Sunflower (*Helianthus annuus*) Long-Chain Acyl-Coenzyme A Synthetases Involved in Seed Triacylglycerol Biosynthesis

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Abstract

Long chain fatty acid synthetases (LACSs) activate the fatty acid chains produced by plastidial *de novo* biosynthesis to generate acyl-CoA derivatives, important intermediates in lipid metabolism. Oilseeds, like sunflower, accumulate high levels of triacylglycerols in their seeds to nourish the embryo during germination. This requires that sunflower seed endosperm supports very active glycerolipid synthesis during development. Sunflower seed plastids produce large amounts of fatty acids, which must be activated in order to be incorporated into triacylglycerols through the action of LACSs. We cloned two different LACS genes from developing sunflower endosperm, *HaLACS1* and *HaLACS2*, which displayed sequence homology with Arabidopsis *LACS9* and *LACS8* genes, respectively. These genes were expressed at high levels in developing seeds and exhibited distinct sub-cellular distributions. We generated constructs in which these proteins were fused to green fluorescent protein and performed transient expression experiments in tobacco cells. The *HaLACS1* protein associated with the external envelope of tobacco chloroplasts, whereas *HaLACS2* was strongly bound to the endoplasmic reticulum. Finally, both proteins were overexpressed in *E. coli* and recovered as active enzymes in the bacterial membranes. Both enzymes displayed similar substrate specificities, with a very high preference for oleic acid and weaker activity towards stearic acid. Based on our findings, we discuss the role of these enzymes in sunflower oil synthesis.

Keywords: Acyl-CoA synthetase; oil biosynthesis; *Helianthus annuus*; sub-cellular location; substrate specificity.

Introduction

Triacylglycerol (TAG) synthesis in oilseeds is a complex process that involves several cell compartments (Ohlrogge and Browse, 1995). The carbon source for TAGs consists of carbohydrates that are imported from leaves and split into a variety of metabolites via the glycolytic pathway. In turn, these compounds are transported into plastids and converted into malonyl-CoA, the basic unit of *de novo* fatty acid synthesis (Rawsthorne, 2002; Pleite et al., 2005). Fatty acid synthesis takes place in the plastid stroma through the action of the fatty acid synthase complex, and it is terminated by the hydrolysis of acyl-ACP intermediates, a process catalysed by acyl-acyl carrier protein (ACP) thioesterases (Ohlrogge and Jaworsky, 1997). The free fatty acids released by thioesterases can move easily through distinct cellular compartments, yet they are only detected at very low levels in developing seeds, as they are rapidly converted to their corresponding acyl-CoAs by long chain acyl-CoA synthetases (LACS, EC 6.2.1.3: Schnurr et al., 2002). Although initially controversial (Thomas et al., 1982; 1983), the existence of free fatty acids as intermediates of fatty acid synthesis was confirmed in labelling experiments (Pollard and Ohlrogge, 1999).

Long chain acyl-CoAs are central metabolites in many biosynthetic and degradative pathways, including beta oxidation, acyl turnover of membrane lipids and many reactions involving the exchange or transference of a fatty acid moiety. LACS are AMP-binding proteins that form part of the carboxyl-CoA ligase superfamily, collectively known as acyl activating enzymes (Shockey and Browse, 2011). These enzymes catalyse a reaction in which one molecule of free fatty acid is esterified to its free coenzyme A, with the consumption of one ATP equivalent. The mechanism underlying this reaction has been well described and it involves the production of an acyl-AMP intermediate that is later used to acylate the coenzyme A molecule (Groot et al., 1976). These enzymes are membrane-bound and they usually contain transmembrane domains in their sequences.

Plants usually contain several LACS that participate in different metabolic processes and that are distributed distinctly. For example, nine LACS genes (*AtLACS1-AtLACS9*) have been described in Arabidopsis and their role in plant metabolism has been extensively studied (Shockey et al., 2002). Proteins *AtLACS6* and *AtLACS7* are localized in the peroxisome and they play important roles in the activation of fatty

acids for beta oxidation in seeds. As such, these LACS are essential for Arabidopsis seed germination (Fulda et al., 2002; 2004). AtLACS1 and AtLACS2 are found in several different organs and in the endoplasmic reticulum, and they are involved in cuticle development (Schnurr et al., 2004; Lu et al., 2009). AtLACS1 and AtLACS4 mediate the synthesis of lipids specific to the pollen coat (tryphine) and thus, they are essential for plant viability (Jessen et al., 2011).

Oilseeds have a strong metabolic flux into TAGs, a continuous supply of acyl-CoAs generated by plastidial synthesis is fed into the acyltransferases of the endoplasmic reticulum, which mediate the incorporation of fatty acids into the three positions of the glycerol backbone. The AtLACS9 gene, which is expressed strongly in seeds and encodes a protein located in the plastid membrane was initially considered the main mediator of this process. However, reverse genomics experiments using an AtLACS9 knockout mutant of Arabidopsis revealed that although these seeds exhibit a significant reduction in plastidial LACS, they display a normal phenotype, indicating that several LACS isoforms are involved in this process (Schnurr et al., 2002). A more recent study demonstrated that AtLACS1, located in the endoplasmic reticulum, is also involved in TAG synthesis (Zhao et al., 2010), further evidence that several different LACS isoforms are involved in this process.

The accumulation of high levels of TAGs in developing sunflower seeds requires high rates of fatty acid and glycerolipid synthesis. Thus, here we have examined fatty acid activation in developing sunflower seeds. We cloned and characterized two *HaLACS* genes (*HaLACS1* and *HaLACS2*) that are expressed strongly in this tissue. These genes were then heterologously overexpressed in *E. coli*, from which they were recovered as active enzymes in the membrane fraction of the bacteria. In addition, the proteins were fused to fluorescent tags and expressed in tobacco cells to study their sub-cellular distribution, which revealed *HaLACS1* and *HaLACS2* to be expressed in plastids and the endoplasmic reticulum, respectively. In the context of these findings, we discuss the role of these enzymes in the synthesis of sunflower oil, and compare and contrast our results with those previously reported for other oilseeds.

