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ORIGINAL ARTICLE

Sunflower (*Helianthus annuus*) fatty acid synthase complex: β-hydroxyacyl-[acyl carrier protein] dehydratase genes

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Abstract

Main conclusion **Two sunflower hydroxyacyl-[acyl carrier protein] dehydratases evolved into two different isoenzymes showing distinctive expression levels and kinetics' efficiencies.**

β-Hydroxyacyl-[acyl carrier protein (ACP)]-dehydratase (HAD) is a component of the type II fatty acid synthase complex involved in 'de novo' fatty acid biosynthesis in plants. This complex, formed by four intraplastidial proteins, is responsible for the sequential condensation of two-carbon units, leading to 16- and 18-C acyl-ACP. HAD dehydrates 3-hydroxyacyl-ACP generating trans-2-enoyl-ACP. With the aim of a further understanding of fatty acid biosynthesis in sunflower (*Helianthus annuus*) seeds, two β-hydroxyacyl-[ACP] dehydratase genes have been cloned from developing seeds, *HaHAD1* (GenBank HM044767) and *HaHAD2* (GenBank GU595454). Genomic DNA gel blot analyses suggest that both are single copy genes. Differences in their expression patterns across plant tissues were detected. Higher levels of *HaHAD2* in the initial stages of seed development inferred its key role in seed storage fatty acid synthesis. That *HaHAD1* expression levels remained constant across most tissues suggest a housekeeping function. Heterologous expression of these genes in *E. coli* confirmed both proteins were functional and able to interact with the bacterial complex 'in vivo'. The large increase of saturated fatty acids in cells expressing *HaHAD1* and *HaHAD2* supports the idea that these *HAD* genes are closely related to the *E. coli FabZ* gene. The proposed three-dimensional models of *Ha*HAD1 and *Ha*HAD2 revealed differences at the entrance to the catalytic tunnel attributable to Phe166/Val1159, respectively. *Ha*HAD1 F166V was generated to study the function of this residue. The 'in vitro' enzymatic characterization of the three HAD proteins demonstrated all were active, with the mutant having intermediate K_m and V_{max} values to the wild-type proteins.

Keywords Fatty acid synthase (FAS) · β-Hydroxyacyl-[ACP] dehydratase (HAD) · Oil biosynthesis · Substrate specificity · Sunflower

Abbreviations

ACP	Acyl carrier protein
DAF	Days after flowering
DAG	Days after germination
ENR	Enoyl-ACP reductase
FAS	Fatty acid synthase
HAD	β-Hydroxyacyl-ACP dehydratase

Introduction

The first steps of 'de novo' fatty acid biosynthesis in plants are localized in plastids and mitochondria, where the multienzymatic complexes: acetyl-CoA carboxylase

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(ACCase) and fatty acid synthase (FAS) work co-ordinately (Yasuno et al. 2004; Harwood 2005). Plant and bacteria FAS complexes differ from the Type I complex in mammals which is coded for by a single gene. A heterodimer of the latter's resulting polypeptide synthesizes fatty acids (Smith et al. 2003). In plant plastids and bacteria, the type II FAS complex, generating mostly up to 16- or 18-carbon fatty acids, is an easily dissociable, multisubunit complex consisting of monofunctional enzymes each encoded by a discrete gene (Høj and Mikkelsen 1982; Shimakata and Stumpf 1982a). This complex is formed by four enzymes a β-ketoacyl-[ACP]-synthase (KAS I, II or III), a β-ketoacyl-[ACP]-reductase (KAR), a β-hydroxyacyl-[ACP]-dehydratase (HAD) and an enoyl-[ACP]-reductase (ENR). The enzyme carrying out the third step of each cycle of fatty acid synthesis, HAD, is responsible for converting β-hydroxyacyl-ACP to trans-2-enoyl-ACP, by releasing a water molecule.

