acyl-ACPs when assayed with them.

Acyl carrier proteins (ACPs) are small (~9 kDa), soluble, acidic proteins involved in fatty acid synthesis in plants and bacteria. ACPs bind to fatty acids through a thioester bond, generating the acyl-ACP lipoproteins that are substrates for fatty acid synthase (FAS) complexes, and that are required for fatty acid chain elongation, acting as important intermediates in de novo fatty acid synthesis in plants. Plants, usually express several ACP isoforms with distinct functionalities. We report here the cloning of three ACPs from developing sunflower seeds: *HaACP1*, *HaACP2*, and *HaACP3*. These proteins were plastidial ACPs expressed strongly in seeds, and as such they are probably involved in the synthesis of sunflower oil. The recombinant sunflower ACPs were expressed in bacteria but they were lethal to the prokaryote host. Thus, they were finally produced using the GST gene fusion system, which allowed the apo-enzyme to be produced and later activated to the holo form. Radio-labelled acyl-ACPs from the newly cloned holo-ACP forms were also synthesized and used to characterize the activity of recombinant sunflower FatA and FatB thioesterases,

important enzymes in plant fatty acids synthesis. The activity of these enzymes changed significantly when the endogenous ACPs were used. Thus, FatA importantly increased its activity levels, whereas FatB displayed a different specificity profile, with much high activity levels towards saturated acyl-CoA derivatives. All these data pointed to an important influence of the ACP moieties on the activity of enzymes involved in lipid synthesis.

**Keywords** Acyl carrier protein · Sunflower, *Helianthus annuus* · Acyl-ACP thioesterase · Fatty acid synthesis

## Abbreviations

ACP	Acyl carrier protein
DAF	Days after flowering
DAG	Diacylglycerol
FAS	Fatty acid synthases
PBS	Phosphate-buffered saline
TAG	Triacylglycerol

## Introduction

Fatty acid synthesis is catalyzed by fatty acid synthase (FAS, EC 2.3.1.85) complexes (Wakil 1989). Plants and bacteria use type II FAS complexes, which are made up of discrete peptides that catalyze the individual steps in the elongation reaction (White et al. 2005). In these discrete type II systems, the acyl chains under elongation bind to a small (~9.5 kDa), soluble, acidic proteins, the acyl carrier proteins (ACP), and thus, all the metabolic intermediates of FAS from malonate to long chain fatty acids exist as acylated-ACPs in plant cells. Moreover, acyl-ACPs are the substrates for other important enzymes within plant lipid

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metabolism, such as stearoyl-ACP desaturases (EC 1.14.99.6), plastidial acyltransferases (EC 2.3.1.15) and acyl-ACP thioesterases (EC 3.1.2.14; Ohlrogge and Jaworski 1997). ACP proteins are highly conserved across different eukaryote and plant species, and their structure has been resolved by the solution of NMR and X-ray crystallography, consisting of four  $\alpha$ -helices connected by three loops. Furthermore, functional ACPs (*holo*-ACPs) contain a 4'-phosphopanthoic acid (4-PP) molecule as the prosthetic group that binds to a serine residue located at the junction of the  $\alpha 1\alpha 2$  loop near the N-terminal region of helix II (Holak et al. 1988; Li et al. 2003). This is an essential moiety as fatty acids bind to the thiol group present in this molecule. Moreover, the presence of this prosthetic group stabilizes the structure of the protein, allowing greater flexibility during the interaction with the FAS complex (Kim et al. 2006). Inactive *apo*-ACP is transferred to the 4-PP group from a coenzyme-A molecule in a reaction that is catalyzed by the *holo*-ACP synthase (EC 2.7.8.7; Lambalot and Walsh 1995).

ACPs are strongly expressed in bacteria and unicellular algae, where they are often encoded by a single gene. In plants, several ACP isoforms are found and they are expressed at distinct levels in different tissues (Battey and Ohlrogge 1990; Rawlings and Cronan 1992). Thus, there are eight genes encoding different ACPs in *Arabidopsis thaliana*, five expressed in plastids and three expressed in the mitochondria (Beisson et al. 2003). Mitochondrial ACPs are involved in the synthesis of lipoic acid and importantly, they have diverged from the plastidial ACPs involved in fatty acid synthesis (Hiltunen et al. 2009). All ACPs are encoded by nuclear genes, and thus, ACPs must be transported to their corresponding organelle as they mature after translation. To date, the influence of ACPs on the different aspects of intraplastidial fatty acid synthesis has not been investigated in depth. In this regard, ACPs from different plants expressing unusual acyl-ACP desaturases ( $\Delta^4$  and  $\Delta^6$  palmitoyl-ACP desaturases from *Coriandrum sativum* and *Thunbergia alata*, respectively) have been purified and studied in terms of their activity and specificity (Suh et al. 1999). In assays of substrate competition, the unusual fatty acids were produced faster from the endogenous palmitoyl-ACP derivative. Furthermore,  $\Delta^4$  desaturase from *C. sativum* displayed fourfold and tenfold more activity with coriander palmitoyl-ACP than with the spinach and *E. coli* equivalents, respectively. These differences were not observed when coriander  $\Delta^9$ -stearate desaturase was assayed with the different ACPs, which indicated a role of ACP in the production of unusual monoenes in these species. Despite these interesting results, there are few studies into the influence of specific ACP isoforms on the different plastidial enzymes and usually, commercial or recombinant *E. coli* ACP is used in biochemical studies.

Here, the genes encoding for three different ACPs were isolated from developing sunflower seeds (*HaACP1*, *HaACP2*, and *HaACP3*) and they were overexpressed in bacteria. These ACPs were used to synthesise different acyl-ACPs to study their effect on the specificity of FatA and FatB acyl-ACP thioesterases from sunflower (*HaFatA* and *HaFatB*). Acyl-ACP thioesterases are important enzymes in plant fatty acid synthesis, as they terminate the plastidial reactions on acyl-ACPs by hydrolyzing the acyl-ACP derivatives (Ohlrogge and Jaworski 1997). At this point the free ACP protein is reused and the released free fatty acids are exported out of the plastids to be incorporated into glycerolipids in the endoplasmic reticulum. Thus, these enzymes to some extent determine the fatty acid composition of the TAGs that accumulate in the seeds, and they are therefore of interest in plant biotechnology (Voelker 1996; Thelen and Ohlrogge 2002). Two thioesterase gene families have been described in plants: FatA, which displays high specificity towards oleoyl-ACP; and FatB, with a broader substrate specificity profile and strong activity towards oleoyl-ACP and palmitoyl-ACP (Jones et al. 1995). While FatA is broadly accepted as an essential house-keeping enzyme (Moreno-Pérez et al. 2012), FatB is necessary to supply the lipids in the cell with the necessary amount of saturated fatty acids (Bonaventure et al. 2003). However, in all these studies of specificity, the ACP forms used for enzymatic characterization came from bacteria (*E. coli*) or other plant sources (spinach), and no studies on the impact of the endogenous ACP forms on acyl-ACP thioesterases have been reported to date.