Experimental Methods

Plant Material

Sunflower seeds from the CAS-6 sunflower line were germinated in wet perlite at 25°C and then transferred to a germination chamber for 2 weeks. Subsequently, the seedlings were moved to growth chambers equipped with fertigation lines and maintained at 25°/15°C (day/night) with a 16 h photoperiod and a photon flux density of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ until vegetative tissues or developing seeds were harvested.

Cloning of cDNAs encoding sunflower LACSs

Developing sunflower seeds (approximately 0.4 g) were harvested 15 days after flowering (DAF), and they were ground in liquid N₂ using a precooled sterile mortar and pestle. The seed mRNA was isolated using the Micro-FastTrack Kit (Invitrogen, Groningen, The Netherlands) in 33 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]) and cDNAs were obtained using a Ready-To-Go TPrimed First Strand Kit (Amersham Bioscience, Roosendaal, The Netherlands).

Arabidopsis thaliana LACS8 and LACS9 protein sequences were used to search sunflower expressed sequence tags (ESTs) in public databases in order to find putative mRNAs encoding sunflower LACS homologues using the TBLASTN algorithm (Altschul et al., 1997). ESTs corresponding to the 5' and 3' ends, and to internal fragments were identified. To match the fragments corresponding to the same mRNA by PCR, several forward and reverse primers were designed: LACS-A-F, LACS-B-F, LACS-D-R and LACS-E-R (Table 1: all the primers were synthesized by Eurofins MWG Operon, Germany). 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 by SECUGEN (Madrid, Spain). Using the BLAST software (Altschul et al., 1997), two different clones were identified, *HaLACS1* and *HaLACS2*, generated with the LACS-A-F/LACS-D-R and LACS-B-F/LACS-E-R primer pairs, respectively. While the first sequence was complete, including the ATG and STOP codons, the 3' end was absent from the second sequence. The 3' end of the *HaLACS2* cDNA was obtained by PCR using the external oligo FA2Z (Table 1), complementary to the sequences incorporated during the initial cDNA synthesis, and the specific internal oligo *HaLACS2*-3rac (Table 1). The PCR fragments were cloned,

sequenced and assembled to obtain DNA sequences encoding two long chain acyl-CoA synthetase isoforms, *HaLACS1* (2098 bp) and *HaLACS2* (2136 bp). These cDNA sequences were deposited in GENBANK under Accession numbers HM490305 and HM490306, respectively.

Analyses of cDNAs and proteins

Sequences homologous to the predicted sequences for sunflower LACSs were retrieved using the BLASTP program (www.ncbi.nlm.nih.gov). The amino acid sequences for LACS proteins deposited at GENBANK were aligned using the ClustalX v.2.0.10 program under the default settings (Larkin et al., 2007). These alignments were used to generate a phylogenetic tree based on the neighbor-joining algorithm (Saitou and Nei, 1987), and the resulting 'phenogram' was created using the MEGA 5.0 program (Tamura et al., 2007).

Genomic DNA analysis

Genomic DNA was isolated from sunflower leaf tissue by the CTAB method (Murray and Thomson, 1980), and aliquots (20 µg) were digested with restriction enzymes and resolved on a 0.8 % agarose gel. The gel was soaked in 250 mM HCl for 30 min, washed three times in distilled water and transferred to a Hybond-N+ nylon membrane (GE Healthcare Life Science). The filters were probed with 1,570 bp and 1,080 bp [α -³²P]dCTP-labelled ("Ready-to go" labelling kit: GE Healthcare Life Science) *HaLACS1* and *HaLACS2* gene-specific DNA fragments obtained by PCR amplification using *HaLACS1S-F/HaLACS1S-R* and *HaLACS2S-F/HaLACS2S-R-3'* primer pairs. Hybridization was performed overnight at 65°C in 0.2 mM potassium buffer, 250 mM SDS and 1 mM EDTA. The filter was then washed twice for 20 min at the same temperature in 2X SSC buffer containing 0.1% SDS. Radioactive filters were visualized and quantified using a Cyclone TM Storage Phosphor System (PerkinElmer, Boston, MA, USA) and Optiquant TM image analysis software (Packard Co., Meriden, CT, USA).

Quantitative real-time PCR

The cDNAs from developing seeds (12, 15, 20, 25 and 30 DAF), roots, stems, green cotyledons (2, 5 and 7 days after imbibition) and leaves were obtained from 1-5 µg of

total RNA as described previously. The cDNAs were analysed by quantitative real-time PCR (qRT-PCR) using specific primer pairs (Table 1: QHaLACS1-F and QHaLACS1-R for HaLACS1; and QHaLACS2-F and QHaLACS2-R for HaLACS2), and SYBR Green I (QuantiTect SYBR Green PCR Kit, Qiagen, Crawley, UK) in a MiniOpticon system to monitor the resulting fluorescence (Bio-Rad). 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 of 94°C for 15 s; 61°C for 30 s; and 72°C for 30 s. Calibration curves were drawn up using sequential dilutions of the cDNAs. The Livak method (Livak and Schmittgen, 2001) was applied to calculate the comparative expression of the samples. The sunflower *HaACT1* actin gene (GenBank Accession number FJ487620) served as a reference gene, for which a specific primer pair was used (Table 1: QHaActin-F4 and QHaActin-R4).