Two genes coding for HAD have been identified in Arabidopsis, At2g22230 and At5g10160. Microarray assays showed both genes are highly expressed during lipid biosynthesis in seed development (Schmid et al. 2005). Plant HAD enzymes have been previously purified from other plant tissues such as spinach leaves and safflower seeds (Shimakata and Stumpf 1982a, b), and several putative genes have been identified in public databases but, up to now, only one of the Arabidopsis *HAD* genes (At2g22230) has been heterologous expressed in *Escherichia coli*, purified and its activity demonstrated by mass spectrometry (Brown et al. 2009).

Some bacteria such as *Escherichia coli*, on the other hand, contain two β-hydroxyacyl-[ACP]-dehydratase enzymes: FabA is required for unsaturated fatty acid synthesis and has a dual dehydratase/isomerase function acting primarily on C10 substrates (Heath and Rock 1996), and FabZ with only a dehydratase function that is the principal enzyme involved in fatty acid elongation from C4 to C18 (Mohan et al. 1994). The sequences of FabA and FabZ are remarkably similar, but can be distinguished by an active site Asp in FabA versus a Glu in FabZ (White et al. 2005). Li et al. (2009) have cloned and analysed different *Arachis hypogea* FAS genes, among them the β-hydroxyacyl-[ACP]-dehydratase. The deduced protein sequence showed similarities with the bacteria *E. coli* β-hydroxyacyl-[ACP] dehydratase, *Ec*FabZ, maintaining a high level of conservation in the catalytic residues. Such comparisons suggest that *A. hypogea* HAD has an analogous function to *Ec*FabZ. Similar analyses and conclusions were reached for the Arabidopsis HAD proteins (Brown et al. 2009).

The *Pseudomonas aeruginosa*, *Plasmodium falciparum* and *Helicobacter pylori* FabZ dehydratase structures have been intensely studied (Kimber et al. 2004; Zhang et al. 2008; Maity et al. 2011). Contrariwise, a plant HAD protein remains to be crystallized. The FabZ enzyme belongs to the hot-dog protein family, whose monomer structure consists of six β-pleated sheets surrounding a long α-helix, resulting in an L shape. Initially, an antiparallel dimeric structure is required that is formed by the interaction of the β3-sheet of one monomer with the last two turns of the α3-helix of a second monomer. The dimers then form hexamers. The catalytic residues, histidine and glutamic acid are located at the dimeric interface (White et al. 2005), specifically in the long, narrow hydrophobic tunnel formed upon dimerization. In *P. aeruginosa* FabZ a highly mobile tyrosine residue, near to the ACP binding domain, blocks the entrance of the catalytic tunnel. When this residue rotates 120° the tunnel opens allowing entrance of the substrate, which is then susceptible to dehydration (Kimber et al. 2004).

Mass spectrophotometric analyses of *At*HAD demonstrated the ability of the enzyme to use either crotonyl-ACP or 3-hydroxybutyryl-ACP as substrates (Brown et al. 2009). Two other plant HADs, from spinach and safflower, have been biochemically characterized in plant extracts. Their enzymatic activities, monitored spectrophotometrically using crotonyl-ACP as substrate, showed very similar K_m values of 9.7 and 9.0 μM, respectively. The reversible reaction was favored in the 'in vitro' assays (Shimakata and Stumpf 1982a, b) as in bacteria, such as *P. falciparum*. Only when the next enzyme in the FAS cycle, ENR, is included in the experiment, did the direction of the reaction change to that observed 'in vivo' (Sharma et al. 2003). In some bacteria and protozoa species, including *P. falciparum*, *H. pylori* and *Toxoplasma gondii*, the readily available substrate crotonyl-CoA has been used instead of crotonyl-ACP for characterization of β-hydroxyacyl-[ACP]-dehydratase activity (Sharma et al. 2003; Liu et al. 2005; Dautu et al. 2008).