As indicated above, here three forms of ACP expressed in developing sunflower embryos were cloned and characterized: *HaACP1*, *HaACP2* and *HaACP3*. These cofactors were expressed in *E. coli*, purified and used to synthesize the corresponding acyl-ACPs to assay recombinant *HaFatA* and *HaFatB* activities. The resulting specificity and kinetic profiles of these enzymes with endogenous ACPs was compared with those obtained using recombinant ACP from *E. coli* (*EcACP*). The results obtained demonstrate that the protein part of the acyl-ACP substrates were as determinant as the acyl moiety to define enzyme specificity. The consequences of this finding for earlier and future studies on ACP-dependent enzymes are discussed.

## Materials and methods

### Plant material

Sunflower seeds from the CAS-6 common sunflower line (Sunflower Collection of Instituto de la Grasa, CSIC, Seville, Spain) were germinated in wet perlite at 25 °C and then moved to a germination chamber for 2 weeks.

Subsequently, the seedlings were transferred to growth chambers, and grown in bags endowed with fertilizer supply at 25°/15 °C (day/night). They were then grown on a 16 h photoperiod, and with a photon flux density of 250 μmol m<sup>-2</sup> s<sup>-1</sup> until the vegetative tissues or developing seeds were harvested.

### Cloning sunflower ACP cDNAs

Developing sunflower embryos (~0.3 g) were harvested at 15–18 days after flowering (DAF), and they were ground in liquid N<sub>2</sub> in a precooled sterile mortar. The mRNA was isolated from the developing embryos as described by González-Thuillier et al. (2015), and cDNAs were obtained using a Ready-To-Go TPrimed First Strand Kit (Amersham Bioscience, Roosendaal, The Netherlands). *Arabidopsis thaliana* ACP protein encoded by the At3g05020 gene (strongly expressed in seeds) was used to search sunflower expressed sequence tags (ESTs) in public databases to identify putative mRNAs encoding sunflower ACP homologues using the TBLASTN algorithm (Altschul et al. 1997). Two ESTs encoding whole ACPs were detected, as well as a partial sequence of a third gene. Specific primers were designed to amplify the former two ESTs (ACP1-F/ACP1-R and ACP2-F/ACP2-R: Table 1), which were named *HaACP1* and *HaACP2*. A partial sequence of the 5' end of the third gene (*HaACP3*) was obtained using the Smart<sup>TM</sup>-RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) and the specific internal reverse oligonucleotides, ACP3-rac1rev and ACP3-rac2rev (Table 1). The 3' end of the *HaACP3* cDNA was obtained by PCR using the externally added FA2Z primer, complementary to the sequences incorporated during the initial cDNA synthesis, and the specific internal primers ACP3-rac1for and ACP3-rac2for (Table 1). Once the complete sequence of *HaACP3* was determined the whole gene was amplified using the ACP3-F/ACP3-R primer pair (Table 1). The PCR fragments obtained using different combinations of these forward and reverse primers were cloned into the pMBL-T vector (Genaxxon BioScience GmbH, Biberach, Germany) and several clones were sequenced on both strands (SECUGEN, Madrid, Spain).

The *HaACP1*, *HaACP2* and *HaACP3* genes were amplified without the peptide signal using the pair of primers pGEXACP1-F/pGEXACP1-R, pGEXACP2-F/pGEXACP2-R and pGEXACP3-F/pGEXACP3-R (Table 1), respectively. For cloning into the pGEX-4T (GST) expression vector (GE Healthcare), the PCR products contained the restriction sites for the enzymes *Bam*HI and *Xho*I at their 5' and 3' ends, respectively. The resulting vectors pGEX-4::HaACP1, pGEX-4::HaACP2 and pGEX-4::HaACP3 were transformed into competent *E. coli* BL21 strain to

**Table 1** Sequence of the oligonucleotides used in this work

Name	Sequence <sup>a</sup>
ACP1-F	5'-ATTGTACCTCCGCCGATC-3'
ACP1-R	5'-CAAAAGACGGGATCTTTCTGATC-3'
ACP2-F	5'-GCCGATTATTCTCGAATTCTTG-3'
ACP2-R	5'-CAACAAAAACAAGAAAAAGTATACCAC-3'
ACP3-rac1rev	5'-ACCTTGTTGACCGTCTCAGGTGTCCG-3'
ACP3-rac2rev	5'-GAAGCCCGGTAAGCGTCTGATTCG-3'
ACP3-rac1for	5'-CGGTTGAGATTGTGATGGGACTGGAAG-3'
ACP3-rac2for	5'-GAGCATTGCAACCGTTCAGGATGCAGC-3'
FA2Z	5'-AACTGGAAGAATTCGCGG-3'
ACP3-F	5'-TTTCATGCCCTCTCTTTCATGC-3'
ACP3-R	5'-CGAAACGAGCTTCTCAATCAG-3'
QHaACP1-F	5'-AACCTGATCTCTCCTCCAC-3'
QHaACP1-R	5'-CGTGAAAAAGTTTGTGACATTGTAA-3'
QHaACP2-F	5'-ACCTTCATGCCAATGCCTGG-3'
QHaACP2-R	5'-TAGACAAGTTTGCAACATAGTGAG-3'
QHaACP3-F	5'-TTCATGCCCTCTCTTTCATGCCAT-3'
QHaACP3-R	5'-CACGCTTCCGGGTTTGCTGT-3'
QHaActin-F4	5'-GCTAACAGGGAAAAAGATGACTC-3'
QHaActin-R4	5'-ACTGGCATAAAGAAAGCAAGCAGC-3'
pGEXACP1-F	5'-CCGGATCCATGGCCAAGCCAGAGAC-3'
pGEXACP1-R	5'-CGCTCGAGTTACGCAGAGTC-3'
pGEXACP2-F	5'-CCGGATCCATGGCCAACAGAACAG-3'
pGEXACP2-R	5'-CCCTCGAGTTACGCACTGTCTTTC-3'
pGEXACP3-F	5'-CCGGATCCATGGCGACACCTGAGACGG-3'
pGEXACP3-R	5'-CCCTCGAGTTATTTTTCGAAACGAGC-3'
acpS-F	5'-GACGCATGCGCAATATTAGGTTTAGGCAC-3'
acpS-R	5'-GTCAAAGCTTAACCTTCAATAATTACCGTGG-3'

<sup>a</sup> Restriction sites are indicated in bold

produce the *apo*-ACPs fused to a glutathione-S-transferase protein (GST), as described by Smith and Johnson (1988).

### Cloning *E. coli* holo-ACP synthase

The *E. coli* holo-ACP synthase (*Eca*cpS) was cloned as described by Lambalot and Walsh (1995), after amplifying it from *E. coli* using the *acpS*-F and *acpS*-R primers (Table 1) that included the *Sph*I and *Hind*III sites. The resulting DNA fragment was cloned into the pQE80L plasmid where a poly histidine tag was added to facilitate its purification by Ni-NTA affinity chromatography.