Expression of recombinant sunflower LACS in E. coli

Primers with internal *Bam*HI and *Kpn*I restriction sites (BamHaLACS1-F and KpnHaLACS1-R, respectively) were designed to amplify the entire coding sequence of *HaLACS1* by PCR (Table 1). The resulting PCR product was subcloned into the *Bam*HI-*Kpn*I sites of pQE-80L (Qiagen, Hilden, Germany) to produce a fusion protein with a hexahistidine tag at the N-terminus. Using a similar approach, the full *HaLACS2* cDNA was cloned into pQE-80L as an *Nde*I-*Kpn*I fragment using the BamHaLACS2-F and KpnHaLACS2-R primer pair (Table 1). Ligation into the correct reading frame was confirmed by sequencing and the resulting constructs were designated pQELACS1 and pQELACS2, respectively. The recombinant plasmids were introduced and expressed in the LACS-deficient *E. coli* strain K27 (Overath et al., 1969). The predicted molecular mass of the resulting recombinant proteins 6(His)*HaLACS1* and 6(His)*HaLACS2* was 77.7 kDa.

To obtain the recombinant proteins, overnight cultures of transformed bacteria were used to inoculate 1 L of LB medium supplemented with 100 µg/L ampicillin. The mixture was then incubated at 37°C until an OD₆₀₀ of 0.6 was reached, after which the cultures were induced with 0.7 mM IPTG (isopropyl-*B-D*-thiogalactopyranoside). The cultures were maintained overnight with shaking and the cells were collected by centrifugation at 4000 x g for 15 min. The cell pellets were resuspended in 10 mL of 50 mM Tris [pH 8.0], 1 mg/mL lysozyme, 0.1 mg/mL DNase, 5 mM DTT and lysed by

sonication at 0°C using a Branson sonicator (Model SLPe; Thomas Scientific), applying 10 pulses of 20 s. The lysed bacterial suspension was centrifuged for 40 min at 20,000 x g and the resulting supernatant was centrifuged for 2 h at 100,000 x g. The pellets containing the membranes were resuspended in 50 mM Tris [pH 8.0], 5 mM EDTA, 5 mM DTT and stored at 80°C for subsequent enzyme assays.

LACS activity

The LACS assays were carried out in a mixture containing: 100 mM Bis-tris-propane [pH 7.6], 10 mM MgCl₂, 5 mM ATP, 2.5 mM DTT, 1 mM CoA, 2 mM fatty acid and 0.3 µg of *E. coli* membrane protein. Enzyme activity was assayed at room temperature for 60 min and terminated by the addition of 100 µL of 10% (v/v) acetic acid in isopropanol. Subsequently, 20 nmol of 17:0-CoA was added as the internal standard and the samples were purified by solid extraction onto C18 cartridges (250 mg), as described previously (Mangino et al., 1992). Purified acyl-CoAs were then derivatized to their fluorescent ethyl etheno analogues and analysed by HPLC using a modified version of the method described by Larson and Grahan (2001).

Construction of plasmids encoding green fluorescent protein-tagged LACSs and transient transformation of tobacco suspension cells

Bioinformatics programs were used to predict the sub-cellular localization of sunflower LACS, to identify target regions that could potentially be disrupted by translational fusion to the green fluorescent protein (GFP) and to identify putative transmembrane regions, including: TargetP1.1 (<http://www.cbs.dtu.dk/services/TargetP/>); ChloroP1.1 (<http://www.cbs.dtu.dk/services/ChloroP/>); TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>); and OCTOPUS (<http://octopus.cbr.su.se/>).

Full-length open reading frames (ORFs, minus the stop codon) of *HaLACS1* and *HaLACS2* were amplified by PCR using appropriate forward and reverse primers that introduced 5' and 3' *NheI* sites (*NheLACS1-F/NheLACS1-R* and *NheHaLACS2-F/NheHaLACS2-R*, respectively: Table 1). The resulting PCR products were subcloned into pMBL T (Genaxxon BioScience GmbH, Biberach, Germany) and the plasmids were digested with *NheI*. The *NheI* DNA fragments were then ligated into *NheI*-digested

pUC18/*Nhe*I-mGFP, a plant expression plasmid that contains the 35S promoter and a unique *Nhe*I restriction site immediately adjacent to the 5' end of the GFP (Simpson et al., 2008). The pRTL2/RFP plasmid that encodes red fluorescent protein (RFP), and the pRTL2/TIC40-RFP plasmid that consists a construct with the 40 kDa component of the translocon at the inner membrane of *Arabidopsis* chloroplasts (TIC40) fused to the N-terminus of RFP, were generated as described previously (Dhanoa et al., 2009).

Tobacco cells were cultured in suspension (*Nicotiana tabacum* cv. Bright Yellow-2 [BY-2]) and prepared for bombardment as described previously (Lingard et al. 2008). Transient (co-)transformations were performed with a Biolistic® PDS-1000/HE particle delivery system (Bio-Rad), using 5 µg of plasmid DNA encoding either HaLACS1-mGFP or HaLACS2-mGFP and 2.5 µg of plasmid DNA encoding either RFP or TIC40-RFP. Bombarded cells were incubated for 6 h to allow the gene products introduced to be expressed and sorted, and to ensure that any potential negative effects due to (membrane) protein overexpression were diminished. The cells were then fixed in 4% (w/v) formaldehyde and permeabilized with 0.01% (w/v) pectolyase Y-23 (Kyowa Chemical Products, Osaka, Japan) followed by 0.3% Triton X-100 (v/v) (Sigma-Aldrich Ltd.). Where necessary, Alexa 594-conjugated concanavalin A (ConA; Molecular probes, Eugene, OR) was added to permeabilized BY-2 cells for 20 min at a final concentration of 5 µg ml⁻¹.

Confocal Microscopy

Confocal laser scanning microscopy (CLSM) images of BY-2 cells were acquired with the LEICA TCN NT software package (Version 2.61) on a Leica DM RBE microscope using a 63x Plan Apochromat oil-immersion objective and a TCS SP2 scanning head (Leica). Confocal images were acquired as a z-series of representative cells and single optical sections that were saved as 512 x 512-pixel digital images. The fluorescence images shown in this work are representative of >20 cells from at least three independent transformation experiments.

