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**Characterization and function of a sunflower (*Helianthus
annuus* L.) Class II acyl-CoA-binding protein**

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Abstract

Acyl-CoA-binding proteins (ACBP) bind to long-chain acyl-CoA esters and phospholipids, enhancing the activity of different acyltransferases in animals and plants. Nevertheless, the role of these proteins in the synthesis of triacylglycerols (TAGs) remains unclear. Here, we cloned a cDNA encoding *HaACBP1*, a Class II ACBP from sunflower (*Helianthus annuus*), one of the world's most important oilseed crop plants. Transcriptome analysis of this gene revealed strong expression in developing seeds from 16 to 30 days after flowering. The recombinant protein (*rHaACBP1*) was expressed in *Escherichia coli* and purified to be studied by *in vitro* isothermal titration calorimetry and for phospholipid binding. Its high affinity for saturated palmitoyl-CoA (16:0-CoA; K_D 0.11 μ M) and stearoyl-CoA (18:0-CoA; K_D 0.13 μ M) esters suggests that *rHaACBP1* could act in acyl-CoA transfer pathways that involve saturated acyl derivatives. Furthermore, *rHaACBP1* also binds to both oleoyl-CoA (18:1-CoA; K_D 6.4 μ M) and linoleoyl-CoA (18:2-CoA; K_D 21.4 μ M) esters, the main acyl-CoA substrates used to synthesise the TAGs that accumulate in sunflower seeds. Interestingly, *rHaACBP1* also appears to bind to different species of phosphatidylcholines (dioleoyl-PC and dilinoleoyl-PC), glycerolipids that are also involved in TAG synthesis, and while it interacts with dioleoyl-PA, this is less prominent than its binding to the PC derivative. Expression of *rHaACBP* in yeast altering its fatty acid composition, as well as the composition and size of the host acyl-CoA pool. These results suggest that *HaACBP1* may potentially fulfil a role in the transport and trafficking of acyl-CoAs during sunflower seed development.

Keywords: acyl-CoA; acyl-CoA binding protein; Phosphatidic acid; Phosphatidylcholine; Phospholipids; Sunflower

1. Introduction

Acyl-CoA metabolites play a central role in many biological processes, such as vesicular trafficking, gene expression or lipid metabolism [1]. Moreover, the content and composition of these acyl-CoA esters modulates the activity of numerous enzymes [1-4]. These acyl-CoA esters are essential substrates in the biosynthesis of different molecules, including sphingolipids, phospholipids and triacylglycerols (TAGs). In plants, the biosynthesis of fatty acids (FAs) takes place in the plastid, where the FA synthase complex uses the malonyl-acyl carrier protein to produce long-chain FA derivatives through successive cycles of condensation, reduction and dehydration [5]. A large proportion of these FAs (18:1 >> 16:0 > 18:0) are exported and rapidly esterified to their acyl-CoA derivatives at the plastid membrane by a long chain acyl-CoA synthetase [6-8]. These acyl-CoAs are then made available to be incorporated into glycerolipids through the action of the acyltransferase in the Kennedy pathway, or through the acyl editing pathway in which they are incorporated into phosphatidylcholine (PC) by lysophosphatidylcholine:acyl-CoA acyltransferases (LPCATs). Moreover, acyl-CoAs can be further modified through elongation in the cytosol by FA elongases. Oleate chains esterified to PC can be desaturated via oleate and linoleate desaturases to produce polyunsaturated FAs (PUFAs) [9]. These PUFAs can be exchanged and again incorporated in the acyl-CoA pool by the acyl editing pathway involving LPCATs [10], or they may be transferred to a diacylglycerol (DAG) molecule by a phosphatidylcholine:diacylglycerol acyltransferase (PDAT), producing TAGs in a reaction that does not involve the acyl-CoA pool [11]. In the case of sunflower a relevant contribution of PDAT to TAG synthesis was reported by Banas et al. [12]. The acyl-CoA pool is also utilized in the production of TAGs by acyl-CoA:diacylglycerol acyltransferase (DGAT) [2].

The first acyl-CoA-binding protein (ACBP) was identified in the rat brain as a diazepam-binding inhibitor [13], and further ACBPs were then later isolated as small 10 kDa proteins in the cow [14] and yeast [15]. The overexpression of ACBPs in animals and plants augments the amount of acyl-CoA esters, which enhances the production of phospholipids and TAGs [15-19]. In addition, ACBPs stimulate the activity of the different acyltransferases that are necessary for phospholipid synthesis [20-22]. In yeast, overexpression of either bovine or yeast ACBP augments the intracellular acyl-CoA pool *in vivo* [23], while depletion of ACBPs modifies the acyl-CoA composition and alters their morphology [24, 25]. Moreover, the expression of a

1 small ACBP from canola in Arabidopsis oilseed upregulated and modulated the acyl-
2 CoA pool, and modified the FAs produced [19].

3 In Arabidopsis, there are six genes that encode ACBP proteins and these fall
4 into four different classes. Class II ACBPs (*AtACBP1* and *AtACBP2*) are membrane
5 proteins that possess ankyrin repeats in their C-terminal domain that facilitate
6 protein-protein interactions [26-28]. Class I (*AtACBP6*) and Class III (*AtACBP3*)
7 ACBPs only contain the acyl-CoA-binding (ACB) domain, yet they differ in their size
8 and subcellular localization [29]. Thus, *AtACBP3* is targeted to the extracellular
9 space [30] and is larger than the cytosolic *AtACBP6* (10 kDa) [31]. Class IV ACBP
10 are the largest members of this family (*AtACBP4* and *AtACBP5*) and they are found
11 in the cytosol [32, 33]. They possess conserved kelch motifs, which potentially
12 participate in protein-protein interactions [28, 32]. More recently, ACBP families have
13 been identified in other crop plants like rice [34] and *Brassica napus* [18, 21, 35, 36].

14 Both *AtACBP1* and *AtACBP2* localize to the plasma membrane and
15 endoplasmic reticulum (ER), and they fulfil key roles in seed development [26, 27].
16 For example, *AtACBP1* participates in cuticle formation [37], and it regulates seedling
17 development and germination [38]. In this regard, the Arabidopsis *acbp1/acbp2*
18 double mutant was embryonic lethal, which indicates an essential role of *AtACBP1*
19 and *AtACBP2* in plant development [39]. More recently, *AtACBP1* was shown to
20 modulate the synthesis and FA composition of sterol through protein-protein
21 interactions, serving as a lipid modulator of gene expression [40, 41].

22 Here, we set out to identify and clone a cDNA that encodes ACBP from
23 developing sunflower seeds. The transcript isolated encodes a Class II *HaACBP1*,
24 which was expressed strongly during seed development and oil synthesis. The
25 recombinant *HaACBP1* (*rHaACBP1*) demonstrated high affinity towards the main
26 classes of acyl-CoA esters and phospholipids present in sunflower seeds. Moreover,
27 when this protein was overexpressed in yeast, the lipid composition and acyl-CoA
28 pool of its heterologous host was altered by its activity.

31 2. Materials and methods

33 2.1. Biological materials

1 Sunflower (*Helianthus annuus* L.) seeds from the CAS-6 wild-type sunflower
2 line were germinated in wet perlite at 25 °C and incubated in a germination chamber
3 for 2 weeks. The seedlings were then moved to turf bags in growth chambers
4 equipped with fertirrigation on 25/15 °C (day/night), 16 h photoperiod cycles, and with
5 a photon flux density of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, until the vegetative tissues or developing
6 seeds were harvested. Flowers were bagged to avoid crossed pollination. No hand
7 pollination was necessary.

8 The *HaACBP1* was expressed heterologously in the *Saccharomyces*
9 *cerevisiae* W303-1A strain (MAT a, ade2-1, his3-11, leu2,3-112, trp1-1, ura3-1, can1-
10 100) to study its activity.

11 12 2.2. Cloning a cDNA encoding sunflower ACBP1

13 Developing sunflower seeds (approximately 0.4 g) were harvested 15 days
14 after flowering (DAF), and ground in liquid nitrogen (N_2) using a precooled sterile
15 mortar and pestle. Seed mRNA was isolated from the total RNA using the Micro-
16 FastTrack Kit (Invitrogen, Groningen, The Netherlands) in 33 μL TE buffer (10 mM
17 Tris-HCl [pH 8.0], 1 mM EDTA) and cDNAs were synthesised using a Ready-To-Go
18 TPrimed First Strand Kit (Amersham Bioscience, Roosendaal, The Netherlands).

19 The TBLASTN algorithm [42] was used to analyse the cDNA encoding
20 *Arabidopsis thaliana* ACBP1 (AtACBP1) and AtACBP2, and to search the sunflower
21 expressed sequence tags (ESTs) available in public databases to identify mRNAs
22 putatively encoding sunflower Class II ACBP homologues. As a result, ESTs
23 corresponding to the 3' terminal fragment of the *HaACBP1* cDNA were identified and
24 their corresponding 5' end was amplified by RACE [43]. In this procedure, a
25 Universal Primer Mix (UPM) and Nested Universal Primer (NUP) were used as the
26 forward primers, while the ACBP-5rac1 and ACBP-5rac2 oligomers derived from the
27 known sequence were used as the reverse primers (see Table S1 for the primer
28 sequences, all of which were synthesized by Eurofins MWG Operon, Germany).
29 ACBP-1F and ACBP-1R were then used to amplify the full coding sequence by PCR,
30 and this full-length sequence encoding *HaACBP1*, including the ATG and STOP
31 codons, was deposited in GenBank under accession number KJ826407.

32 The putative transmembrane region of *HaACBP1* was predicted using the
33 Consensus Constrained TOPology prediction server (CCTOP,
34 <http://cctop.enzim.ttk.mta.hu>) [44]. Primers were designed with internal *SphI* and

KpnI restriction sites (*SphI*ACBP1-F and *KpnI*ACBP1-R, respectively) to amplify a fragment encoding HaACBP1 without the anchor sequence. The PCR product was cloned into the pMBL-T Easy vector (Genaxxon BioScience GmbH, Biberach, Germany) and several clones were sequenced on both strands by SECUGEN (Madrid, Spain) to confirm the identity of the clone using the BLAST software [42]. The plasmid bearing *HaACBP1* was then digested with *SphI* and *KpnI*, and the DNA fragment obtained was ligated into the *SphI*-*KpnI* sites of pQE-80L (Qiagen, Hilden, Germany) to produce a fusion protein with a (His)₆-tag at its *N*-terminus. Removal of the first 30 amino acids of *HaACBP1* facilitated the purification of the *rHaACBP1* in the soluble fraction, like the recombinant AtACBP1 [45]. Sequencing confirmed the resulting construct was in the correct reading frame and it was designated as pQE80-*HaACBP1*. The recombinant plasmid was expressed in the *Escherichia coli* BL21(DE3)Star pLysS strain.