### RT-qPCR studies of gene expression

The cDNAs from vegetative tissues (leaf, stem and roots), developing seeds (12, 15, 20, 25, and 30 DAF) and cotyledons (2, 5, and 7 days after imbibition) were obtained as described above. The expression of the three sunflower ACP genes in these tissues was quantified by RT-qPCR using gene-specific primer pairs: QHaACP1-F/IQHaACP1R for *HaACP1*; QHaACP2-F/QACP2-R for

HaACP2; and QHaACP3-F/QHaACP3R for HaACP3 (Table 1). The fluorescence resulting from the SYBR Green I system (QuantiTect™ SYBR® Green PCR Kit; Qiagen, Crawley, UK) was monitored in a MiniOpticon apparatus (Bio-Rad, Hercules, CA). The reaction mixture was heated to 50 °C for 2 min and then to 95 °C for 15 min before subjecting it to 40 PCR cycles: 94 °C for 15 s; 61 °C for 30 s; and 72 °C for 15 s. The calibration curves were drawn up using sequential dilutions of the cDNAs and the Livak method was employed to calculate the comparative expression of the samples (Livak and Schmittgen 2001). Amplification of the sunflower *HaACT1* actin gene with QHaActin-F4/QHaActin-R4 primers (GenBank Accession number FJ487620) served as a reference (Table 1).

### Protein expression in *E. coli* and affinity purification

Different recombinant proteins were produced by overexpressing the corresponding gene in the pGEX-4T-1 (GE Healthcare) or pQE80L (Qiagen) vectors using *E. coli* K27 or BL21 strains, respectively. As a general rule, bacteria were grown at 37 °C until the optical density at 600 nm reached a value of 0.5. The cultures were then induced with 0.5 mM of isopropyl-beta-D-thiogalactopyranoside (IPTG) and allowed to grow for an additional 5–6 h. Then, bacteria were recovered by centrifugation, resuspended in Phosphate-buffered saline [PBS: 20 mM K<sub>2</sub>HPO<sub>4</sub> (pH 7.3), 140 mM NaCl, 2.7 mM KCl] and treated with 1 mg/mL lysozyme for 1 h at 37 °C. The bacteria were then sonicated on ice using a probe sonicator (Branson B12), applying 10 pulses of 10'' (20 kHz, 75 W) with pauses of 45''. Then 1 mg/mL of DNase was added, it was incubated for 30 min at 37 °C and the lysate was centrifuged for 20 min at 12,000×g using the resulting crude extracts for subsequent protein purification.

The polyhistidine tagged *holo*-ACP synthases were purified from *E. coli* (*EcacpS*) by affinity chromatography, loading 5 mL of the crude extract onto a 1 mL column of Ni-NTA agarose [equilibrated in 50 mM sodium phosphate (pH 7.5), 300 mM NaCl and 30 mM imidazole] having previously filtered it through a 0.45 µm membrane. The column flow through was collected and passed twice more through the column to maximize protein recovery, and the column was then eluted with 10 mL of elution buffer to remove all the unbound protein. Subsequently, the purified proteins were recovered by eluting the column with 50 mM sodium phosphate (pH 7.5), 300 mM NaCl and 250 mM imidazole, collecting four fractions of 0.5 mL that were assayed for their protein content. The columns were then regenerated by passing 10 mL of distilled water, and they were stored at 4 °C in 20 % ethanol/water. The imidazole associated with the purified proteins was

removed using Millipore ultrafiltration units of 30 or 3 K depending on their size, and the histidine tagged *holo*-ACP synthase from *E. coli* was stored in 50 µL aliquots at –80 °C.

To purify the recombinant HaACPs, the cleared lysate was loaded onto a GSA-agarose affinity column (GE Healthcare) containing 1 mL of the hydrated GSA-agarose equilibrated in PBS. A volume of 1 mL of the crude extract was loaded onto the column and incubated for 1 h at room temperature on a rocking plate. The column flow through was discarded and it was washed with an additional 5 mL of PBS. Subsequently, 1 mL of PBS containing 10 units of Thrombin (GE Healthcare) was added to the columns, and they were again incubated for 1 h at room temperature on the rocking plate. The column flow through containing the sunflower *apo*-ACPs was then collected and the columns were washed with additional 1 mL of PBS. The columns were then regenerated with 5 mL of 10 mM glutathione dissolved in 50 mM Tris-HCl (pH 8). The thrombin present in the fraction was removed using 30 K ultrafiltration (Millipore) units, and the *apo*-ACPs were then concentrated using 3 k ultrafiltration units (Millipore) prior to activation.

### Activation of the mature sunflower ACPs

The activation reaction was carried out on 400 µg of *apo*-ACP in 50 mM Tris (pH 8.8), 1 mM CoA, 5 mM DTT, 10 mM MgCl<sub>2</sub> and 200 nmol of *EcacpS* in a final volume of 0.5 mL, and it was run for 4 h at 37 °C. In these conditions virtually all the *apo*-ACP was activated to the *holo*-ACP form, it was confirmed on native PAGE gels and with a radioactive assay based on the acyl-ACP synthetase reaction (Kuo and Ohlrogge 1984).

### ACP Production in *E. coli*

The *EcACP* clone used in this work was kindly supplied by Dr Penny von Wettstein-Knowles (University of Copenhagen). This gene was cloned into the pQE80L vector and overexpressed in *E. coli*. The polyhistidine *EcACP* was purified by Ni-NTA agarose affinity chromatography and fully activated by a reaction with *EcacpS*, as described above.

### Preparation of radiolabelled acyl-ACP substrates

Labelled acyl-ACP substrates were prepared using a recombinant acyl-ACP synthetase from *E. coli* kindly supplied by Dr J. Shanklin (Brookhaven National Laboratory). Acylation reactions were carried out on 50 µg of *holo*-ACP, with 180 kBq (~0.1 µmol) of (1-<sup>14</sup>C) fatty acid ammonium salt [(<sup>3</sup>H) fatty acid in the case of 16:1<sup>Δ9</sup>], 5 mM ATP, 2 mM DTT, 400 mM LiCl, 10 mM MgCl<sub>2</sub>,



100 mM Tris–HCl (pH 8.0) and 10  $\mu$ g acyl-ACP synthetase. Reactions were carried out in a final volume of 500  $\mu$ L at room temperature for 3–6 h, and the acyl-ACPs were then purified and concentrated by ion exchange chromatography on DEAE-Sepharose (as described by Rock and Garwin 1979).

### Production of recombinant *HaFatA* and *HaFatB* thioesterases

The sunflower thioesterase genes have been cloned and studied previously by us (Serrano-Vega et al. 2003, 2005). The genes encoding the mature *HaFatA* and *HaFatB* polypeptides were cloned into the pQE80L vector for overexpression in *E. coli*. In the case of *HaFatB*, the domain involved in membrane anchoring was removed to recover the enzyme in the soluble fraction. Recombinant *HaFatA* and *HaFatB* thioesterases tagged with a 3' poly-histidine tail were recovered in the soluble phase and purified on a Ni–NTA affinity column, as indicated above. Purified thioesterases (up to 85 % purity when assessed by SDS-PAGE) were used for activity assays.

### Acyl-ACP thioesterase assays

Thioesterase activity was assayed in a final volume of 0.1 ml containing 50 mM Tris–HCl (pH 8.0), 5 mM DTT plus 0.2–12  $\mu$ g of the protein preparation, and an amount of acyl-ACP substrate ranging from 0.02 to 0.08 nmol (30–170 Bq approx.). The reactions were run at room temperature for 5 min and quenched by adding 0.25 ml of 1 M acetic acid in 2-propanol. The free fatty acids were then extracted twice with 0.3 ml hexane, and the radioactivity in the pooled organic phase was determined in a calibrated liquid scintillation counter (Rackbeta II; LKB). The data from thioesterase assays were fitted to the Hill equation by non-linear least-squares regression analysis using OriginPro 8 software and they were correlated at  $P < 0.005$  as determined by Student's *t* test. Both the  $V_{\max}$  and  $K_m$  were derived from these curves. Protein concentrations were measured using the colorimetric Bradford (1976) method.