Results and Discussion

Cloning of LACS genes from developing sunflower seeds

Two genes encoding LACS were cloned from developing sunflower seeds using PCR primers designed from the sequences of the sunflower EST homologues of *Arabidopsis thaliana* LACS8 and LACS9 (see Materials and Methods). Both these genes, *HaLACS1* and *HaLACS2*, were 2.1 kb long, encoding two proteins of 697 and 711 aa, with molecular weights of 76.3 and 77.6 kDa, respectively, and that shared 62.02% sequence identity (Fig. 1). The *HaLACS1* protein displayed significant sequence identity (74.2 %) with the plastidial LACS9 from *Arabidopsis* (Shockey et al., 2002: Fig. 1) and with the more phylogenetically distant LACS9 from *Oryza sativa* (73.8 %). The *HaLACS2* protein exhibited the strongest sequence homology (69.9%) with *Arabidopsis* LACS8 (Fig. 1), which was recently revealed to be an endoplasmic reticulum-binding enzyme expressed at high levels in *Arabidopsis* seeds (Zhao et al., 2010), and it also shared 69.2% identity with the corresponding rice homologue.

Analysis of these proteins revealed the presence of the acyl-activating enzyme consensus motif (PROSITE PS00455 [LIVMFY]XX[STG][STAG]G[ST][STEI][SG]X [PASLIVM][KR], shown in bold in Fig. 1), which appears to be crucial for ATP binding and adenylate formation (Conti et al., 1996). Putative active site residues based on the structures of other family members are also shown in the alignment (Fig. 1; Gulick et al., 2003; Reger et al., 2008; Hughes and Keatinge-Clay, 2011).

Using the sequences described here and other known Viridiplantae LACS8 and LACS9 protein sequences, a phylogenetic tree was generated using the sequence of the zebrafish LACS homologue as an outgroup with which to root the tree (Fig. 2). This sequence, which shares 47% identity with both LACS isoforms, was selected as a representative of the Vertebrata, and it exhibits higher sequence identity with them than other plant LACS isoforms such as *AtLACS6* and *AtLACS7*. The genes *HaLACS1* and *HaLACS2* grouped with the other plant homologues of *AtLACS9* and *AtLACS8*, respectively, and were more closely related to the LACS isoforms from *Solanum lycopersicum* than to other known LACs. The *Helianthus* and *Solanum* genera are grouped within in Asteridae subclass, which is consistent with conventional species of trees where Monocot (Poaceae species) and Dicot species are differentiated from the Briophyta and Lycopodiophyta species. However, unlike the Magnoliophyta, the sequences from the Briophyta and Lycopodiophyta species revealed no clear distinction between LACS8 and LACS9 isoforms, suggesting that the development of

the two distinct LACS isoforms coincided with the divergence of these two phylogenetic groups.

Expression of sunflower LACS

The two sunflower LACS studied here were expressed in all the tissues analysed (Fig. 3), *i.e.*: vegetative tissues such as leaves, roots and stems; developing seeds; and germinating cotyledons. *HaLACS1* was expressed very strongly in developing seeds, in agreement with a role of this enzyme in sunflower oil synthesis. Remarkably, the expression of this enzyme did not change significantly over the period studied (12 to 30 DAF). The *HaLACS2* enzyme was also expressed in developing seeds, but more weakly than *HaLACS1*. Unlike the plastidial isoform, *HaLACS2* expression varied during seed development, reaching a maximum 20-25 DAF period that coincided with the highest rate of lipid synthesis. Previous studies of germinating cotyledons reported that the LACS isoforms located in peroxisomes are those that are most closely implicated in the mobilization of fatty acids associated with oil seed germination (Fulda et al., 2004). However, the enzymes studied here were also expressed in developing cotyledons, where *HaLACS2* was expressed more strongly than *HaLACS1*. In developing vegetative tissues, both *HaLACS1* and *HaLACS2* were expressed at similar levels in the roots and stems, while *HaLACS2* expression predominated in leaves.

Genomic organization of sunflower LACS genes

In Southern blots of genomic DNA, *HaLACS1* and *HaLACS2* gene-specific probes recognised single indicative of the presence of only one copy the genes analysed within the sunflower genome (Fig. 4). These findings were consistent with previous observations in other plant species with a fully sequenced genome (*i.e.*: in the *Ricinus communis*, *Vitis vinifera* and *Arabidopsis thaliana* species included in the phylogenetic tree in Fig. 2).

Expression in E. coli and substrate specificity

The LACS proteins were overexpressed in *E. coli* and recovered as active enzymes in the microsomal fractions of the bacteria. This strain is deficient in LACS activity and thus, all the activity recorded can be attributed to the exogenous proteins (Shockey et

al., 2002). Although both peptides had been endowed with a poly-His terminal sequence to facilitate their purification, they failed to bind to the Nickel-sepharose column. Several attempts were made to solubilise these enzymes using Triton X-100, Tween-80 and deoxycholate as surfactants, yet the recovery of the active soluble enzyme was poor in all cases and again, the enzymes were eluted after failing to bind to the Nickel column indicating that they formed aggregates that prevented the histidine tag from interacting with the Ni-NTA matrix. Enzymatic studies were thus carried out using the crude microsomal fraction, as previously described for other LACS isoforms (Shockey et al., 2002).