To assess the activity of HaACBP1 following its heterologous expression in *S. cerevisiae*, the full-length cDNA sequence encoding HaACBP1 was amplified with the *SpeI*ACBP1-F and *XmaI*ACBP1-R primers (Table S1). According to the Kozak optimization sequence [46], one nucleotide in the forward primer was replaced in order to improve its expression (Table S1). The PCR fragment was digested with *SpeI* and *XmaI*, before it was cloned into the yeast p416GPD expression vector [46] and transformed into the *S. cerevisiae* W303-1A strain by electroporation [47].

2.3. Analysis of the *HaACBP1* protein

HaACBP1 homologous protein sequences were retrieved using the BLASTP program (www.ncbi.nlm.nih.gov). Alignment with other amino acid sequences encoding ACBPs deposited at GenBank was performed using the ClustalX v.2.0.10 program under default settings [48]. These alignments were used to generate a phylogenetic tree based on the neighbor-joining algorithm [49]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was calculated according to Felsenstein [50], with evolutionary distances computed using the Poisson correction method [51] and based in the units of the number of amino acid substitutions per site. This analysis involved 181 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 610 positions in the final dataset. The resulting 'phenogram' was drawn using the MEGA X program [52].

2.4. Modelling the three-dimensional structure of HaACBP1

Homology modelling studies of the putative HaACBP1 protein structure were carried out with IntFOLD version 5.0 [53, 54], using the following PDB entries as templates for the final HaACBP1 tertiary structure: 5jhqA, 5ijmA, 3v31A, 2cquA, 3ui2A, 3c5rA, 5d68A and 2f8yA. All of these entries correspond to proteins including ACB and/or ankyrin repeat domains. The model obtained (Figure 2) has a confidence of CERT and a p-value of 8.994×10^{-5} . Furthermore, molecular docking was performed with SwissDock [55, 56] and FunFOLD [57] using coenzyme-A (ZINC8551087) as the substrate. The UCSF Chimera package was used for critical residue mapping and model visualization [58].

2.5. RT-qPCR analysis of HaACBP1 expression

Total RNA was isolated from developing sunflower seeds at 16, 20, 25 and 30 DAF, roots, stems, green cotyledons (1, 4 and 7 days after imbibition; DAI) and leaves as described above (see section 2.2), which served as a template (5 µg) to generate cDNAs and analyse them by real-time quantitative PCR (RT-qPCR) using specific primer pairs (QHaACBP1-F and QHaACBP1-R, Table S1) and SYBR Green I (QuantiTect SYBR Green PCR Kit, Qiagen, Crawley, UK). The reactions were carried out in a MiniOpticon system to monitor the resulting fluorescence (Bio-Rad), maintaining the reaction mixture at 95 °C for 5 min before subjecting it to 40 PCR cycles of: 94 °C for 15 s; 59 °C for 30 s; and 72 °C for 30 s. Calibration curves were drawn up using sequential dilutions of the cDNAs and the Livak method [59] was applied to calculate the relative expression in the samples. The sunflower actin gene, HaACT1 (GenBank Accession number FJ487620), was used as an internal reference to normalize the relative amount of cDNAs from the samples, amplified with the specific QHaActin-F4 and QHaActin-R4 primer pair (Table S1).

2.6. Expression and purification of rHaACBP1 in E. coli

Cultures of *E. coli* BL21(DE3)Star pLysS cells harbouring pQE80-HaACBP1 were grown at 37 °C with vigorous shaking in 300 mL of LB media (1% Bacto Tryptone, 0.5% yeast extract and 1% NaCl [pH 7]) supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. The expression of the recombinant protein was induced by adding 0.4 mM isopropylthio-β-D-galactoside (IPTG) when the

bacterial culture reached an OD₆₀₀ value of 0.4. After a further 5 h incubation at 37 °C, the cells were harvested by centrifugation and resuspended in 8 mL native lysis buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 10 mM imidazole). The cells were sonicated on ice, applying 10 cycles of 10 s bursts at 200-300 W with a 10 s cooling period in between. The cleared lysate was obtained by centrifugation (10,000 g for 1 h at 4 °C) and purified on a 1 mL NTA-Ni agarose column (QIAGEN). The protein extract was loaded onto a previously equilibrated column and the column was then washed with 5 volumes of washing buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 20 mM imidazole). The bound proteins were finally eluted with 2 mL of native elution buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 250 mM imidazole) and the sample eluted was dialyzed against the Isothermal titration calorimetry (ITC) binding buffer (50 mM K₂HPO₄ [pH 7.6], 0.3 mM EDTA and 175 mM KCl: [60]). The purified protein (Figure S3) was concentrated using Amicon Ultra-15 Centrifugal Filters (Millipore) and the protein concentration was determined by the Bradford method [61].

2.7. Isothermal titration calorimetry (ITC) study of acyl-CoA ester binding

ITC experiments were performed on a MicroCal iTC₂₀₀ system (MicroCal). The acyl-CoA esters (Avanti Polar Lipids) and rHaACBP1 protein used in this study were prepared in ITC binding buffer and degassed under vacuum with stirring to avoid bubble formation, following the manufacturer's instructions (GE Healthcare). The acyl-CoA ester in solution (250 µM) was injected into 500 µL of the protein solution (12.5 µM) in the cell at 30 °C. Each injection volume was 1.5 µL with an interval of 150 s between injections. All the experimental data were analysed using Microcal ORIGIN7 software (OriginLab Corporation, Northampton, USA) and the parameters of the dissociation constant (K_D) were obtained by non-linear regression fitting of the isotherm data based on a one-set of sites model [62].

2.8. Protein-lipid binding assays

The binding between rHaACBP1 protein and lipids was examined following previous protocols [63]. In these assays, various phospholipids and lysophospholipids standards purchased to Sigma-Aldrich were spotted onto a nitrocellulose filter (Amersham, GE), and incubated for 1 h at room temperature in the dark. The filters were then blocked for 1 h in Tris-Buffered saline buffer (TBS: 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.4], 140 mM NaCl, 2.7 mM KCl) supplemented

1 with 1% non-fat milk (Bio-Rad) and then incubated for 2 h with rHaACBP1 (final
2 concentration 1 $\mu\text{g/mL}$) in blocking buffer. Subsequently, the filters were washed
3 twice with TTBS (TBS supplemented with 0.1% Tween 20), for 10 min each wash,
4 and then probed for 1 h at room temperature with anti-(His)₆ antibodies (Amersham,
5 GE). After three washes with TTBS, ECL signals were detected using the Western-
6 Blotting Detection Kit (Amersham, GE) to determine the protein-lipid binding.

8 2.9. Yeast lipid analysis

9 The mutant W303-1A *S. cerevisiae* strain was transformed with the
10 p416GPD::*HaACBP1* construct or the empty plasmid as a control [64]. The cultures
11 were grown for 24 h in (SC)-Ura medium supplemented with 2% (w/v) glucose and
12 then diluted to an OD₆₀₀ of 0.4 in the same medium. The yeast culture was incubated
13 for 48 h to obtain a stationary phase cell culture and then diluted to an OD₆₀₀ of 2 in
14 200 ml of the same medium. The cells were then collected by centrifugation at 5,000
15 x g for 10 min and washed twice with 10 mL of distilled water. For extraction, 2 mL of
16 methanol plus 1 mL of glass beads (710-1180 μm ; Sigma-Aldrich, USA) was added
17 to the yeast pellets, which were then vortexed at maximum power for 10 min.
18 Subsequently, 4 mL of chloroform was added to the tubes with 1 mL NaCl (0.9 %
19 w/v), and the tubes were then shaken and centrifuged at 2500 x g for 2 min. The
20 lower phase was transferred to a clean tube while the resulting residue was extracted
21 twice with 2 mL of chloroform following the same procedure and the chloroform lower
22 phases were combined. The combined chloroform phase was re-extracted with 1 mL
23 of methanol plus 0.5 mL 0.9% NaCl in a step involving shaking and centrifugation at
24 2500 x g for 2 min. The upper phases were discarded and the chloroform phase
25 containing the lipids was evaporated under nitrogen at 40 °C. The lipid residues were
26 then dissolved in 1 mL of chloroform and stored at -20 °C under nitrogen.

27 Glycerolipid species were determined by high-performance liquid
28 chromatography (HPLC). A volume of 0.4 mL of the total lipid extract was loaded
29 onto a LiChrolut 0.5 g silica gel cartridge (Merck, Germany) using a vacuum
30 manifold, and then equilibrated with 2 mL of chloroform, as described previously [65].
31 In this method, non-polar lipids (TAGs and DAG) were eluted with 10 mL of
32 chloroform and then the polar lipids were eluted with 10 mL of methanol. Both
33 fractions were dried to completeness under nitrogen and then resuspended in 1 mL
34 hexane or 1 mL hexane/2-propanol (3:2) for non-polar or polar lipids, respectively.

1 Non-polar and polar lipids were analysed on a direct phase LiChrospher 100 Diol (5
2 μm) column (Merck, Germany), applying both the official Cd11d-96 AOCS method
3 and the method described elsewhere [66]. The chromatography system consisted of
4 a Waters 2695 Module (Milford, MA) equipped with a Waters 2420 ELSD. The data
5 were processed using the Empower software and the ELSD was regularly calibrated
6 using commercial high-purity standards purchased from Sigma-Aldrich for each lipid.