### Bioinformatics

ACP protein sequences from public databases were aligned to identify regions of homology using the ClustalX v2.1 program (Larkin et al. 2007) and a phylogenetic tree was constructed using MEGA 5.0 software (Tamura et al. 2011). The prediction of the transit peptides of the three *HaACPs* was carried out using the ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>) and Signal IP (<http://www.cbs.dtu.dk/services/SignalP/>) programmes. The tertiary

structure of sunflower ACPs were modelled using the SWISSMODEL server (Guex and Peitsch 1997) based on the structure of *Spinacia oleracea* (spinach) ACP (UniProtKB/Swiss-Prot: P07854.2; Zornetzer et al. 2006).

## Results

### ACPs from sunflower

Three new sunflower ACPs were cloned from developing sunflower seeds that were named *HaACP1*, *HaACP2* and *HaACP3*. These genes were around 400 bp long and they encoded proteins of 139, 132, and 139 amino acids with molecular weights of 14.71, 14.15, and 14.76 kDa, and with isoelectric points of 4.69, 4.48, and 5.01, respectively. They were deposited in the gene bank with accession numbers HM013970 (*HaACP1*), HM13971 (*HaACP2*) and HM013972 (*HaACP3*). These sequences were launched into the public ChloroP and Signal IP prediction servers, which confirmed that the three proteins were targeted to chloroplasts, and that each of them had predicted transit peptides of approximately 55 amino acids (Fig. 1). The mature *HaACP1* and *HaACP2* proteins displayed 80 % identity, whereas they maintained 60 % identity with *HaACP3*, also displaying high levels of identity when aligned with ACP from Arabidopsis (At3g05020) and *E. coli* (Fig. 1).

### Structural analysis of *HaACPs*

The three-dimensional structures of *HaACPs* were modelled using the SWISS-MODEL server (Guex and Peitsch 1997) and the data in the RSC PDB protein database (Berman et al. 2000). The structure of the *HaACPs* corresponded to that of a globular protein with four  $\alpha$ -helices that generate a hydrophobic region at the surface of the protein (Fig. 2). The serine residue that binds to the 4-PP moiety remains in the external region of the protein.

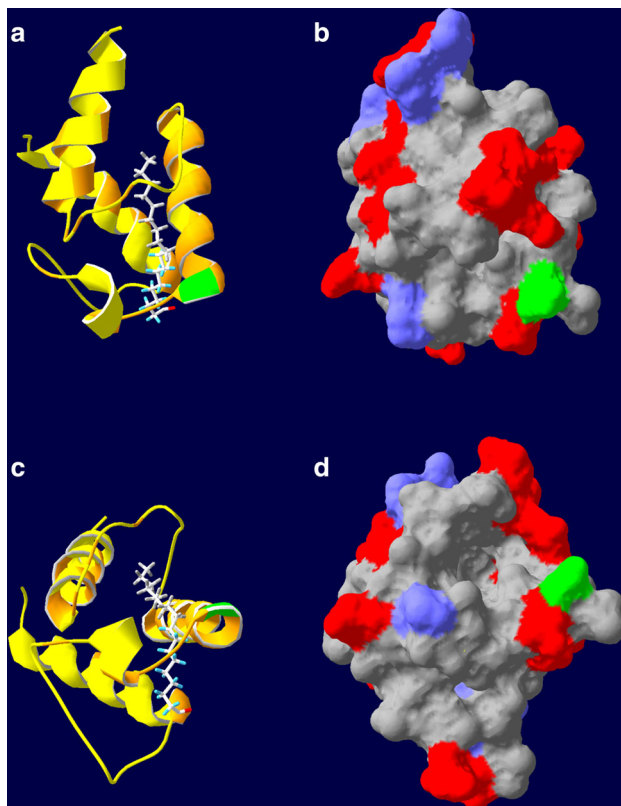
### Phylogenetic analysis of *HaACPs*

A phylogenetic tree was drawn up to study the relationship between the three new ACPs and those described in other plant species using the whole sequences of the ACP proteins (Fig. 3). All three forms were situated within the group of chloroplastic/plastidic ACP isoforms, clearly differentiated from the mitochondrial forms. *HaACP1* and *HaACP2* clustered together in the tree, very close to *HaACP3*. Moreover, all three resided in a group near to the ACPs from tomato (*Solanum lycopersicum*) and castor bean (*Ricinus communis*).

<i>HaACP1</i>	MAAFTGASSTLISPPHISRQCLASTRISGLKMOVFSNSGRSNLPSVFR----RLQISCAAKPETVEKVCDDIVKK	71
<i>HaACP2</i>	MAAFTGSS--TLPS-----CQCLAPTRVSGLKMOVFSNMGRNTNLPVLSFR----RLQISCAAKPETVDKVCNIVRK	64
<i>HaACP3</i>	MASVAGFS--TFMPSLSCHKPNQTLTRASNFNAVSLAINGKSFPSLKFSSAPSRFRVCCAATPETVNKVCNIVRK	73
<i>AtACP4</i>	MASLSTTS-LSFKAP--STTISQVLRKASSQSQSVTFGRFTSSTKSLR-----LQISCAAKAETVQKVSQVSDIVKE	65
<i>EcACP</i>	-----MSTIEERVKKIIGE	14
	. ::* .*: :	
▼		
<i>HaACP1</i>	QLALKDDVSVAGDSKFFATLGADSLDVEIVMALEEAFFGITVEEESAQSITATVQDAADLIEKLCCKDSA----	139
<i>HaACP2</i>	QLALKEDMSVSGESKFFAALGADSLDVEIVMGLEEEFFGITVEEESAQNITATVQDAADLIEKILEKDSA----	132
<i>HaACP3</i>	QLAVPEDTAVTGESKFFATLGADSLDVEIVMGLEEEFFGISVEEESAQSITATVQDAADLIEKLVSKK-----	139
<i>AtACP4</i>	QLALAADVPLTAEKFFSALGADSLDVEIVMALEEKFNISVEESDAQNTTICEAADLIEDLVQKKPAAETS	137
<i>EcACP</i>	QLGVKQEEVTNNAASFVEDLGADSLDVELVMALEEEFDTEIPDEKAEKITTVQAADIDYINGHQA-----	78
	**.: : * . *****:*.*** * . : :.***:*.*** * * * * :	

**Fig. 1** Alignment of the sunflower ACPs. Alignment of the deduced amino acid sequences of the *H. annuus* acyl carrier proteins, *HaACP1*, *HaACP2*, and *HaACP3*, along with the closely related sequences from *A. thaliana*, *AtACP4* (gil15234875), and *E. coli*, *EcACP* (gil955473707). Identical residues are highlighted in black and highly conserved residues in light grey. The serine residue

involved in 4'-phosphopanthoic acid binding is indicated with a black triangle and those residues participating in the acyl binding site are indicated with a rectangular box. The putative N-terminal plastid transit peptides of *HaACP1*, *HaACP2* and *HaACP3* are underlined (56, 49 and 58 amino acids, respectively)