The *HaLACS1* enzyme displayed high activity towards monounsaturated fatty acids, with the highest rates of activation observed for palmitoleic and oleic acid (Fig. 5). Polyunsaturated fatty acids like linoleic and linolenic acids were also esterified at a high rate by this enzyme. By contrast, *HaLACS1* displayed low specificity towards saturated fatty acids, such as palmitic and stearic acids, and very low or undetectable activity for very long chain saturated fatty acids. This profile matched well with the enzyme location, as the main fatty acid exported from the de novo synthesis was oleic acid liberated from acyl-ACP derivatives via FatA thioesterase (Serrano-Vega et al., 2005). Compared with *HaLACS1*, *HaLACS2* displayed lower absolute activities in the crude microsomal preparations, exhibiting the highest activities towards palmitic and linoleic acids (Fig. 5). Low activity towards stearic and very long chain fatty acids was also evident. This profile fits well with a role in the activation of free fatty acids released from membrane lipids by acyl-hydrolase enzymes, which contribute to the acyl exchange between polar lipids, the acyl CoA pool and triacylglycerols. The specificity profiles observed were similar to those reported for the LACS isoforms from *Arabidopsis* (Shockey et al., 2002), which displayed high relative activity towards oleic and linoleic acids and low activity towards stearic acid. These low rates of activation of stearic acid may restrict its metabolism and accumulation. Sunflower mutants with altered fatty acid compositions have been described, including mutants with high stearic acid content (Fernández-Moya et al., 2005), in which certain limitations to the accumulation of stearic acid have been detected, possibly reflecting the inability of the enzymatic machinery to accept stearyl derivatives as substrates (Aznar-Moreno et al., 2013). This hypothesis is consistent with the substrate specificities of sunflower LACS

observed here, which were low for stearic acid and may therefore restrict stearic acid accumulation in sunflower.

Subcellular localization of HaLACS1 and HaLACS2

The subcellular localization of sunflower LACSs was analysed by microscopy in transiently transformed tobacco BY-2 cells cultured in suspension, a well-characterized system to study protein localization (Brandizzi et al., 2003). Specifically, cells were transformed transiently by biolistic bombardment with HaLACS1 or HaLACS2 fused at their C-termini to green fluorescent protein (GFP), and analysed by confocal laser-scanning microscopy (CLSM). The position of the appended GFP moiety was selected because both proteins are predicted to possess a single membrane-spanning domain at their N-termini, based on the Aramemnon database (<http://aramemnon.uni-koeln.de/>), as seen in their *Arabidopsis* counterparts (Shockey et al., 2002). Hence, both HaLACS1 and HaLACS2 were linked to GFP at their C-termini (rather than at their N-termini) to decrease the potential negative effects on membrane association and/or protein targeting.

Transiently-expressed HaLACS1-GFP displayed a reticular and circular fluorescence pattern that was distinct from that of the co-expressed red fluorescent protein (RFP) in the cytosol, but that partially co-localized with Tic40-RFP (translocon of the inner chloroplast envelope) and concanavalin A (ConA), an endoplasmic (ER) reticulum marker (Fig. 6, rows 1-3). Overall, these results suggest a dual sub-cellular localization of HaLACS1 in plastids and the ER in BY-2 cells. Its localization in plastids is entirely consistent with a role of this enzyme in the immediate activation of free fatty acids released by plastidial acyl-ACP thioesterases, as well as previous localization and proteomic studies of *Arabidopsis* LACS9 in plastid envelopes (Schnurr et al., 2002; Joyard et al., 2010). However, the localization of HaLACS1-GFP in the ER of BY-2 cells is a novel finding, and may reflect the biogenetic and functional relationship between the ER and plastids in membrane trafficking via ER-plastid membrane contact sites (reviewed in Wang and Benning, 2012). Alternatively, the presence of HaLACS1-GFP in the ER may be due to ectopic (over)expression and subsequent mis-sorting from plastids to the ER. However, this would seem unlikely as numerous cells (n>50) were evaluated at various time points after biolistic bombardment to offset any potential

negative effects of (membrane) protein overexpression and in no instances was HaLACS1-GFP exclusively observed in plastids. We are currently investigating the mechanism underlying the apparent trafficking of HaLACS1 to both plastids and the ER.

A parallel series of sub-cellular localization studies with HaLACS2-GFP revealed that this fusion protein, unlike HaLACS1-GFP, was exclusively localized in the ER. Transiently-expressed HaLACS2-GFP did not co-localize with Tic40-RFP (Fig. 6, row 4), but rather it co-localized with ConA (Fig. 6, row 5). These data are consistent with the reported sub-cellular localization of *Arabidopsis* LACS8, which possesses the highest sequence similarity with HaLACS2 (Zhao et al., 2010). *Arabidopsis* LACS8 and LACS9 are expressed at high levels in developing seeds and they were previously thought to be involved in the *de novo* fatty acid synthesis associated with seed oil biogenesis. However, reverse genetics studies have revealed that *Arabidopsis* plants with disrupted *LACS9* display a normal phenotype, suggesting that this enzyme is not essential for oil synthesis. Furthermore, when backcrosses of different *Arabidopsis* LACS mutants were studied, significant decreases in seed TAG synthesis were observed in only one *lacs1/lacs9* double mutant (Zhao et al., 2010). This result was somewhat unexpected as *Arabidopsis* LACS1 was thought to be involved in the synthesis of surface lipids. LACS8 enzyme has no clear role in plant lipid metabolism. However, given its expression in the ER, its ubiquity in different tissues and its substrate specificity towards 16:0 and 18:2, the main fatty acids in sunflower phosphatidylcholine (Cantisán et al., 1999), this enzyme may be involved in the acyl turnover of glycerolipids from the ER.