7 To determine the yeast FA composition, 0.1 mL of the total yeast lipid extract
8 was trans-esterified to their FA methyl esters (FAMES) in a methylation reaction
9 carried out for 1 h after the addition of 2 mL of methanol/toluene/sulphuric acid
10 (88/10/2; v/v/v). Methyl esters were then extracted with 2 mL of heptane and
11 analysed by gas-liquid chromatography (GLC). The chromatography system
12 consisted of a Hewlett–Packard 6890 gas chromatograph (Palo Alto, CA, USA)
13 coupled to a flame ionization detector and using a Supelco SP-2380 fused-silica
14 capillary column (30 m length, 0.25 mm i.d., 0.20 μm film thickness: Supelco,
15 Bellefonte, PA, USA). The carrier gas used was hydrogen at 28 cm s⁻¹, the
16 temperature of the detector and injector was maintained at 200 °C, the oven
17 temperature was 170 °C and the split ratio was 1:50. Peaks were identified by
18 comparing their retention times with those of the corresponding commercial
19 standards (Sigma-Aldrich).

21 2.10. Acyl-CoA analysis

22 Acyl-CoAs were extracted from the transformed yeast and their acyl-etheno
23 derivatives were obtained as described by Larson and Graham [67]. The O.D. of the
24 cultures was adjusted with fresh medium and 50 mL of culture was centrifuged at
25 1500 g for 10 min to pellet the cells. Heptadecanoyl-CoA (1 nmol; Avanti polar lipids)
26 was added as an internal standard, and the cells were washed with 10 mL of distilled
27 water and pelleted again. The cell pellets were lysed by vortexing at the maximum
28 power for 2 min with 250 μL of freshly prepared extraction buffer (2 mL 2-propanol, 2
29 mL KH_2PO_4 25 mM [pH 7.2], 50 μL glacial acetic acid and 80 μL BSA 50 mg/mL) plus
30 1 mL of glass beads (710-1180 μm ; Sigma-Aldrich, USA). The lipids were then
31 extracted 3 times with 200 μL hexane saturated with 2-propanol and water 1:1 v/v.
32 Subsequently, 5 μL of saturated ammonium sulphate was added and the protein was
33 precipitated by adding 600 μL methanol/chloroform 2:1 v/v. The tubes were vortexed
34 and incubated at room temperature for 20 min, and then finally centrifuged at 13,000

g for 2 min. The aqueous supernatants were then transferred to fresh tubes and dried under nitrogen at 40 °C. The dried residues were dissolved in 300 µL chloroacetaldehyde reagent (0.5 M chloroacetaldehyde solution diluted in 0.15 M trisodium citrate/citric acid [pH 4.0], 0.5% w/v SDS) and the derivatization reaction was carried out at 85 °C for 20 min in dark.

Acyl-CoA etheno derivatives were analysed by HPLC using a Waters 2695 separation module with an XBridge C18 column (250 x 0.5 mm, 5 µm: Waters, Spain) and a Multi λ Fluorescence Detector 2475 (Waters, Spain), and using a modified version of the quaternary gradient system described previously [67]. The solvents consisted of A: 1% acetic acid; B: 90% acetonitrile, 1% acetic acid; C: 0.25%, trimethylamine, 0.1% tetrahydrofuran; D: 90% acetonitrile. The flow rate was 0.75 mL/min and the analysis temperature was 40 °C. The gradient elution profile followed the steps: 0–7.0 min, A–B 90:10 to A–B 20:80; 7.0–7.1 min, A–B 20:80 B to A–C 20:80; 7.1– 9.0 min, A–C 20:80 to C–D 90:10; 9.0–34.0 min, C–D 90:10 to C– D 25:75; 34.0–35.0 min, C–D 25:75 to D 100; 35.0–39.0 min, D 100; 39.0–40.0 min, D 100 to A–B 90:10; 40.0–45.0 min, A–B 90:10. The detector was set to an excitation wavelength of 230 nm and a detection wavelength of 420 nm. The peaks were quantified attending to the internal standard signal and the data were processed using the Empower Login software.

3. Results and discussion

3.1. Sequence analysis of *HaACBP1*

Although a sunflower Class I ACBP was characterized previously [68], no such Class II proteins have been reported. Here, we identified and studied the function of a Class II ACBP in this oil crop, proteins believed to be essential regulators of plant metabolism as evident through the embryonic lethality of the *Arabidopsis acbp1/acbp2* double mutant [39]. When the *Arabidopsis thaliana* AtACBP1 and AtACBP2 protein sequences were used to probe the sunflower expressed sequences tags (ESTs) deposited at the NCBI using the BLASTP algorithm [42], an incomplete EST for *HaACBP* (EL478536.1) was identified and its corresponding *N*-terminal region was amplified by RACE [43] with the appropriate primers (Table 1S). The full coding sequence of *HaACBP1* was amplified from a developing sunflower kernel

1 cDNA library and cloned into the PQE80 plasmid for expression in *E. coli*. The 1.05
2 kb *HaACBP1* cDNA amplified encoded a putative protein of 349 amino acids, with a
3 predicted molecular weight of 39.33 kDa, 61% solubility and a pI of 4.43. We took
4 advantage of the recently available sunflower genome ([69];
5 <https://www.heliagene.org/HanXRQ-SUNRISE/>) to identify the corresponding gene
6 (HanXRQChr15g0485641), *HaACBP1*. This is the only complete Class II ACBP
7 present in sunflower genome. In this regard, other gene was found
8 (HanXRQChr08g0225131) coding for an incomplete protein of this class that only
9 displays the n-terminal side of an ACBP losing homology in the rest of the sequence.
10 Phylogenetic analysis with a set of 181 Viridiplantae Class II ACBP protein
11 sequences was performed to examine their relationship with *HaACBP1* (Figure 1).
12 The *HaACBP1* sequence was included in the Asteraceae family along with other
13 closely related species like *Lactuca sativa* and *Cynara cardunculus* (78% and 76%
14 identity, respectively). This family of ACBP1 sequences was closely related to those
15 of the Solanaceae family (68% identity approx.), order Lamiales, or species in the
16 Asterids group including *Camellia sinensis*, *Daucus carota*, *Coffea arabica* or
17 *Ipomoea*. Adjacent to this group lay the sequences from species of the Brassicales
18 order, including four copies of Class II ACBP described in *Brassica napus* [35] and
19 those from Arabidopsis, AtACBP1 and AtACBP2. All dicot species appeared to be
20 separate from the monocots and mosses, with sequences from the latter used to root
21 the phylogenetic tree. The palm oil tree is a monocot of great economic importance.
22 The ACBPs of this species were recently studied by Amiruddin et al. [70], resulting a
23 single gene coding for class II ACBP. This gene clustered within the Polales I group in
24 Figure 1, together with those from other species within the Arecacea family. The
25 deduced amino acid sequence for *HaACBP1* was aligned with that of the Class II
26 ACBPs from Arabidopsis and other plants included in the phylogenetic tree (Figure
27 S1), revealing strong homology in the ACB domain typical of ACBPs (Figure S1,
28 green box) and in the ankyrin repeat known to mediate protein-protein interactions
29 (Figure S1 red box), [27]. The CoA-binding site in the ACB domain [71] was highly
30 conserved in all the sequences analysed (residues Y132, Y135, K136, K158 and
31 Y177 in sunflower). Moreover, a hydrophobic sequence in the N-terminal domain
32 (Figure S1, yellow box) allows the protein to anchor to the plasma and ER
33 membranes [45, 72]. This membrane anchoring was supported by the prediction of
34 its subcellular location using DeepLoc [73] (Supplementary Figure S2), which

proposed a very likely localisation of *HaACBP1* at the ER membrane. However, experimental evidence is still pending to confirm the exact location of this protein.

The *HaACBP1* amino acid sequence was used to predict its tertiary structure with IntFOLD version 5.0 [53, 54]. In accordance with the sequence alignments (Figure S1) and previously characterized ACBPs [35, 74], two different domains were again identified in sunflower ACBP1 (Figure 2): (i) an ACB domain (in green) with its typical four helix structure (panels A and B); and (ii) the ankyrin repeats with helices oriented in parallel. In addition, CoA interaction was predicted using SwissDock [55, 56] and FunFOLD [57] (Figure 2), corroborating the implication of residues Y132, Y135, K136, K158 and Y177 (in purple) in this process.

3.2. Studies on sunflower ACBP1 expression

The transcriptional profile of *HaACBP1* mRNA was analyzed in developing seeds, leaves, stems, roots and seedlings, and *HaACBP1* expression was detected in all the tissues assayed, with the highest levels of the transcript in the developing seeds and stems (Figure 3). This pattern is consistent with the broad expression of its homologue, *AtACBP1*, which is involved in stem cuticle synthesis, and that promotes abscisic acid signaling during seedling development and seed germination [37, 38]. Strong *HaACBP1* expression in sunflower seeds could also indicate it fulfils a role in the biosynthesis of lipids in oil-accumulating tissues. In general, the expression of *HaACBP1* was weaker in vegetative tissues (roots and leaves) and germinating cotyledons relative to that in the stems. Its homologue in rice (*OsACBP4*) is also expressed in seedlings and developing seeds but unlike *HaACBP1*, *OsACBP4* is strongly expressed in the leaves rather than the stems [34]. In the case of *B. napus*, the gene *BnACBP1* was expressed at high levels in developing embryos together with the class I form *BnACBP6* [75] suggesting an involvement in oil synthesis. In palm oil tree on the contrary, *EgACBP1* was expressed in oil accumulating tissues (mesocarp and developing endosperm) although it was not the predominant form, which were *EgACBP6*, *EgACBP3* and *EgACBP4*, ACBPs of the class I and III [75]. All these results points that ACBPs of the class II are generally expressed in oil accumulating tissues, although their level of expression with respect to other forms could vary along plant species and families.

3.3. Expression of sunflower rHaACBP1 in Escherichia coli

The *in vitro* characterization of HaACBP1 was facilitated by its overproduction in *E. coli* and its purification by affinity chromatography. To favour recombinant HaACBP1 purification in the soluble fraction, the first 30 amino acids of the protein were removed using a similar strategy to that employed in the purification of recombinant AtACBP1 [45], with a (His)₆-tag fused to its *N*-terminal end. Here, we have to consider that His tag could modify protein properties. However, this method has been broadly used to study the properties of a large variety of proteins and enzymes. The protein was expressed in *E. coli* and the different fractions obtained during purification were analysed by SDS-PAGE (Figure S3). The protein was strongly expressed in the soluble phase and it was purified almost to homogeneity. The purified protein was recovered and dialyzed prior to performing the ITC and filter-binding assays.