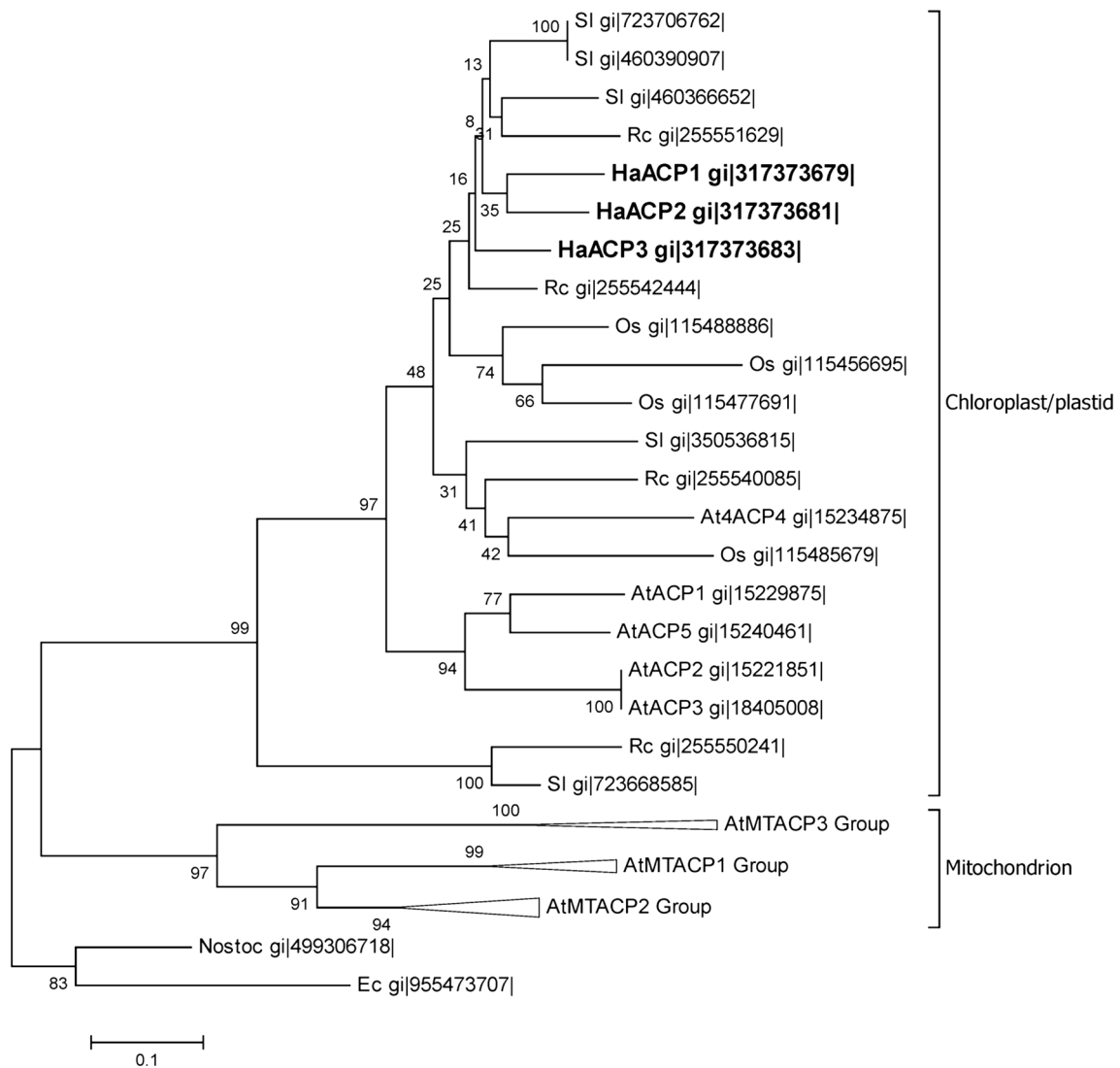
**Fig. 2** Structure of the sunflower ACP. Proposed structural model for sunflower acyl carrier protein *HaACP1* modelled from the *Spinacia oleracea* ACP (2fvf; Zornetzer et al. 2006). Models **a** and **c** represent ACP ribbon diagrams with stearate. Models **b** and **d** show views of the molecular surfaces. The serine residue bound to 4'-phosphopanthoic acid is shown in green in the molecular surface models, and positive and negative residues are shown in blue and red, respectively

## Gene expression

The expression of the *HaACPs* in different vegetative tissues, in germinating cotyledons and in developing seeds was studied by RT-QPCR at different developmental stages (Fig. 4). The three *HaACPs* were expressed in all the organs studied. In vegetative tissues, *HaACP1* and *HaACP2* predominated in the roots and stems, whereas *HaACP1* and *HaACP3* were the genes with most transcripts in green leaves. There was a little change in ACP expression in germinating cotyledons at 1, 4, and 7 days after imbibition (DAI), with a clear predominance of the *HaACP1* and *HaACP2* forms over that of *HaACP3*. The strongest expression of these three proteins was found in the developing embryos in seeds, especially during the period of oil accumulation 16–25 days after anthesis (DAA), when all the three ACP forms were expressed at similar levels, indicating their involvement in the process of oil synthesis.

## Influence of *HaACPs* on the substrate specificity of *HaFatA* and *HaFatB* thioesterases

The influence of the protein group of the acyl-ACP lipoprotein substrate on *HaFatA* and *HaFatB* thioesterase substrate specificity was examined by assaying these enzymes at a constant substrate concentration and comparing the data with those obtained with acyl-ACPs containing *EcACP*. Recombinant *HaFatA* displayed the highest thioesterase activity towards 18:1-ACP derivatives in all cases (Fig. 5a), although the final level of activity reached with that substrate depended strongly on the ACP