Conclusions

Activation of fatty acids to form acyl-CoA esters is necessary for plant glycerolipid synthesis. Because *de novo* fatty acid synthesis occurs in the plastids and chloroplast, it has been proposed that plastidial LACS mediate fatty acid export in the chloroplast membrane (Koo et al., 2004). However, reverse genomics approaches have demonstrated that plastidial AtLACS9 is not essential for *Arabidopsis* viability, and that at least one other LACS is involved in the activation of fatty acids synthesized by plastids and in oil accumulation. This finding is compatible with the diffusion of free fatty acids released by acyl-ACP thioesterases and their activation in different cellular

compartments. We cloned two LACS isoforms from developing sunflower seeds with different sub-cellular distributions. The first was homologous to AtLACS9 and it was located in the envelope of the plastids, displaying high activity towards oleic acid and other monounsaturated fatty acids, and apparently contributing to the activation of *de novo*-synthesized fatty acids. The second isoform was homologous to Arabidopsis AtLACS8 and it was located in the ER. This enzyme displayed higher activity towards palmitic and linoleic fatty acids, and its function was not so clear, although it may be involved in the acyl turnover in glycerolipids of the ER. Finally, the low specificity of both enzymes towards stearic acid may be related to the decreased metabolic flux to TAGs observed in high stearic sunflower mutants.

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Tables

Table 1. Sequences of the PCR primers used here.

Primer name	Sequence ^a
LACS-A-F	5'-CGAGACGGAGGTTACTACCG-3'
LACS-B-F	5'-CGACCCTGATCTGTGATTCCAAGC-3'
LACS-D-R	5'-CTCAAGGCAACCGTCTTTATGG-3'
LACS-E-R	5'-GGAGGGACTACTAAGGCAACACAG-3'
HaLACS2-3rac	5'-ACCGGGGACATCGGTAGGTTTC-3'
FA2Z	5'-AACTGGAAGAATTCGCGG-3'
HaLACS1S-F	5'-CGAGACGGAGGTTACTACCG-3'
HaLACS1S-R	5'-TCAAGAAAGATAAACTTTGCC-3'
HaLACS2S-F	5'-GGGACGGAATAGTCCGTTTAAGC-3'
HaLACS2S-R	5'-GGAGGGACTACTAAGGCAACACAG-3'
QHaLACS1-F	5'-GAGTGCATATGTAGTTGGTGTCTTGG-3'
QHaLACS1-R	5'-GTTTCGATCCCGGCCCATGC-3'
QHaLACS2-F	5'-GGATGTTGGGGGATCCTTGTC-3'
QHaLACS2-R	5'-CACCGCCAACCTCAACTGGAAC-3'
QHaActin-F4	5'-GCTAACAGGGAAAAGATGACT-3'
QHaActin-R4	5'-ACTGGCATAAAGAGAAAGCACG-3'
BamHaLACS1-F	5'- GGATCC ATGAGTGCATATGTAGTTG-3'
KpnHaLACS1-R	5'- GGTACCT CAATCAAGAAAGATAAACTTTG-3'
BamHaLACS2-F	5'- GGATCC ATGGATGTTGGGGGATCCTTG-3'
KpnHaLACS2-R	5'- GGTACCT TAAACCATATAGCATTGCAAATC-3'
NheHaLACS1-F	5'- GCTAGC ATGAGTGCATATGTAGTTG-3'
NheHaLACS1-R	5'- GCTAGC AGAAAGATAAACTTTGCCAGC-3'
NheHaLACS2-F	5'- GCTAGC ATGGATGTTGGGGGAT-3'
NheHaLACS2-R	5'- GCTAGC ACCATATAGCATTGTC-3'

^a Restriction sites are indicated in bold.

Figure Legends

Figure 1. Alignment of the *HaLACS1* and *HaLACS2* protein sequences with those of *Arabidopsis* LACS8 and LACS9, and with the corresponding rice homologues: sunflower (*Helianthus annuus* L.; gi|317373791| and gi|317373793|); *Arabidopsis* (*Arabidopsis thaliana*; gi|15224012| and gi|15228136|); rice (*Oryza sativa*; gi|115487538| and gi|115463155|). Residues of the acyl-activating enzyme consensus motif are shown in bold and the putative residues involved in the catalytic centre are highlighted with black boxes. The asterisks indicate identical residues, colons indicate conservative changes and the dots indicate weakly conservative changes.

Figure 2. Phylogenetic comparison of plant long-chain acyl-CoA synthetase homologues of *Arabidopsis thaliana* LACS8 and LACS9 proteins. The phylogenetic tree includes the following plant species: Al, *Arabidopsis lyrata*; At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Cs, *Cucumis sativus*; Fv, *Fragaria vesca*; Gm, *Glycine max*; Ha, *Helianthus annuus*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Rc, *Ricinus communis*; Sl, *Solanum lycopersicum*; Sm, *Selaginella moellendorffii*; Vv, *Vitis vinifera*; Zm, *Zea mays*. The zebrafish (*Danio rerio*) sequence was selected from homologous Vertebrate sequences and used as an outgroup to root the tree. GenInfo identifiers (gi) follow the species names.

Figure 3. Expression of *HaLACS1* (white columns) and *HaLACS2* (light grey columns) genes in vegetative tissues and developing seeds from sunflower determined by qRT-PCR. The data represent mean values $\pm$ SD of three independent samples.

Figure 4. Southern blots of sunflower genomic DNA digested with the indicated restriction enzymes and hybridized with *HaLACS1* (A) and *HaLACS2* (B) gene-specific probes. The position of molecular weight standards (in kb) is indicated on the left.

Figure 5. Substrate specificity of the sunflower LACS *HaLACS1* and *HaLACS2*. The data represent the mean $\pm$ SD of three independent determinations.

Figure 6. Representative CLSM images of GFP-tagged sunflower LACS1 or LACS2 expressed transiently (via biolistic bombardment) in tobacco BY-2 cells. Images in column A show LACS1-GFP (rows 1-3) and LACS2-GFP (rows 4 and 5), while those in column B represent the expression of various sub-cellular marker proteins co-expressed in the same cells, namely cytosolic RFP (row 1) and the inner chloroplast envelope protein Tic40-RFP (rows 2 and 4). Images in column B also show the ER in cells transformed with LACS1-GFP or LACS2-GFP, revealed by staining with fluorescent dye-conjugated concanavalin A (ConA: rows 3 and 5). Corresponding merged images of all the cells are shown in column C. Scale bar = 10 μ m.