3.4. The interaction of rHaACBP1 with different acyl-CoA esters

One of the most remarkable properties of ACBPs is their capacity to bind to a wide range of acyl-CoA esters [33, 76], their specificity for these metabolites representing a feature that defines their activity. The affinity of HaACBP1 towards different acyl-CoA esters was studied through ITC, a technique widely used to determine the affinity or binding energies between proteins and metabolites [77], including ACBPs from different plants [78, 79]. As HaACBP1 was expressed strongly in developing seeds (Figure 3), 16:0-CoA, 18:0-CoA, 18:1-CoA and 18:2-CoA, the main acyl-CoAs found in seed tissues [80], were included in this study. The value of K_D given by the ITC studies is a measurement of the affinity of between the protein and the different substrates, being the equilibrium dissociation constant between them. Recombinant HaACBP1 purified from *E. coli* extracts displayed stronger affinity for saturated 16:0-CoA and 18:0-CoA so they displayed lower K_D values (0.11 μ M and 0.13 μ M respectively) than for the monounsaturated 18:1-CoA (K_D 6.36 μ M) or the polyunsaturated 18:2-CoA (K_D 21.4 μ M: Table 1). The affinity determined for 18:3-CoA, the major acyl-CoA found in sunflower leaves [80], was the weakest of those calculated (K_D 25.8 μ M), whereas no binding of 24:0-CoA was detected (Figure S5). Another set of saturated and unsaturated very long chain (VLC)FA acyl-CoAs were also studied (26:0-CoA, 30:0-CoA, 20:4-CoA) and while neither 26:0-CoA nor 30:0-CoA bound to HaACBP1, 20:4-CoA bound to the protein albeit weakly (Figure S5).

1 It is noteworthy that when the thermodynamic characteristics of the
2 interactions were considered, HaACBP1 bound to 16:0-CoA and 18:0-CoA with
3 favourable enthalpies and entropies (for 16:0-CoA, the $\Delta H = -5.3 \text{ kcalmol}^{-1}$ and $-T\Delta S$
4 $= -4.3 \text{ kcalmol}^{-1}$), suggesting both hydrogen bonding and hydrophobic interactions
5 are important for these interactions. As the lipid becomes increasingly unsaturated
6 through to 18:2-CoA, the binding affinity to HaACBP1 drops, driven in particular by
7 the loss of a favourable entropy while the change in enthalpy of the interaction
8 remains unchanged (for 18:2-CoA, the $\Delta H = -5.8 \text{ kcalmol}^{-1}$ and $T\Delta S = -1.4 \text{ kcalmol}^{-1}$).
9 This suggests that the unsaturated lipid has a weaker hydrophobic interaction
10 surface, which would be consistent with what one might expect when comparing
11 saturated and unsaturated lipids binding to hydrophobic patches on the protein
12 surface.

13 The high affinity towards unsaturated 16:0-CoA and 18:0-CoA might indicate
14 HaACBP1 plays a role in metabolic pathways requiring saturated acyl-CoAs, like
15 those involved in the biosynthesis of surface lipids [81] or the synthesis of
16 sphingolipid bases [4]. This function would be in good agreement with the known role
17 of AtACBP1 in Arabidopsis cutin [37]. The synthesis of sunflower oil requires high
18 fluxes of transference of 18:1 and 18:2 to TAG. This transference, is mostly
19 supported by the enzymes PDAT and DGAT [12]. Acyl-CoAs are not substrates for
20 PDAT, so only DGAT would be involved with ACBP proteins. HaACBP1 displayed
21 low affinities towards those fatty acids (6.4 and 21.4 μM), so it is expectable it does
22 not have a direct contribution in this reaction favouring the interaction between the
23 enzyme and the substrates. Furthermore, sunflower glycerol phosphate
24 acyltransferase (GPAT), involved in the first step of *de novo* glycerolipid synthesis
25 has a preference for saturated acyl-CoAs [3]. This involvement in saturated FA
26 metabolism was also supported by the increase in 18:0-CoA levels in siliques of the
27 Arabidopsis *acbp1* mutant, whereas the reduction in most of the polyunsaturated
28 phospholipid species [39] demonstrates how this protein influences FA composition.
29 Moreover, rHaACBP1 was seen to bind monounsaturated and polyunsaturated acyl-
30 CoA 18:1-CoA and 18:2-CoA, the major FAs found in sunflower TAGs [80]. Although
31 AtACBP1 also interacts with acyl-CoA esters [76, 82], the most important difference
32 between HaACBP1 and its homologue AtACBP1 is the affinity of the latter towards
33 VLCFA CoA esters [37]. We found that rHaACBP1 did not bind to saturated (24:0-
34 CoA, 26:0-CoA, 30:0-CoA) and polyunsaturated (24:4-CoA) acyl-CoA esters (Figure

S4 and S5), whereas *AtACBP1* did bind to these CoA esters with reasonable affinity, especially the 24:0-CoA associated with the synthesis of cuticle lipids [37]. Hence, there appears to be functional differences between these proteins according to the plant host. Interestingly, while *AtACBP1* displayed some affinity towards 18:3 [82], the affinity of *rHaACBP1* was weakest towards polyunsaturated 18:3-CoA, which is not present in the acyl-CoA pool from developing sunflower seeds or in its oil composition [80]. This seems to indicate that *HaACBP1* is not involved in the transfer or trafficking of these FAs.

3.5. The affinity of *rHaACBP1* towards different phospholipid species

The affinity of *HaACBP1* for different phospholipids was analysed using *in vitro* filter binding assays. The biochemical affinity of recombinant *Arabidopsis* [39, 76] and rice [34] Class II ACBPs to different phospholipids has been studied. To characterize the interaction of *rHaACBP1* towards different phospholipids, experiments were carried out with PC, phosphatidic acid (PA), lysophosphatidic acid (LPA), and lysophosphatidylcholine (LPC: Figure 4A). These phospholipid classes are important intermediates in the synthesis of glycerolipids through the Kennedy or acyl editing pathways [6, 9], and some of them can be produced by catabolic enzymes like phospholipase D (PLD) or A2 (PLA2). In this regard, previous studies on *AtACBP1* demonstrated that it could modify the levels of PA and LPC when overexpressed in *Arabidopsis* under freezing and ABA-induced stress [63]. *HaACBP1* bound poorly to both PA and LPC derivatives, displaying slightly higher activity towards PA(14:0, 14:0) and PA(18:1, 18:1 - Figure 4A). The binding capacity of this protein was stronger towards PC species, especially PC (14:0, 14:0), PC(18:1, 18:1) and PC(18:2, 18:2), the latter present in remarkable amounts in developing sunflower seeds [66]. Poor interactions were observed towards saturated PC (16:0, 16:0) and PC (18:0, 18:0). This profile was similar to that found in sunflower *HaACBP6* [68] but it contrasted with the Class II *Arabidopsis* ACBPs that have higher affinities towards PA and LPC than the sunflower enzyme [39, 63, 83]. Interestingly, the affinity of *HaACBP1* towards glycerolipids esterified to 16:0 and 18:0 was always weak, in contrast to its affinity for saturated acyl-CoAs (Table 1). The strong binding capacity for derivatives esterified to short-chain FAs was also unexpected, as those species are not detected in sunflower. In experiments to further characterize the affinity of this protein for PC and PA species, using serial dilutions of these lipids, *rHaACBP1*

1 displayed stronger affinity for PC species (Figure 4B), particularly PC(18:2,18:2), a
2 glycerolipid especially abundant in developing sunflower seeds [66]. The interaction
3 with PA(18:1,18:1) was weaker than that with PC species.

5 3.6. The impact of recombinant *HaACBP1* production on yeast lipid composition

6 ACBP proteins like *HaACBP1* interact with acyl-CoA esters, which are central
7 intermediates in lipid metabolism. Furthermore, *HaACBP1* is expressed strongly in
8 developing sunflower seeds, in which lipid synthesis is very active, and it is located in
9 the membranes of a variety of structures including the ER, the site of glycerolipid
10 assembly from acyl-CoA and of other precursors like glycerol-3-phosphate and LPC.
11 The expression of exogenous proteins in *Saccharomyces cerevisiae* can be used to
12 study membrane-bound enzymes or proteins related to lipid metabolism, such as
13 desaturases or acyltransferases [84, 85]. Yeast is a eukaryotic microorganism that
14 undergoes cellular compartmentation, it is easy to transform and can be grown in
15 controlled conditions to produce reproducible results. It produces a fairly large
16 quantities of lipids, and it is especially appropriate to express and accommodate
17 proteins targeted to the ER. Hence, the influence of *HaACBP1* on lipid metabolism
18 was assessed in yeast. The yeast genome contains genes encoding two small Class
19 I ACBP proteins similar to *AtACBP6* [86], and it is unclear if this microorganism
20 possesses membrane bound Class II ACBPs homologous to *HaACBP1*. To study the
21 effect of this protein on yeast lipid metabolism, total lipid extracts from yeast cells
22 transformed with the p-416::*HaACBP1* plasmid were compared to cells transformed
23 with the empty p-416 vector as a control. The extract was characterized in terms of
24 the absolute TAG content, by far the most abundant non-polar glycerolipid, and for
25 the total polar lipids (Figure 5A). Transformed and control yeast displayed similar
26 absolute amounts of these lipids, which tended to be slightly more abundant in
27 *HaACBP1* expressing cells, although these overall differences were not significant.
28 The amounts of TAGs in these cultures ranged from 29 to 22 µg/mL of culture,
29 whereas polar lipids were between 5.3 and 8.1 µg/mL, again not significantly different
30 when *HaACBP* was expressed.