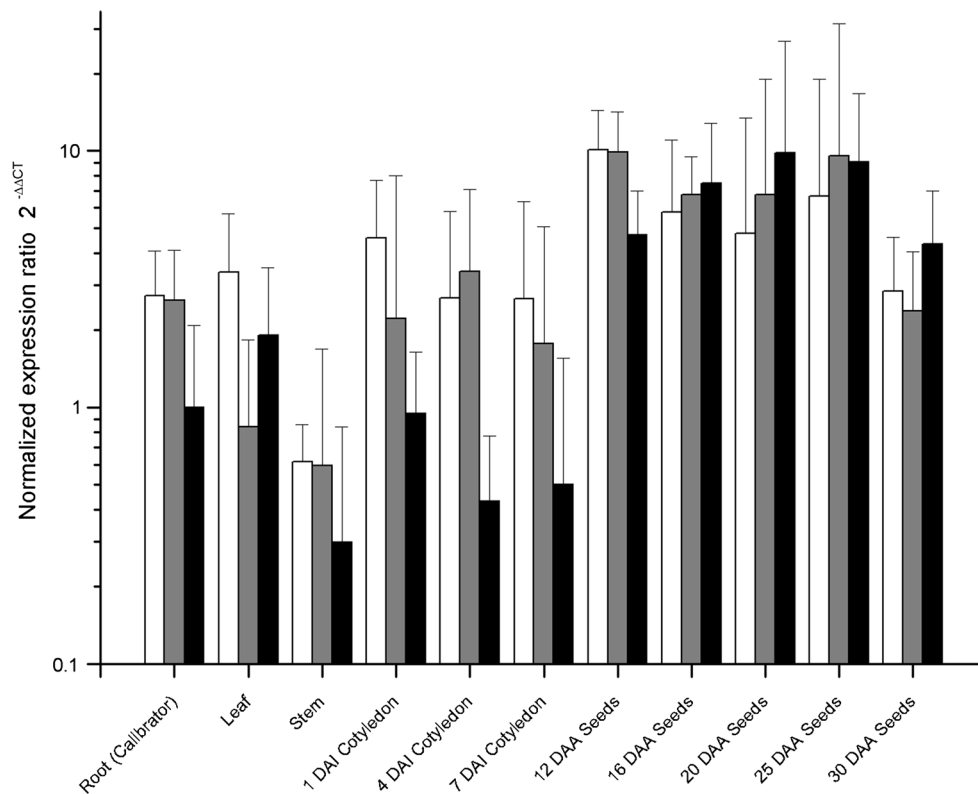
**Fig. 3** Phylogenetic comparison of chloroplast and mitochondrial ACPs using ACPs from *Nostoc* and *E. coli* as an outgroup to root the tree. The tree was constructed using the neighbour-joining method.

The plant species included in the phylogenetic tree are *Ha*, *H. annuus*; *Os*, *Oryza sativa*; *Rc*, *Ricinus communis*; and *Sl*, *Solanum lycopersicum*

form used. Thus, the activity achieved with 18:1-*EcACP* was half that measured with the endogenous 18:1-*HaACP1* and 18:1-*HaACP2*, and fourfold lower than that found with the 18:1-*HaACP3* derivative. Two different patterns were found for the other substrates assayed. Thus, the activity of *HaACP1* and *HaACP2* increased from the 16:0 to the 18:0 derivative, with the 16:1 derivative lying in between these (16:0 < 16:1 < 18:0). Furthermore, acyl-ACP derivatives synthesized with *EcACP* and *HaACP3* displayed higher activity towards 16:1-ACP, which was hydrolyzed at a higher rate than the saturated 16:0 and 18:0 acyl-ACP derivatives.

In the case of *HaFatB*, the specificity profile changed significantly in function of the ACP form assayed (Fig. 5b). Thus, the activity of *EcACP* was much lower than that

measured with the endogenous forms of ACP, also displaying a very different specificity profile. The *HaFatB* enzyme displayed strong hydrolytic activity towards saturated derivatives when acyl-*HaACPs* were used as the substrates. Thus, the strongest activity was found for 16:0-*HaACP2*, followed by 16:0-*HaACP1* and 16:0-*HaACP3*. Activities displayed by the enzyme towards 18:0-*HaACP* derivatives were also high, this time the 18:0-*HaACP3* being the substrate hydrolysed at the highest rate. The activities detected were much lower when monounsaturated 16:1 and 18:1 acyl-ACPs were assayed. In contrast, the profile obtained with *EcACP* was very different. Accordingly, the levels of activity were much lower than those found with the endogenous substrates, as much as 10-fold lower in the case of 16:0-ACP, and the enzyme



**Fig. 4** Expression of the *HaACP1* (white bar), *HaACP2* (grey bar) and *HaACP3* (black bar) genes in different sunflower tissues. *DAI* days after imbibitions, *DAA* days after anthesis

displayed similar activity towards 16:0, 18:0 and 18:1-ACPs, and a weaker activity on the 16:1-ACP derivative.

#### Kinetics parameters of *HaFatA* and *HaFatB* thioesterases

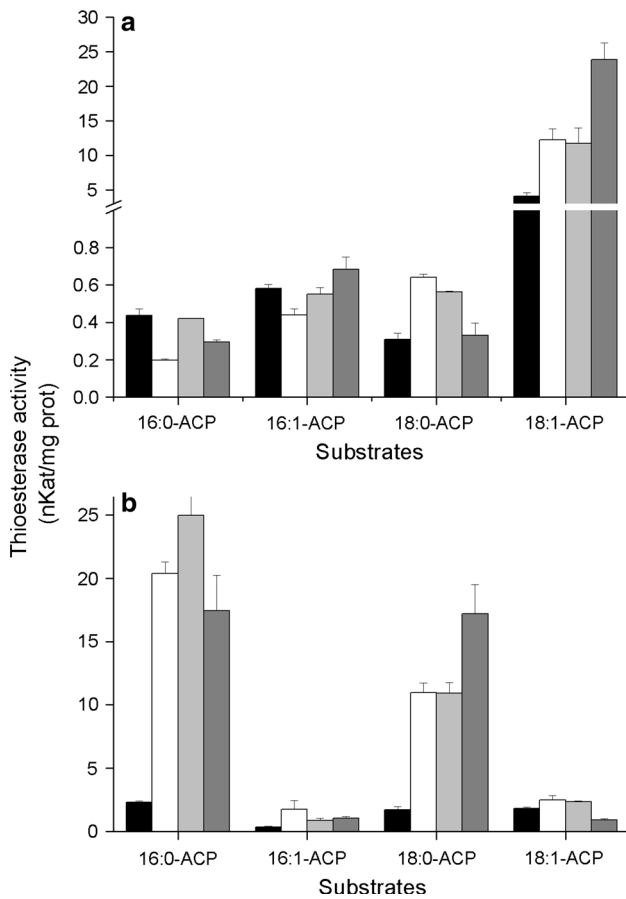
Certain kinetic parameters of the *HaFatA* and *HaFatB* thioesterases towards the acyl-ACP substrates synthesized with the different ACP forms were determined. In the case of *HaFatA*, the maximum  $K_{cat}$  values corresponded to the 18:1-ACP substrates, as expected, with similar values for all the three forms of the 18:1-*HaACPs* between 17.3 and 25.5  $s^{-1}$  (Table 2). These values were four to sixfold higher than for the saturated 16:0-ACP and 18:0-ACP acyl intermediates. The  $K_m$  values were similar for all substrates assayed and ranged from 2.1 to 5.2  $\mu M$ , so the specificity factors ( $K_{cat}/K_m$ ) followed a similar pattern to the  $K_{cat}$  values. When the kinetic parameters of *HaFatB* with the different *HaACP* derivatives were assessed (Table 3), the maximum  $K_{cat}$  values corresponded to the saturated 16:0-ACP and 18:0-ACP acyl-ACP derivatives, with lower values towards 18:1-ACP derivatives. Similarly, the maximum  $K_{cat}$  values were recorded for 18:0 derivatives, in a range between 20 and 26  $s^{-1}$  for the three *HaACP* forms. The values for 16:0 derivatives were lower but still

remained high, around 9  $s^{-1}$ . The  $K_m$  values were in a micromolar order, being slightly higher for 18:0-*HaACPs* and lower for 18:1-*HaACPs*. The kinetic characterization of *HaFatB* was also carried out with *EcACP* derivatives to better assess the impact of using endogenous forms of that protein. The kinetic profile of the *HaFatB* using the bacterial ACP form was quite different (Table 3) with  $K_{cat}$  values remaining in a much lower range than those found with *HaACPs*. Thus, the  $K_{cat}$  values for 16:0-*EcACP* were around 20-fold lower than those found for 16:0-*HaACP*. Such differences were even higher in the case of 18:0 derivatives (80-fold higher) and even greater for the 18:1 derivatives. The maximum  $K_{cat}$  values of this enzyme were obtained with 18:1-*EcACP* and 16:0-*EcACP*. Again, the  $K_m$  values all stayed within a micromolar range.