Figure 1.

HaLACS1	-----MSAYVVGV LAPLLLTALRNLLKE-KRRGLPADVSGEPGYAIRNYRFTSPVETAWAGI	57
AtLACS9	-----MIPYAAGVIVPLALTLVLQKSKKE-KKRGVVVDVGGEPGYAIRNHRFTEPVSSHWEHI	57
OslACS9	-----MNPYFVGLLVPIAVSLLLRKRRMVGKMRALPVDVGGEPGYAIRNYRFKQPVETHWEGV	58
HaLACS2	MDVGGSLSTFMKDKS----YGAIAVLFAVAVPLVLSMFVGKKV-KQRGVPVEVGGEPGFMNRVSRKLLVDVPWEGA	75
AtLACS8	MEDSGVNPMDSPSKGSDFGVYGIIGGGIVALLVPVLLSVLNGTKKG-KKRGVP IKVGGEGYTMHRAPELVDVPWEGA	80
OslACS8	MSILQRIATSDVPLVKEYGLPGIIGAILLAVVIPIMLSSIFN--KKG-KKRAVQADVGGEGAGLAMNRSRFSMLVEVPWEGA	78
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HaLACS1	ETLADLFEQACKKHGDKNLLGTRKIIISRETEVNQDGRSFEKLHLGLDYEWMTYGVQVYQVVCNFASGLVQIGHKSGERVAIFA	138
AtLACS9	STLPELFEISCNAHSDRVFLGTRKLIISREIETSEDGKTFEKLHLGLDYEWLTFGKTLAEVCDFAASGLVQIGHKTEERVAIFA	138
OslACS9	TTLAELFEQSCDKYVNMPLLGTRKLIISREQESSLDGRSFEKLHLGLDYEWLTKCYAEVFKSVCNFASGLIRLGHQKTDRAIFA	139
HaLACS2	TTMAALFEQSCCKHSQHRFLGTRKLIERSFIDSGGGRKFEKLHLGLDYEWTFQGVFERVCNFASGLIQLGHDPDTRIAIFS	156
AtLACS8	ATMPALFEQSCCKYKSDRLLGTRREFIDKEFITASDGRKFEKLHLGLDYEWQSYGEVFERVCNFASGLVNVGHNVDDRAVIFS	161
OslACS8	TTMAALFEMASKKYPRHRCGLGTRKLIINREFVESPDGRKFEKLHLGLDYEWDTYAEAFSRACNFSGLIKLGHQRDSRAAIFA	159
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HaLACS1	DTREEWFIALQACFRNRVTVTMYASLGEEAICHSLNETEVTTVICGNKELKKLIDISGQIDTVQRVICMDDEVYSSPF-L	218
AtLACS9	DTREEWFISLQGCFRNRVTVTIYSSLGEEALCHSLNETEVTTVICGSKELKKLMDISQQLTVKRVICMDDEFFPS----	214
OslACS9	ETRAEWQIALQACFRQNTVTIYASLGEEALCHSLNETEVTTVVCGQKELKKMIDISGQLDTVKRVIIYINEEGISAIEVL	220
HaLACS2	DTRAEWLIAFEGCFRQNTVTIYASLGDDALHSLNETKVTSLICDSKLLKKVASVSSLETVTNVIYFESDNTTEALS--	235
AtLACS8	DTRAEWFIAPQGCFRQNTVTIYASLGEEALYSLNETRVSTLIDCSKQLKKLSAIQSSLKTKVKNIIYIEEDGVVDASSD	242
OslACS8	DTRTEWIIAAGQCFRQNTVTIYASLGEDALVHSLNETQVSTLIDCSKQLKKLPAISSKLQSVKHVIYIEDEPVEAEVLN	240
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HaLACS1	TDGSSSWKIPFFSEVEEIGRENAVEADLPLPADVAVVMTSGSTGLPKGVMMTHGNVLATVSAVMTIVPGLGNDVYLAYL	299
AtLACS9	-DVNSNWMATSFDTVQKLGRENVPDNPFLSADVAVIMYTSGSTGLPKGVMMTHGNVLATVSAVMTIVPDLGKRDIYMAYL	294
OslACS9	AQKSTSWIEPFEDVGRIGDTPVDANMPLPSDVAVIMYTSGSTGLPKGVMMTHRNVLATLSAVMTIVPEIGKKDVLAYL	301
HaLACS2	--KIGDWQISSFSEVEKMGRRNSPVKARLPKIKKDIIVIMYTSGSTGLPKGVMMTHGNVATAAAVMTIVPIGIGTSDVYMAYL	314
AtLACS8	VNSMGDITVSSISEVEKLGQKNAVQPIPSKNGVAVIMYTSGSTGLPKGVMTIHGNVLATAAGVMKVVPKLDKNDTYIAYL	323
OslACS8	--QMKHWTTFSGFEVEELGKTSHTDARLPSSTDVAVIMYTSGSTGLPKGVMTIHGNMVATTAAVMTIIPKLTGDDVYLAYL	319
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HaLACS1	PLAHILELAAENLIAAVGSSIGYGSPLTLTDTSSKIKRGTKGDASVLRPTLMAAVPAALDRVRDGVRRKKVDSAGGLSKTLF	380
AtLACS9	PLAHILELAAESVMATIGSAIGYGSPLTLTDTSNKIKKGTGKDVLTALKPTIMTAVPAALDRVRDGVRRKKVDAKGGLSKKLF	375
OslACS9	PLAHILELAAEALIAAVGASIGYGSPLTLTDTSNKIKKGTGKDSALKPTLMTAVPAALDRVRDGVRRKKVDTKGGVAKQLF	382
HaLACS2	PLAHIFELAAETVMITAGSSIGYGSALTTLTDTSNKIKKGTMGDATAALKPTLMAVPAALDRVRDGVRRKKVVEEKGGLTKTLF	395