31 To study the possible impact of the protein on lipid synthesis, the composition
32 of the polar lipid species of the yeast was evaluated. The most abundant polar lipid
33 species detected were PC, phosphatidylethanolamine (PE) and phosphatidylinositol
34 (PI), with minor amounts of other species like phosphatidylserine (PS),

phosphatidylglycerol (PG), cardiolipin (CL), PA and LPC. Expression of *HaACBP1* in yeast did not affect the proportions of the main polar lipid species (Figure 5B), with PC and PE still representing around 35% and 25 % of the total polar lipids, respectively. The proportion of PI was higher in yeast expressing *HaACBP1* although these differences were not significant. The only statistically significant differences detected were in terms of PA and LPC, which were lower in *HaACBP1* transformants. These glycerolipids are non-structural components of the yeast membrane, and they represent the intermediates of their associated synthetic and degradation pathways. Thus, PA is an intermediate in the *de novo* synthesis of glycerolipids through the Kennedy pathway and it is a product of the ABA-mediated stress responses through the action of PLD on PC [87]. PLD α 1 promotes ABA signal transduction by producing PA, an important lipid messenger in that signalling pathway [88]. By contrast, LPC is the primary substrate of the acyl editing pathway due to the activity of LPCAT [10]. The decrease in these species revealed that *HaACBP1* has an impact on these pathways, both of which depend on acyl-CoAs as substrates. Thus, *HaACBP1* can potentially bind both phospholipids and the acyl-CoA substrates, favouring the acyl exchange reaction catalysed by LPCAT.

The *in vitro* assays carried out with *HaACBP1* pointed to a weak interaction with LPC and PA (Figure 4A), which could be sufficient to interfere with the reactions involving these lipid species. Indeed, studies on *Arabidopsis* seedlings with analogous ACBPs also displayed an important effect on these lipid species, which accumulate more PA when they overexpressed *AtACBP1* upon ABA treatment, whereas the *acbp1* mutant accumulates lower PA [26]. An interaction between *AtACBP1* and *AtPLD α* was demonstrated through a yeast two-hybrid screen. The effect of ACBP1 in yeast has not been studied, yet the data might indicate there is an interaction between *HaACBP1* and the endogenous PLD α of yeast [89] that could provoke a decrease in PA (Figure 5B). The same might occur with PLA2, leading to a decrease in LPC.

There is evidence that *HaACBP1* affects the FA composition of total lipids (Figure 5C), as evident through the increase in palmitate at the expense of oleate, whereas other FA components remained in the same proportions in both control and recombinant cultures. This effect suggests *HaACBP1* may potentially interact with the FA synthase of *S. cerevisiae* to decrease the rate of elongation from 16:0-CoA to 18:0-CoA, which is finally desaturated to 18:1-CoA. This hypothesis is consistent with

1 the high affinity of HaACBP1 towards 16:0-CoA, such that the protein could
2 sequester 16:0-ACP from the FA synthase complex, making it available to other
3 enzymes that catalyse the transfer of FAs to glycerolipids or other complex lipid
4 species.

6 *3.7 The effect of HaACBP1 on the yeast acyl-CoA pool*

7 The analysis of the yeast host lipids was complemented by determining the
8 size and composition of the acyl-CoA pool, the metabolites that interact most tightly
9 with ACBPs. The expression of HaACBP1 clearly affected the total amount of acyl-
10 CoAs, expanding the pool size in yeast 2-fold from 38 to 71 pmol acyl-CoA/mL
11 culture (Table 2). Importantly, the pool composition also changed, with control yeast
12 mainly containing 16:0, 16:1, 18:0 and 18:1-CoA derivatives, with only minor
13 amounts of the 20:1, 20:0 and 22:0 VLCFAs. The main components were 16:0-CoA
14 and 18:1-CoA, accounting for 44.2% and 34.8% of the total acyl-CoA derivatives. It
15 should be noted that the 18:1-CoA pool includes 18:1^{Δ9} plus 18:1^{Δ11} as these cannot
16 be separated by the chromatographic system used. Indeed, these acyl-CoA species
17 are the most commonly reported in yeast [90]. Expression of the recombinant
18 HaACBP1 in yeast caused important changes in the relative proportions of the two
19 main components in this pool, significantly increasing the proportion of 16:1-CoA at
20 the expense of 18:1-CoA, which accounted for 61.1 and 25.0 % of the pool,
21 respectively. The concentration of 20:1-CoA also diminished significantly, probably
22 as a consequence of the deployment of its precursor 18:1-CoA. Therefore, the
23 heterologous expression of HaACBP1 yielded an important increase in the C16
24 derivatives in the acyl-CoA pool, which is compatible with the increase of palmitic in
25 the FAs of yeast (Figure 5C). This phenomenon again fits well with the hypothesis
26 that HaACBP1 diverts 16:0-CoA from yeast FAS to other pathways. The composition
27 of the acyl-CoA pool indicates that this 16:0-CoA is very actively desaturated to 16:1-
28 CoA, yet enzymes transferring acyl chains to glycerolipids prefer 16:0, which is better
29 represented in glycerolipids and less in acyl-CoAs in which 16:1-CoA tends to
30 accumulate. In this regard, we have no data on the interaction of HaACBP1 with 16:1
31 acyl-CoA or 16:1 glycerolipid derivatives.

32 Together, these data suggest that HaACBP1 was functionally active in yeast
33 and that it exerted an effect on the acyl-CoA pool. The incremental effect of
34 HaACBP1 is in good agreement with previous studies when yeast ACBP or bovine

class I ACBP was expressed in yeast, expanding the acyl-CoA pool [21, 76], indicating that the function of these proteins is likely stabilized to accommodate acyl-CoA metabolites in the yeast cytosol. In this regard, HaACBP1 had exerted the same effect but being a membrane-bound protein, its action occurs at the organelle to which is targeted, including the ER, plasma, or the vesicle systems [26, 71]. In the case of the HaACBP1 the probable location in ER membranes (Supplementary figure S2) reinforces its role in the process of reserve lipids synthesis in seeds. This result is in agreement with recently published data in which the class II forms from *B. napus* ACBP *BnACBP2* was expressed at the maximum level during the oil accumulation period of embryo development [75].

4. Conclusions

A Class II ACBP protein (HaACBP1), structurally homologous to AtACBP1, was cloned from the sunflower plant. This protein was ubiquitously expressed in sunflower, and it was expressed most strongly in stems and developing seeds, in contrast to the Arabidopsis homologue (AtACBP1) that was most strongly expressed in seeds alone. The recombinant purified protein displayed high specificity for saturated and monounsaturated acyl-CoAs in *E. coli*, with lower affinity towards polyunsaturated acyl-CoAs and no interaction with VLCFA derivatives. In terms of glycerolipids, HaACBP1 showed high affinity towards different forms of PC, including forms present in high proportions in developing sunflower seeds like PC(18:1,18:1) and PC(18:2,18:2). This phenotype differs from AtACBP1, which associated more specifically with PA and LPC. In this regard, the substrate specificity of HaACBP1 resembled Class I (HaACBP6) from sunflower [68]. When expressed in yeast, HaACBP1 altered the lipid composition. Although it did not significantly affect TAG levels, the modification of PA and LPC suggests an interaction with endogenous yeast enzymes that affect the metabolites of those glycerolipids, such as endogenous PLD α or LPCAT. The expression of HaACBP1 in yeast also induced an increase in C16 FAs at the expense of 18:1 FAs, indicating that this enzyme interacts with the endogenous FAS complex. The impact exerted by the protein on yeast acyl-CoAs was also consistent with previous results, increasing the pool of these metabolites in line with the activity of other ACBPs [86] and increasing the proportion of C16 derivatives in the lipid yeast fraction. Overall, these results indicate that

HaACBP1 appears to be involved in acyl transfer associated with the acyl-CoA pool, especially in the trafficking of saturated FAs during seed development.

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References

- [1] D.S. Neess, H. Bek, S.F. Engelsby, F. Gallego, N.J. Færgeman, Long-chain acyl-CoA esters in metabolism and signaling: Role of acyl-CoA binding proteins, *Prog. Lipid Res.* 59 (2015) 1-25.
- [2] J.A. Aznar-Moreno, P. Denolf, K. Van Audenhove, S. De Bodt, S. Engelen, D. Fahy, J.G. Wallis, J. Browse, Type 1 diacylglycerol acyltransferases of *Brassica napus* preferentially incorporate oleic acid into triacylglycerol, *J. Exp. Bot.* 66 (2015) 6497-6506.
- [3] M. Payá-Milans, J.A. Aznar-Moreno, T. Balbuena, R. Haslam, S.K. Gidda, J. Pérez-Hormaeche, R.T. Mullen, J.J. Thelen, J.A. Napier, J.J. Salas, R. Garcés, E. Martínez-Force, M. Venegas-Calcrón, Sunflower *HaGPAT9-1* is the predominant GPAT during seed development, *Plant Sci.* 252 (2016) 42-52.
- [4] M. Chen, E.B. Cahoon, M. Saucedo-García, J. Plasencia, M. Gavilanes-Ruíz, Plant sphingolipids: structure, synthesis and function, in: H. Wada, N. Murata (Eds.), *Lipids in photosynthesis*, Springer, Dordrecht, 2009, pp. 77-115.
- [5] J.J. Salas, E. Martínez-Force, J.L. Harwood, M. Venegas-Calcrón, J.A. Aznar-Moreno, A.J. Moreno-Perez, N. Ruiz-López, M.J. Serrano-Vega, I.A. Graham, R.T. Mullen, R. Garcés, Biochemistry of high stearic sunflower, a new source of saturated fats, *Prog. Lipid Res.* 55 (2014) 30-42.
- [6] J. Ohlrogge, J. Browse, Lipid biosynthesis, *Plant Cell* 7 (1995) 957-970.
- [7] L.F. Zhao, V. Katavic, F.L. Li, G.W. Haughn, L. Kunst, Insertional mutant analysis reveals that long-chain acyl-CoA synthetase 1 (*LACS1*), but not *LACS8*, functionally overlaps with *LACS9* in *Arabidopsis* seed oil biosynthesis, *Plant J.* 64 (2010) 1048-1058.