#### Discussion

In this regards, it is unclear whether the different plastidial isoforms of ACPs have different characteristics and functions in plants. Here, three plastidial ACP isoforms were cloned from developing sunflower seeds and thus, they are very probably involved in the synthesis of fatty acids associated to TAG accumulation in that species. The





**Fig. 5** Substrate specificity of sunflower *HaFatA* (a) and *HaFatB* (b) thioesterases with different acyl-ACP derivatives. Acyl-ACPs were synthesized with ACP from *E. coli* (*EcACP*; black bar) and the three forms of ACP from sunflower (*HaACP1*, white bar; *HaACP2*, light grey, and *HaACP3*, dark grey)

**Table 2** Kinetic parameters of the recombinant sunflower acyl-ACP thioesterase *HaFatA* with different acyl-ACP substrates

Acyl-ACP	$K_m$ ( $\mu\text{M}$ )	$K_{cat}$ ( $\text{s}^{-1}$ )	$K_{cat}/K_m \text{ s}^{-1} \mu\text{M}^{-1}$
<i>HaACP1</i> -16:0	$4.5 \pm 1.2$	$3.5 \pm 0.5$	0.78
<i>HaACP2</i> -16:0	$4.9 \pm 0.4$	$4.2 \pm 0.1$	0.85
<i>HaACP3</i> -16:0	$5.1 \pm 1.9$	$3.9 \pm 0.9$	0.76
<i>EcACP</i> -16:0 <sup>a</sup>	1.3	0.5	0.40
<i>HaACP1</i> -18:0	$4.2 \pm 0.9$	$3.6 \pm 0.5$	0.85
<i>HaACP2</i> -18:0	$4.9 \pm 0.8$	$4.0 \pm 0.6$	0.81
<i>HaACP3</i> -18:0	$4.6 \pm 1.7$	$6.0 \pm 1.1$	1.3
<i>EcACP</i> -18:0 <sup>a</sup>	0.9	0.4	0.40
<i>HaACP1</i> -18:1	$2.1 \pm 1.2$	$25.5 \pm 1.1$	12.3
<i>HaACP2</i> -18:1	$2.4 \pm 0.9$	$17.3 \pm 1.5$	7.4
<i>HaACP3</i> -18:1	$2.8 \pm 0.2$	$24.0 \pm 1.0$	8.7
<i>EcACP</i> -18:1 <sup>a</sup>	2.1	11.4	5.4

Data represent the average  $\pm$  SD from at least three independent determinations

<sup>a</sup> Data taken from Serrano-Vega et al. (2005)

**Table 3** Kinetic parameters of recombinant sunflower acyl-ACP thioesterase *HaFatB* with different acyl-ACP substrates

Acyl-ACP	$K_m$ ( $\mu\text{M}$ )	$K_{cat}$ ( $\text{s}^{-1}$ )	$K_{cat}/K_m \text{ s}^{-1} \mu\text{M}^{-1}$
<i>HaACP1</i> -16:0	$4.0 \pm 1.7$	$9.6 \pm 1.9$	2.4
<i>HaACP2</i> -16:0	$5.1 \pm 1.5$	$9.4 \pm 1.8$	1.9
<i>HaACP3</i> -16:0	$4.7 \pm 0.3$	$9.6 \pm 0.5$	2.0
<i>EcACP</i> -16:0	$0.84 \pm 0.2$	$0.52 \pm 0.06$	0.62
<i>HaACP1</i> -18:0	$9.6 \pm 3.6$	$20.4 \pm 8.0$	2.1
<i>HaACP2</i> -18:0	$6.7 \pm 0.1$	$21.5 \pm 0.2$	3.2
<i>HaACP3</i> -18:0	$12.8 \pm 0.3$	$26.5 \pm 6.3$	2.1
<i>EcACP</i> -18:0	$1.1 \pm 0.2$	$0.27 \pm 0.01$	0.24
<i>HaACP1</i> -18:1	$0.9 \pm 0.3$	$0.7 \pm 0.1$	0.85
<i>HaACP2</i> -18:1	$1.4 \pm 0.1$	$0.60 \pm 0.05$	0.43
<i>HaACP3</i> -18:1	$2.0 \pm 0.7$	$0.18 \pm 0.05$	0.09
<i>EcACP</i> -18:1	$1.3 \pm 0.2$	$1.19 \pm 0.1$	0.91

Data represent the average  $\pm$  SD from at least three independent determinations

modelling of the three sunflower ACPs reveals that they display an appropriate functional structure, without displaying any unexpected or aberrant domain.

The three sunflower ACPs are ubiquitously expressed proteins whose transcripts are found in all the tissues assayed, although all they were expressed more strongly in the developing seeds. This is consistent with the role of these proteins in the synthesis of fatty acids, which takes place in all plant tissues but especially in the oil accumulating embryos (Fig. 4). Hence, *HaACP3* may be more strongly implicated in the synthesis of fatty acids associated to TAG accumulation in seeds due to display of higher expression levels in developing seeds and lower ones in vegetative tissues. Differentiation between them was also supported by their position in the phylogenetic tree (Fig. 3). In this regard, data of Arabidopsis plastidial ACP expression available on <http://aralip.plantbiology.msu.edu/> shows that there are some forms of ACP that are specifically expressed in developing seeds (*At3g05020* and *At5g27200*), vegetative tissues (*At4g25050*) and with ubiquitous expression (*At1g54630* and *At1g54580*), pointing to a relative specialization of certain forms of these proteins that would be in agreement with results in sunflower.

The heterologous expression of these genes in *E. coli* was initially carried out in the protein expression plasmid pQE80L. However, the induction of protein expression provoked the death of the bacteria, probably because the *HaACP*s interact with the endogenous fatty acid metabolism and they hinder the native lipid synthesis. Moreover, the concentration of these recombinant proteins in the soluble fraction was very low when expressed in yeast (data not shown). Since it was necessary to produce

milligram amounts of these proteins, we produced fusion proteins in the pGEX-4T-1 plasmid that generated recombinant ACPs fused to the large soluble GST protein. This system is particularly suited to express proteins targeted to inclusion bodies or those that cause lethality. Thus, the peptide resulting of the fusion of ACP and Glutathione S-transferase did not interacted with *E. coli* metabolism and was expressed strongly in the soluble phase of the bacteria and they could be purified by affinity chromatography. In that form, the 4-pp group was not esterified to the ACPs, so after the *HaACP* moieties were released by digestion of the fusion protein with thrombin, the resulting apo-*HaACP* had to be activated in vitro to the holo forms. That was achieved by incubation with the recombinant *EcacpS* in the presence of CoA, resulting in the quantitative conversion to holo-*HaACP*s. These recombinant holo-*HaACP*s were used to produce different acyl-ACP substrates to assay the sunflower acyl-ACP thioesterases.