AtLACS8	PLAHVFLEAEIIVFTSGSAIGYGSAMTLTDTSNKVKKGTGKDVSAALKPTIMTAVPAALDRVRDGVRRKKVVEEKGGMATLFL	404
OslACS8	PLAHVFELAAETVMLASGAAIGYGSALTMTDTSNKIKKGTGKDVSAALKPTLMISVPAALDRIRDAVFKKVGEKGGTLTKLFL	400
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HaLACS1	NLAYNRRLSAINGSWLGAWGLEKLLWNLYVFRKVRILGGRIRFILSGGAPLSADTQRFINICFGAPIGOGYGLTETCAGG	461
AtLACS9	DBAYARRLSAINGSWFGAWGLEKLLWDVLVFRKIRAVLGGQIRYLLSGGAPLSGDTQRFINICVGAPIGOGYGLTETCAGG	456
OslACS9	DVAYNRRLAAVNGSWLGAWGLEKLLWDMVLVFKKIRAVLGGQIRFVLSGGAPLSGDTQRFINICLVGPIGOGYGLTETCAGG	463
HaLACS2	NLAYQRRLAVDGSWLGAWGLEKLLWDVIFVKIKQVSLGGKRMFLCGGAPLAADTQRFINICVGAPIGOGYGLTETCAGA	476
AtLACS8	DBAYKRRLAAVNGSWFGAWGLEKMLWDVIFVKIKQVSLGGKRMFLVGGAPLSGDTQRFINICMGSPIGOGYGLTETCAGA	485
OslACS8	DIAVKRNLGAIEGWSWFERMIWNLIFKPIRSMGLGGRIRFVLCGGAPLSGDTQRFINICLVGPIGOGYGLTETCAGA	481
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HaLACS1	TFSEYDDTSVGRVGAPLPCSYIKLINWPEGGYLTSDDPMPRGEIVIGGPNVTLGYPKNEDEKTEVYKVDERGLRWFTGDI	542
AtLACS9	TFSEFEDTSVGRVGAPLPCSFVKLVDAEGGYLTSDDKPMRGEIVIGGSNITLGYPKNEEKTEVYKVDEKGMRFWFTGDI	537
OslACS9	TFSEYDDPSVGRVGAPLPCSYIKLIDWSEGGYLTSDPMPRGEIVIGGPNVTGKYPKNEAKTNEVYKDEKGMRFWFTGDI	544
HaLACS2	AFSEADDNSVGRVGPPPCVYIKLVSWDEGGYLTSDKPMRGEIVVGGYSVTAGYFNNEEKTEVYKVDESGMRWFTGDI	557
AtLACS8	TFSEWDDPAVGRVGPPPCGYVKLVSWEEGGYRISDKPMRGEIVVGGNSVTAGYFNNEEKTEVYKVDEKGTWFTGDI	566
OslACS8	AFSEWDDTSVGRVGPPPCCYVKLVSWEEGGYKISDPMRGEIVVGGYITKGYFNNEAKTNEVYKVDERGMRFWFTGDI	562
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HaLACS1	GQFHKDGCLEIDRKKDIVKLOHGEYVSLGKVEAVLSVSPYVDNIMVYANSFHSYCAIVVASQSALESWALQKGIKVVDV	623
AtLACS9	GRFHDPDGCLEIDRKKDIVKLOHGEYVSLGKVEAALSISPYVENIMVHADSFYSYCVALLVASQHTVEGWASKQGDFAFNF	618
OslACS9	GRFHDPDGCLEIDRKKDIVKLOHGEYVSLGKVEAALIVSPYVENIMIHADPFHSYCVALLVAAHNELENWASQQGVTYTDF	625
HaLACS2	GRFHDPDGCLEIDRKKDIVKLOHGEYVSLGKVEAALASSKYVENVMHADPFHSYCVALLVPPHGVLEQWQAQSGAGISYKDF	638
AtLACS8	GRFHDPDGCLEIDRKKDIVKLOHGEYVSLGKVEAALGSSNYVDNIMVHADPINSYCVALLVPPSRGALEKWAEEAGVKHSEF	647
OslACS8	GQFHPDGCLEIDRKKDIVKLOHGEYVSLGKVESALTTSNVVDSIMVYADPFHNYCVALLVPAHQALEQWQAQNSGINSYKFN	643
	* : * * * * : * * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * : * * *	
HaLACS1	ASLCEMSETVKEVYGSVLVKAAKTGRLEKFEIPAKIKLLPDAPWTPESGLVTAALKLKRDIIKRTFSDELAKFYLS	697
AtLACS9	EELCTKEQAVKEVYASLVKAQKSRLEKFEIPAKIKLLASPWTPEGLVTAALKLKRDVIRREFSEDLTKLYA-	691
OslACS9	VDLCQKPEAVKEVLGSLSKAAQARLEKFEIPAKIKLISEPWTPEGLVTAALKLKREVLKTYEDDLAKLYA-	698
HaLACS2	ADLCKKESVSEVLQSLNKAQDAKLEKFEIPAKIKLMPEPWTPEGLVTAALKLKREQLKAKFKDDLQMLY-	711
AtLACS8	AELCEKEAVKEVQSLTKAGAKAALKEKFEIPAKIKLSEPWTPEGLVTAALKIKREQIKSKFKDELSKLYA-	720
OslACS8	DELCHNDQAKEVQSLSKAAKAARLEKFEIPAKIVLLPEPWTPEGLVTAALKLKREQIKTKFKDDLKLYH-	716
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Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.