- [8] J.A. Aznar-Moreno, M. Venegas-Calcrón, E. Martínez-Force, R. Garcés, R. Mullen, S.K. Gidda, J.J. Salas, Sunflower (*Helianthus annuus*) long-chain acyl-coenzyme A synthetases expressed at high levels in developing seeds, *Physiologia Plantarum* 150 (2014) 363-373.
- [9] J. Shanklin, E.B. Cahoon, Desaturation and related modifications of fatty acids, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49 (1998) 611-641.
- [10] P.D. Bates, A. Fatihi, A.R. Snapp, A.S. Carlsson, C. Lu, Acyl editing and headgroup exchange are the major mechanisms that direct polyunsaturated fatty acid flux into triacylglycerols, *Plant Physiol.* 160 (2012) 1530-1539.
- [11] U. Stahl, A.S. Carlsson, M. Lenman, A. Dahlqvist, B.Q. Huang, W. Banas, A. Banas, S. Stymne (2004). Cloning and functional characterization of a Phospholipid : Diacylglycerol acyltransferase from *Arabidopsis*, *Plant Physiol.* 135 (2004) 1324-1335.
- [12] W. Banaś, A.S. Garcia, A. Banaś, S. Stymne, S. Activities of acyl-CoA: diacylglycerol acyltransferase (DGAT) and phospholipid: diacylglycerol acyltransferase (PDAT) in microsomal preparations of developing sunflower and safflower seeds, *Planta*, 237 (2013) 1627-1636.
- [13] A.C. Guidotti, M. Forchetti, M.G. Corda, D. Konkell, C.D. Bennett, E. Costa, Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors, *Proc. Nat. Acad. Sci. USA*, 80 (1983) 3531-3535.
- [14] I.B. Mogensen, H. Schulenberg, H.O. Hansen, F. Spener, J. Knudsen, A novel acyl-CoA-binding protein from bovine liver - Effect on fatty-acid synthesis, *Biochem. J.* 241 (1987) 189-192.
- [15] T.M. Rose, E.R. Schultz, G.J. Todaro, Molecular cloning of the gene for the yeast homolog (ACB) of diazepam binding inhibitor/endozepine/acyl-CoA-binding protein, *Proc. Nat. Acad. Sci. USA*, 89 (1992) 11287-11291.
- [16] Y.Y. Yang, P.H. Pritchard, J. Bhuiyan, D.W. Secombe, M.H. Moghadasian, Overexpression of acyl-CoA binding protein and its effects on the flux of free fatty acids in McA-RH 7777 cells, *Lipids* 36 (2001) 595-600.
- [17] H. Huang, B.P. Atshaves, A. Frolov, A.B. Kier, F. Schroeder, Acyl-coenzyme A binding protein expression alters liver fatty acyl-coenzyme A metabolism, *Biochem.* 44 (2005) 10282-10297.

- [18] S. Oikari, T. Ahtialansaari, M.V. Heinonen, T. Mauriala, S. Auriola, K. Kiehne, U.R. Folsch, J. Janne, L. Alhonen, K.H. Herzig, Downregulation of PPARs and SREBP by acyl-CoA-binding protein overexpression in transgenic rats, *Pflug Arch. Eur. J. Phy.* 456 (2008) 369-377.
- [19] O.P. Yurchenko, S.D. Singer, C.L. Nykiforuk, S. Gidda, R.T. Mullen, M. Moloney, R.J. Weselake, Production of a *Brassica napus* low molecular mass acyl-CoA-binding protein in *Arabidopsis* alters the acyl-CoA pool and acyl composition of oil in seeds, *Plant Physiol.* 165 (2014) 550-560.
- [20] H. Fyrst, J. Knudsen, M.A. Schott, B.H. Lubin, F.A. Kuypers, Detection of acyl-CoA-binding protein in human red-blood-cells and investigation of its role in membrane phospholipid renewal, *Biochem. J.* 306 (1995) 793-799.
- [21] L. Kannan, J. Knudsen, C.A. Jolly, Aging and acyl-CoA binding protein alter mitochondrial glycerol-3-phosphate acyltransferase activity, *BBA-Mol. Cell Biol. L.* 1631 (2003) 12-16.
- [22] O.P. Yurchenko, C.L. Nykiforuk, M.M. Moloney, U. Stahl, A. Banas, S. Stymne, R.J. Weselake, A 10-kDa acyl-CoA-binding protein (ACBP) from *Brassica napus* enhances acyl exchange between acyl-CoA and phosphatidylcholine, *Plant Biotech. J.* 7 (2009) 602-610.
- [23] S. Mandrup, R. Jepsen, H. Skott, J. Rosendal, P. Hojrup, K. Kristiansen, J. Knudsen. Effect of heterologous expression of acyl-CoA-binding protein on acyl-CoA level and composition in yeast, *Biochem. J.* 290 (1993) 369-374.
- [24] B. Gaigg, T.B.F. Neergaard, R. Schneiter, J.K. Hansen, N.J. Faergeman, N.A. Jensen, J.R. Andersen, J. Friis, R. Sandhoff, H.D. Schroder and J. Knudsen. Depletion of acyl-coenzyme A-binding protein affects sphingolipid synthesis and causes vesicle accumulation and membrane defects in *Saccharomyces cerevisiae*, *Mol. Biol. Cell* 12 (2001) 1147-1160.
- [25] N.J. Faergeman, S. Feddersen, J.K. Christiansen, M.K. Larsen, R. Schneiter, C. Ungermann, K. Mutenda, P. Roepstorff, J. Knudsen, Acyl-CoA-binding protein, ACBP1, is required for normal vacuole function and ceramide synthesis in *Saccharomyces cerevisiae*, *Biochem. J.* 380 (2004) 907-918.
- [26] M.L. Chye, B.Q. Huang, S.Y. Zee, Isolation of a gene encoding *Arabidopsis* membrane-associated acyl-CoA binding protein and immunolocalization of its gene product, *Plant J.* 18 (1999) 205-214.

- [27] H.Y. Li, M.L. Chye, Arabidopsis acyl-CoA-binding protein ACBP2 interacts with an ethylene-responsive element-binding protein, AtEBP, via its ankyrin repeats, *Plant Mol. Biol.* 54 (2004) 233-243.
- [28] Z.Y. Du, M.L. Chye, Interactions between Arabidopsis acyl-CoA-binding proteins and their protein partners, *Planta* 238 (2013) 239-245.
- [29] S.C. Lung, M.L. Chye, Arabidopsis acyl-CoA-binding proteins regulate the synthesis of lipid signals, *New Phytol.* 223 (2019) 113-117.
- [30] K.C. Leung, H. Y. Li, S. Xiao, M. H. Tse, M. L. Chye, Arabidopsis ACBP3 is an extracellularly targeted acyl-CoA-binding protein, *Planta* 223 (2006) 871-881.
- [31] Q.F. Chen, S. Xiao, M. L. Chye, Overexpression of the Arabidopsis 10-kilodalton acyl-coenzyme A-binding protein ACBP6 enhances freezing tolerance, *Plant Physiol.* 148 (2008) 304-315.
- [32] H.Y. Li, S. Xiao, M.L. Chye, Ethylene- and pathogen-inducible Arabidopsis acyl-CoA-binding protein 4 interacts with an ethylene-responsive element binding protein, *J. Exp. Bot.* 59 (2008) 3997-4006.
- [33] S. Xiao, Q.F. Chen, M.L. Chye, Light-regulated Arabidopsis ACBP4 and ACBP5 encode cytosolic acyl-CoA-binding proteins that bind phosphatidylcholine and oleoyl-CoA ester, *Plant Physiol. Biochem.* 47 (2009) 926-933.
- [34] W. Meng, Y.C.F. Su, R.M.K. Saunders, M.L. Chye, The rice acyl-CoA-binding protein gene family: phylogeny, expression and functional analysis, *New Phytol.* 189 (2011) 1170-1184.
- [35] N.H. Raboanatahiry, G.Y. Lu, M. Li, Computational prediction of acyl-CoA binding proteins structure in *Brassica napus*, *Plos One* 10 (2015) 6.
- [36] N.H. Raboanatahity, B. Wang, L. Yu, M. Li., Functional and structural diversity of Acyl-coA binding proteins in oil crops, *Front. Gen.* 9 (2018) 182.
- [37] Y. Xue, S. Xiao, J. Kim, S.C. Lung, L. Chen, J.A. Tanner, M.C. Suh, M.L. Chye, Arabidopsis membrane-associated acyl-CoA-binding protein ACBP1 is involved in stem cuticle formation, *J. Exp. Bot.* 65 (2014) 5473-5483.
- [38] Z.Y. Du, M.X. Chen, Q.F. Chen, S. Xiao, M.L. Chye, Arabidopsis acyl-CoA-binding protein ACBP1 participates in the regulation of seed germination and seedling development, *Plant J.* 74 (2013) 294-309.

- 1 [39] Q.F. Chen, S. Xiao, W.Q. Qi, G. Mishra, J.Y. Ma, M.F. Wang, M.L. Chye, The
2 Arabidopsis acbp1acbp2 double mutant lacking acyl-CoA-binding proteins
3 ACBP1 and ACBP2 is embryo lethal, *New Phytol.* 186 (2010) 843-855.
- 4 [40] S.C. Lung, P. Liao, E.C. Yeung, A.S. Hsiao, Y. Xue, M.L. Chye, Arabidopsis
5 acyl-CoA-binding protein ACBP1 modulates sterol synthesis during
6 embryogenesis, *Plant Physiol.* 174 (2017) 1420-1435.
- 7 [41] S.C. Lung, P. Liao, E.C. Yeung, A.S. Hsiao, Y. Xue, M.L. Chye, Arabidopsis
8 acyl-CoA-binding protein1 interacts with sterol C4-methyl oxidase1-2 to
9 modulate gene expression of homeodomain-leucine zipper IV transcription
10 factors, *New Phytol.* 218 (2018) 183-200.
- 11 [42] S.F. Altschul, T.L. Madden, A.A. Schaffer, J.H. Zhang, Z. Zhang, W. Miller, D.
12 J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein
13 database search programs, *Nucleic Acids Res.* 25 (1997) 3389-3402.
- 14 [43] N.I. Bower, I.A. Johnston, Targeted rapid amplification of cDNA ends (T-
15 RACE)-an improved RACE reaction through degradation of non-target
16 sequences, *Nucleic Acids Res.* 38 (2010) e194.
- 17 [44] L. Dobson, I. Reményi, G.E. Tusnády, CCTOP: a Consensus Constrained
18 TOPology prediction web server, *Nucleic Acids Res.* 43 (2015) W408-W412.
- 19 [45] M.L. Chye, Arabidopsis cDNA encoding a membrane-associated protein with
20 an acyl-CoA binding domain, *Plant Mol. Biol.* 38 (1998) 827-838.
- 21 [46] M. Kozak, An analysis of 5'-noncoding sequences from 699 vertebrate
22 messenger RNAs, *Nucleic Acids Res.* 15 (1987) 8125-8148.
- 23 [47] D.M. Becker, L. Guarente, (1991) High-efficiency transformation of yeast by
24 electroporation, *Methods Enzymol.* , 194: 182-187.
- 25 [48] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H.
26 McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J.
27 Gibson, D.G. Higgins, Clustal W and clustal X version 2.0, *Bioinformatics* 23
28 (2007) 2947-2948.
- 29 [49] N. Saitou, M. Nei, The Neighbor-Joining method - a new method for
30 reconstructing phylogenetic trees, *Mol. Biol. Evol.* 4 (1987) 406-425.
- 31 [50] J. Felsenstein, Confidence limits on phylogenies: An approach using the
32 bootstrap, *Evolution* 39 (1985) 783-791.