All plants possess two types of thioesterases, FatAs and FatBs. In this regard, thioesterases from many very different sources have been characterized in terms of their substrate specificity and their activity towards different acyl derivatives bound to the same ACP (Pollard et al. 1991; Hawkins and Kridl 1998; Salas and Ohlrogge 2002; Wu et al. 2009; Sánchez-García et al. 2010). The effect of the ACP on the activity of these enzymes has not been studied in detail, despite the great variety of ACP forms that can be found in plants. In the present study, we hypothesized that ACP could have an important role on the acyl-ACP thioesterase activity, as it has the acyl moiety. So we investigated the specificity and kinetic parameters of these enzymes using endogenous acyl-ACP derivatives and acyl-*EcACP*s as the control.

From the first studies into plant acyl-ACP thioesterases, the specificity of FatAs indicated that they participate in the export of the 18:1 derivatives to synthesize glycerolipids from the plastids to the cytosol and endoplasmic reticulum. This makes it a very important housekeeping enzyme as most of the C18 fatty acids present in plant tissues are derived from these de novo synthesized fatty acids. From the point of view of oil TAG synthesis in seeds, this enzyme was also of importance since most oil seeds accumulate oleic acid or fatty acids derived from oleic acid by desaturation (linoleic and linolenic) or elongation/desaturation (gondoic and erucic). This means that FatA enzymes have to support very high metabolic fluxes, allowing the synthesis and accumulation of large amounts of storage TAGs. When sunflower FatA was assayed with the *E. coli* and endogenous acyl-ACPs the 18:1-ACP derivatives were those hydrolyzed at the highest rate. However, the specificities and catalytic efficiencies were higher with sunflower ACPs (Fig. 5a; Table 2), which indicated that there was an influence of the protein moiety

of the substrate on this enzyme, and that endogenous ACP should be used to measure the kinetic parameters of these enzymes and at estimating their performance and contribution to metabolic flux.

FatB thioesterases were first described in common plants like *Arabidopsis* by Dörmann et al. (1995, 2000), this enzyme was hypothesized to play a role in the export of saturated fatty acids present in these plants. However, the specificity of *Arabidopsis* FatB showed strong preference for 18:1-ACP and a weaker preference for 18:0-ACP, which challenged this hypothesis and pointed that this enzyme did not complement the specificity of FatA within plant lipid metabolism. However, a decisive approach to FatB functionality was made when a FatB knockout mutant was isolated and characterized in *Arabidopsis*. The analysis of the lipid composition of that mutant showed that the suppression of this enzyme drastically decreased the proportion of saturated 16:0 and 18:0 fatty acids in all plant tissues (Bonaventure et al. 2003). This deficiency in saturated fatty acids caused dwarf phenotype in mutant plants, pointing that saturated fatty acids are essential for normal plant development. Later studies pointed that a deficiency in saturated fatty acids could alter long chain bases synthesis pathway, producing a lack of sphingolipids that could hamper plant growth (Chen et al. 2006). Thus, while this enzyme appears to be involved in the synthesis of these fatty acids, its specificity profile did not support this function. Studies on FatB enzymes from other sources did not clarified these contradictory results. Thus, FatBs from coriander (Salas and Ohlrogge), castor (Sánchez-garcía et al. 2010), *Jatropha curcas* (Wu et al. 2009), *Madhuca longifolia* (Ghosh et al. 2007) and *Macadamia tetraphylla* (Moreno-Pérez et al. 2011) were cloned and characterized yielding profiles similar to those reported by Dörmann et al. (2000) for the *Arabidopsis* form. The data presented in this work showed that FatB from sunflower was much more active with the endogenous saturated acyl-ACP substrates, displaying  $K_{cat}$  values 20-fold larger for 16:0 derivatives and almost 100-fold larger for 18:0 ones (Fig. 5b; Table 3). On the contrary, it displayed lower activities towards 18:1 derivatives. This result indicate that plant FatB thioesterases are probably very specific towards saturated acyl-ACP substrates, and that the specificity profiles reported elsewhere for this enzyme are probably due to the use of non-endogenous ACPs to assay this enzyme activity. This aspect of acyl-ACP thioesterases should be taken into consideration in future when ACP-dependent enzymes are characterized. Another interesting issue is that HaFatB was more efficient at exporting 18:0 than 16:0 in sunflower. In this regard, FatB should be responsible of most of the saturated fatty acid flow that are stored in TAGs by developing seeds, so it is an important enzyme in plant lipid biotechnology. The production of

sunflower oils enriched in stearic acid has been a priority in our group due it could be a source of healthy solid fats. Thus, the partial inhibition of sunflower stearate desaturase produced an increase of the accumulation of stearic acid in sunflower (Pérez-Vich et al. 1999), which means that this plant should have the necessary enzymatic machinery to export this fatty acid from plastids at high rates. Moreover, the stearyl-ACP thioesterase activity of sunflower seeds plastids was measured in vivo by labelling experiments (Pleite et al. 2006), resulting in a considerable capacity of that enzyme to hydrolyze the mentioned substrate, which does not fitted well with the specificity profile reported for FatB from sunflower and other sources, which very often show low levels of activity towards stearyl-ACP. Data in the present work explain that results and supported that most of the export of stearic acid from sunflower seed plastids is undertaken by HaFatB thioesterase, which was highly specific for endogenous stearyl-ACPs. Furthermore, the gene coding for HaFatB could be related with the improved high-stearic traits of some sunflower mutants (Pérez-Vich et al. 1999). On the contrary, the low stearic acid content of common sunflower can be easily explained by the competition of this enzyme with the very active sunflower stearyl-ACP desaturase.

## Conclusions

The main conclusion of this work was that the nature of the ACP protein can alter the specificity and catalytic efficiency of non-divergent enzymes of fatty acid metabolism, as was the case of *HaFatA* and *HaFatB* thioesterases. The endogenous ACPs enhanced *HaFatA* activity with respect to substrates prepared from *EcACP*, and the resulting specificity and kinetic parameters were similar for the three sunflower ACPs. In the case of *HaFatB*, the use of endogenous ACP caused important changes on enzyme specificity, which displayed much stronger activity and catalytic efficiency with these ACPs and was highly specific towards saturated acyl-ACPs. This was in very good agreement with the reverse genetic results obtained in *Arabidopsis* (Bonaventure et al. 2003) and suggests that plant FatB1 like enzymes are highly specialized for the export of de novo synthesized saturated fatty acids from plastids. Moreover, these results demonstrate that the nature of ACPs used is very important when studying intraplastidial biochemistry. Indeed, the results obtained with alternative ACPs could change substantially when using physiological substrates, a fact that must be taken into consideration in future experiments in the field.

**Author contribution statement** Jose A. Aznar-Moreno did the work of cloning, gene and protein expression and

biochemical determinations. Mónica Venegas-Calero supervised and organized the work of cloning and gene expression. Enrique Martínez-Force did the phylogenetic tree and the protein modelling work. Rafael Garcés managed to get funds for the project and also general coordination of tasks. Joaquín J. Salas did the manuscript writing and edition and also supervision and organization of the protein expression and biochemical work.

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