- [51] E. Zuckerkandl, L. Pauling, Evolutionary divergence and convergence, in: V. Bryson, H.J. Vogel (Eds.), *Evolving genes and proteins*, Academic Press, New York, 1965, pp. 97-166.
- [52] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms, *Mol. Biol. Evol.* 35 (2018) 1547-1549.
- [53] L.J. McGuffin, R. Adiyaman, A.H.A. Maghrabi, A.N. Shuid, D.A. Brackenridge, J.O. Nealon, L.S. Philomina, IntFOLD: an integrated web resource for high performance protein structure and function prediction, *Nucleic Acids Res.* 47 (2019) W408-W413.
- [54] L.J. McGuffin, A.M. Shuid, R. Kempster, A.H.A. Maghrabi, J.O. Nealon, B.R. Salehe, J.D. Atkins, D.B. Roche, Accurate Template Based Modelling in CASP12 using the IntFOLD4-TS, ModFOLD6 and ReFOLD methods. *Proteins: Structure, Function, and Bioinformatics*, 86 (2018) 335-344.
- [55] A. Grosdidier, V. Zoete, O. Michielin, Fast docking using the CHARMM force field with EADock DSS, *J. Comput. Chem.* 32 (2011) 2149-2159.
- [56] A. Grosdidier, V. Zoete, O. Michielin, SwissDock, a protein-small molecule docking web service based on EADock DSS, *Nucleic Acids Res.* 39 (2011) W270-277.
- [57] D.B. Roche, S.J. Tetchner, L.J. McGuffin, FunFOLD: an improved automated method for the prediction of ligand binding residues using 3D models of proteins, *BMC Bioinformatics* 12 (2011) 160.
- [58] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF Chimera-a visualization system for exploratory research and analysis, *J. Comput. Chem.* 25 (2004) 1605-1612.
- [59] K.J. Livak, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method, *Methods* 25 (2001) 402-408.
- [60] D.K. Srivastava, S. Wang, K.L. Peterson, Isothermal titration microcalorimetric studies for the binding of octenoyl-CoA to medium chain acyl-CoA dehydrogenase, *Biochem.* 36 (1997) 6359-6366.
- [61] M.M. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248-254.

- 1 [62] R. Miao, S.C. Lung, X. Li, X.D. Li, M.L. Chye,. Thermodynamic insights into an
2 interaction between acyl-CoA-binding protein 2 and lysophospholipase 2 in
3 Arabidopsis, J. Biol. Chem. 294 (2019) 6214-6226.
- 4 [63] Z.Y. Du, S. Xiao, Q.F. Chen, M.L. Chye, Depletion of the membrane-associated
5 acyl-coenzyme A-binding protein ACBP1 enhances the ability of cold
6 acclimation in Arabidopsis, Plant Physiol. 152 (2010) 1585-1597.
- 7 [64] D. Mumberg, R. Muller, M. Funk, Yeast vectors for the controlled expression of
8 heterologous proteins in different genetic backgrounds, Gene 156, (1995)
9 119–122.
- 10 [65] A.M. Nash, E.N. Frankel, Limited extraction of soybean with hexane, J. Am. Oil
11 Chem. Soc. 63 (1986) 244–246.
- 12 [66] J.J. Salas, E. Martínez-Force, R. Garcés, Phospholipid molecular profiles in the
13 seed kernel from different sunflower (*Helianthus annuus*) mutants, Lipids 41
14 (2006) 805-811.
- 15 [67] R. Larson, I.A. Graham, Technical advance: a novel technique for the sensitive
16 quantification of acyl-CoA esters from plant tissues, Plant J. 25, (2001) 115-
17 125.
- 18 [68] J.A. Aznar-Moreno, M. Venegas-Calderón, Z.Y. Du, R. Garcés, J.A. Tanner,
19 M.L. Chye, E. Martínez-Force, J.J. Salas, Characterization of a small acyl-
20 CoA-binding protein (ACBP) from *Helianthus annuus* L. and its binding
21 affinities, Plant Physiol. Biochem. 102 (2016) 141-150.
- 22 [69] H. Badouin, J. Gouzy, C.J. Grassa, F. Murat, S.E. Staton, L. Cottret, C.
23 Lelandais-Briere, G.L. Owens, S. Carrere, B. Mayjonade, L. Legrand, N. Gill,
24 N.C. Kane, J.E. Bowers, S. Hubner, A. Bellec, A. Berard, H. Berges, N.
25 Blanchet, M.C. Boniface, D. Brunel, O. Catrice, N. Chaidir, C. Claudel, C.
26 Donnadieu, T. Faraut, G. Fievet, N. Helmstetter, M. King, S.J. Knapp, Z. Lai,
27 M.C Le Paslier, Y. Lippi, L. Lorenzon, J.R. Mandel, G. Marage, G. Marchand,
28 E. Marquand, E. Bret-Mestries, E. Morien, S. Nambeesan, T. Nguyen, P.
29 Pegot-Espagnet, N. Pouilly, F. Raftis, E. Sallet, T. Schiex, J. Thomas, C.
30 Vandecasteele, D. Vares, F. Vear, S. Vautrin, M. Crespi, B. Mangin, J.M.
31 Burke, J. Salse, S. Munos, P. Vincourt, L.H. Rieseberg, N.B. Langlade, The
32 sunflower genome provides insights into oil metabolism, flowering and Asterid
33 evolution, Nature 546 (2017) 148-152.

- [70] N. Amiruddin, P.L. Chan, N. Azizi, P.E. Morris, K.L. Chan, P.W. Ong, R. Rosli, S.S. Masura, D.J. Murphy, R. Sambanthamurthi, R.P. Haslam, M.L. Chye, J.L. Harwood, E.T.L. Low, Characterization of Oil Palm Acyl-CoA-Binding Proteins and Correlation of Their Gene Expression with Oil Synthesis, *Plant Cell Physiol.*, 61 (2020) 735-747.
- [71] M. Burton, T.M. Rose, N.J. Faergeman, J. Knudsen, Evolution of the acyl-CoA binding protein (ACBP), *Biochem. J.* 392 (2005) 299-307.
- [72] H.Y. Li, M.L. Chye, Membrane localization of Arabidopsis acyl-CoA binding protein ACBP2, *Plant Mol. Biol.* 51 (2003) 483-492.
- [73] J.J. Almagro-Armenteros, C.K. Sønderby, S.K. Sønderby, H. Nielsen, O. Winther, DeepLoc: prediction of protein subcellular localization using deep learning, *Bioinformatics* 33 (2017) 3387–3395.
- [74] J.P. Taskinen, D.M. van Aalten, J. Knudsen, R.K. Wierenga, High resolution crystal structures of unliganded and liganded human liver ACBP reveal a new mode of binding for the acyl-CoA ligand, *Proteins-Structure Function and Bioinformatics* 66 (2007) 229-238.
- [75] P. Liao, H.K. Woodfield, J.L. Harwood, M.L. Chye, S. Scofield, Comparative transcriptomics analysis of Brassica napus L. during seed maturation reveals dynamic changes in gene expression between embryos and seed coats and distinct expression profiles of acyl-CoA-binding proteins for lipid accumulation, *Plant Cell Physiol.*, 60 (2019) 2812-2825.
- [76] S. Xiao, M.L. Chye, New roles for acyl-CoA-binding proteins (ACBPs) in plant development, stress responses and lipid metabolism, *Prog. Lipid Res.* 50 (2011) 141-151.
- [77] J.C.D. Houtman, P.H. Brown, B. Bowden, H. Yamaguchi, E. Appella, L.E. Samelson, P. Schuck, Studying multisite binary and ternary protein interactions by global analysis of isothermal titration calorimetry data in SEDPHAT: Application to adaptor protein complexes in cell signaling, *Protein Sci.* 16 (2007) 30-42.
- [78] A.S. Hsiao, R.P. Haslam, L.V. Michaelson, P. Liao, Q.F. Chen, S. Sooriyaarachchi, S.L. Mowbray, J.A. Napier, J.A. Tanner and M.L. Chye, Arabidopsis cytosolic acyl-CoA-binding proteins ACBP4, ACBP5 and ACBP6 have overlapping but distinct roles in seed development, *Biosci. Rep.* 34 (2014) 865-877.

- 1 [79] W. Meng, A.S. Hsiao, C.J. Gao, L.W. Jiang, M.L. Chye, Subcellular localization
2 of rice acyl-CoA-binding proteins (ACBPs) indicates that OsACBP6::GFP is
3 targeted to the peroxisomes, *New Phytol.* 203 (2014) 469-482.
- 4 [80] J.A. Aznar-Moreno, E. Martínez-Force, M. Venegas-Calderón, R. Garcés, J.J.
5 Salas, Changes in acyl-coenzyme A pools in sunflower seeds with modified
6 fatty acid composition, *Phytochem.* 87 (2013) 39-50.
- 7 [81] L. Samuels, L. Kunst, R. Jetter, Sealing plant surfaces: cuticular wax formation
8 by epidermal cells. *Annu. Rev. Plant Biol.*, 59 (2008) 683-707.
- 9 [82] W. Gao, S. Xiao, H.K. Li, S.W. Tsao, M.L. Chye, *Arabidopsis thaliana* acyl-
10 CoA-binding protein ACBP2 interacts with heavy-metal-binding farnesylated
11 protein AtFP6, *New phytol.* 181 (2009) 89-102.
- 12 [83] W. Gao, H.Y. Li, S. Xiao, M.L. Chye, Acyl-CoA-binding protein 2 binds
13 lysophospholipase 2 and lysoPC to promote tolerance to cadmium-induced
14 oxidative stress in transgenic *Arabidopsis*, *Plant J.* 62 (2010) 989-1003.
- 15 [84] P.S. Covello, D.W. Reed, Functional expression of the extraplastidial
16 *Arabidopsis thaliana* oleate desaturase gene (FAD2) in *Saccharomyces*
17 *cerevisiae*, *Plant Physiol.*, 111 (1996) 223-226.
- 18 [85] L. Aymé, S. Baud, B. Dubreucq, F. Joffre, T. Chardot, Function and localization
19 of the *Arabidopsis thaliana* diacylglycerol acyltransferase DGAT2 expressed in
20 yeast, *PloS one*, 9 (2014) 3.
- 21 [86] J. Knudsen, N.J. Faergeman, H. Skøtt, R. Hummel, C. Børsting, T.M. Rose,
22 M., J.S. Andersen, P. Højrup, P. Roepstorff, K. Kristiansen, Yeast acyl-CoA-
23 binding protein: acyl-CoA-binding affinity and effect on intracellular acyl-CoA
24 pool size, *Biochemical J.*, 302 (1994) 479-485.
- 25 [87] Y. Hong, W. Zhang, X. Wang, Phospholipase D and phosphatidic acid
26 signalling in plant response to drought and salinity, *Plant cell Environm.*, 33
27 (2010) 627-635.
- 28 [88] W. Zhang, C. Qin, J. Zhao, X. Wang, Phospholipase D α 1-derived phosphatidic
29 acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling,
30 *Proc. Nat. Acad. Sci. USA*, 101 (2004) 9508-9513.
- 31 [89] Z. Xie, M. Fang, M.P. Rivas, A.J. Faulkner, P.C. Sternweis, J. Engebrecht,
32 V.A. Bankaitis,. Phospholipase D activity is required for suppression of yeast
33 phosphatidylinositol transfer protein defects, *Proc. Nat. Acad. Sci. USA*, 95
34 (1998) 12346-12351.

- 1 [90] A. Mapelli-Brahm, R. Sánchez, X. Pan, A.J. Moreno-Pérez, R. Garcés, E.
2 Martínez-Force, R.J. Weselake, J.J. Salas, M. Venegas-Calación,. Functional
3 Characterization of Lysophosphatidylcholine: Acyl-CoA Acyltransferase Genes
4 From Sunflower (*Helianthus annuus* L.), Front. Plant Sci. 11 (2020) 403.
5

1 Tables

2
3 **Table 1.** Thermodynamic parameters from the interaction studies of rHaACBP1 with different acyl-CoAs assessed by isothermal
4 titration calorimetry (ITC): 16:0 (palmitoyl-CoA), 18:0 (stearoyl-CoA), 18:1 (oleoyl-CoA), 18:2 (linoleoyl-CoA), 18:3 (linolenoyl-CoA).

Acyl-CoAs	N	ΔH (kcal/mol)	ΔS (cal/mol K ⁻¹)	ΔG (kcal/mol)	K_D (μM)
16:0	0.9	-5.3	14.6	-9.9	0.11
18:0	0.5	-5.8	13.3	-9.8	0.13
18:1	0.8	-1.6	25.9	-9.4	6.4
18:2	1.1	-5.8	5.26	-7.4	21.4

5 All parameters were calculated according to Miao et al. [62]

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8 **Table 2.** Size and composition of the acyl-CoA pool of *S. cerevisiae* cultures transformed with the empty p-416 vector and the
9 plasmid expressing the HaACBP1 protein.

Line	Total ($\mu mol/mL$ culture)	Composition (mol %)						
		16:1-CoA	16:0-CoA	18:1-CoA	18:0-CoA	20:1-CoA	20:0-CoA	22:0-CoA
P-416	38.4 $\pm$ 6.0	44.2 $\pm$ 7.4	10.9 $\pm$ 2.6	34.8 $\pm$ 2.4	5.6 $\pm$ 2.3	2.0 $\pm$ 0.5	0.2 $\pm$ 0.1	2.3 $\pm$ 0.8
HaACBP1	71.7 $\pm$ 11.6*	61.1 $\pm$ 6.7 *	7.6 $\pm$ 2.5	25.0 $\pm$ 3.2*	3.5 $\pm$ 1.1	0.6 $\pm$ 0.4*	0.2 $\pm$ 0.0	2.1 $\pm$ 0.5

10
11 The data correspond to the average of four measurements $\pm$ standard deviations. The asterisks indicate significant differences relative to control cells
12 as assessed by one-way ANOVA with a Tukey post hoc analysis and a significance level of 0.05%.

Figure Legends

Figure 1. Evolutionary relationships of taxa using ACBP1 homolog sequences. The evolutionary history was inferred using the Neighbor-Joining method, applying taxa clustering and scaling according to [49-51]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA X [52]. Bryophyte species *Physcomitrella patens* ACBP1 sequences were used as outgroup to root the phylogenetic tree.

Figure 2. Proposed structural model for HaACBP1 residues from E69 to L319 docking with a CoA molecule. **A** and **B**, ribbon diagrams showing the (i) ACB domain in green, the residues involved in CoA binding in purple, and (ii) the ankyrin repeats in yellow, orange and red (the coloured residues correspond to those shown in the alignment of Figure S1). **C** and **D**, the molecular surface in the same colour code as panels A and B, the CoA molecule is in cyan.

Fig. 3. Quantitative real-time PCR analysis of *HaACBP1* expression in various sunflower tissues: DAF (days after flowering), DAI (days after imbibition). Expression was normalized to that of the sunflower actin gene *HaACT1*, and the error bars represent the standard deviation (n=3).

Fig. 4. Protein-lipid interaction of rHaACBP1 with different phospholipid species. **A**, Binding of rHaACBP1 to phospholipids on a membrane. **B**, Diagram of the lipid species on the membrane shown in panel A. **C**, rHaACBP1 binding to the filter with serial dilutions of phosphatidylcholine (50, 25, 12.5 and 5 μ M). Phospholipids: PA, phosphatidic acid; PC, phosphatidylcholine; LPA, lysoPA; and LPC, lysoPC. Fatty acids: 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid and 18:2, linoleic acid. The fatty acid together with the PC or PA indicates that both positions of the glycerolipid are esterified.

Fig. 5. Effect of HaACBP1 on yeast (*S. cerevisiae*) glycerolipids. The data correspond to the lipids produced in 200 mL of a stationary culture at OD₆₀₀ = 2.0.

1 The data from yeast transformed with the empty plasmid (p-416) is shown in the grey
2 bars and that from the yeast expressing HaACBP1 corresponds to the white bars. **A**,
3 The total triacylglycerols (TAGs) and polar lipids produced in the cultures. **B**, The
4 composition of the polar lipids. **C**, The fatty acid composition of the total lipid extract
5 of the yeast. The data represents the average of four measurements $\pm$ standard
6 deviations and the asterisks indicate significant differences relative to the control
7 cells according to one-way ANOVA with a Tukey *post hoc* analysis and at a
8 significance level of 0.05%: CL, cardiolipin; LPC, lysophosphatidylcholine; PA,
9 phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG,
10 phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Figure 1

0.10

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Expression and function of a sunflower (*Helianthus annuus* L.) Class II acyl-CoA-binding protein

Supplementary material

Table S1. Sequence of PCR primers used in this work. Restriction sites are underlined. Start and stop codons are represented in italics. The nucleotide highlighted in bold was changed according to Kozak [46], in order to optimize expression on yeast.

Primer name	Sequence
ACBP1-5rac 1	5'- CTT C ACAAGCAGCTCGGCTATGTG -3'
ACBP1-5rac 2	5'- CACAGGAATGCCAGCCTCGACAC -3'
UPM	5'- CTAATACGACTCACTATAGGGC -3'
NUP	5'- AAGCAGTGGTAACAACGCAGAGT -3'
ACBP-1F	5'- ATG T TCGACTGGCAACAGTACACTC -3'
ACBP-1R	5'- TCAAAGAGAGGGCGCCTGGGG -3'
<i>Sph</i> IACBP1-F	5'- <u>GCATGC</u> ATGTTTCGAGATCAAAATCTC-3'
<i>Kpn</i> IACBP1-R	5'- <u>GGTACC</u> TCAAAGAGAGGGCGCC -3'
<i>Spe</i> IACBP1-F	5'- <u>ACTAGT</u> ACCATG G TCGACTGGCAACAG -3'
<i>Xma</i> IACBP1-R	5'- <u>CCCGGG</u> TCAAAGAGAGGGCGCC-3'
QHaACBP1-F	5'- CCAATCAATCACCGGCGTCC -3'
QHaACBP1-R	5'- GTTGCCAGTCGAACATTGTG -3'
QHaActin-F4	5'- GCTAACAGGGAAAAGATGACT-3'
QHaActin-R4	5'- ACTGGCATAAAGAGAAAGCACG-3'

Figure S2. Subcellular location prediction of *HaACBP1* proteins by program DeepLoc. from Almagro-Armenteros et al. [72].

Figure S3. Expression and purification of *rHaACBP1*. The picture shows the gel electrophoresis of different fractions of *E. coli* obtained during purification. Protein

1 staining followed with 15% sodium dodecylsulphate-polyacrylamide gel
2 electrophoresis (SDS PAGE). Arrow denotes the 31-kDa *rHaACBP1* band.
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Figure S4. Binding isothermal titration calorimetry of rHaACBP1 with palmitoyl-CoA (16:0-CoA), stearoyl-CoA (18:0-CoA), oleoyl-CoA (18:1-CoA), linoleoyl-CoA (18:2-CoA), linolenoyl-CoA (18:3-CoA) and lignoceroyl-CoA (24:0-CoA). The graphs represented in panels below show the integrated area under each peak plotted against the molar ratio of acyl-CoA to rHaACBP1 at 30 °C. The solid line represents the best fit of the experimental data for the dissociation constant (K_d). Explain all symbols used in fig below

Figure S5. Binding isothermal titration calorimetry of rHaACBP1 with 26:0 CoA, 30:0-CoA, and 20:4-CoA. The graphs represented in panel below show the integrated area under each peak plotted against the molar ratio of acyl-CoA to rHaACBP1 at 30 °C. The solid line represents the best fit of the experimental data for the dissociation constant (K_d).