In the present study we have characterized two different *Helianthus annuus* (sunflower) *HAD* genes and their products with the goal of optimizing the total lipid content in developing seeds. The predicted structural models of these proteins showed some interesting differences between both isoforms. A mutant affecting the opening of the active site pocket in HAD1 was generated to study the effect of the residue occluding the entrance to the FabZ active site tunnel as the homologous residue in the sunflower *HAD* genes differ. The three proteins, heterologously expressed in *E. coli* were functional and their kinetic parameters, determined in vitro, question whether this residue has an analogous function in the sunflower enzymes.

Materials and methods

Plant material

Sunflower (*Helianthus annuus* L.) wild-type line CAS-6 (Sunflower Collection of Instituto de la Grasa, CSIC, Seville, Spain) was grown and materials collected as described by González-Thuillier et al. (2015).

Cloning of cDNAs encoding sunflower HAD

Approximately 0.1 g of each tissue was ground in liquid N_2 with precooled sterile mortar and pestle. Total RNA was extracted using a Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA). To eliminate residual DNA from total RNA, a DNA-free kit (Ambion, Austin, TX, USA) was used, and mRNA was isolated from total RNA (1 μg) using the GenElute mRNA Miniprep kit (Sigma-Aldrich). The corresponding cDNA was synthesized using a Ready-To-Go T-Primed First Strand Kit (GE Healthcare Life Science, Little Chalfont, UK) from DNA-free mRNA.

The *A. thaliana* HAD protein sequence encoded by the At2g22230 gene was used to search sunflower expressed sequence tags (ESTs) publicly available in the NCBI database (http://www.ncbi.nlm.nih.gov/) to find putative mRNAs encoding HAD homologs using the TBLASTN algorithm (Altschul et al. 1997). ESTs corresponding to internal sequences of two genes were identified. These ESTs were aligned using the ClustalX v.2.0.10 program (Larkin et al. 2007) to group those with similar or identical sequences. Polymerase chain reaction (PCR) fragments were amplified with two pairs of primers designed from these groups, F-HAD1/R-HAD1 and F-HAD2/R-HAD2 (Table 1; all the primers were synthesized by Eurofins MWG Operon, Ebersberg, Germany). The 5′-ends were obtained using the Smart^{TM}-RACE cDNA amplification kit (Clontech, Mountain View, CA USA) and specific reverse internal oligonucleotides pairs for each cloned sequence: R-HAD1.2, R-HAD1.3 and R-HAD1.4; and R-HAD2.2, R-HAD2.3 and R-HAD2.4 (Table 1). The 3′-end of the cDNAs was obtained by PCR using the external oligo FA2Z (Table 1), complementary to the sequences incorporated during the initial cDNA synthesis, and specific internal oligos for each cloned sequence: F-HAD1.2 and F-HAD2.2 (Table 1). Once the ends of the two genes were identified the complete sequences were amplified with the specific pair of primers F-atg_HAD1/R-stop_HAD1 and F-atg_HAD2/R-stop_HAD2 for *HaHAD1* and *HaHAD2*, respectively. The PCR fragments were cloned into the pMBL-T vector (Dominon, North Kingstown, RI, USA), transformed into XL1-Blue, and several clones were sequenced on both strands by Secugen SL (Madrid, SP). The identity of the clones was confirmed using the BLASTX algorithm (Altschul et al. 1997), distinguishing two different gene types coding for HAD isoforms, *HaHAD1* and *HaHAD2* (684 base pairs (bp) and 666 bp, respectively). These cDNA sequences were deposited in GENBANK under Accession numbers HM044767 |gi:302634223| and GU595454 |gi:291480628|, respectively.

cDNA and protein sequence analyses

Protein sequences homologous to the predicted sequences of *Ha*HAD1 and *Ha*HAD2 were identified using the BLASTP algorithm (Altschul et al. 1997). Alignment of the amino acid sequences, including the transit peptides, for plastid HAD proteins publicly available at GENBANK was performed using the ClustalX v.2.0.10 program with the default settings (Larkin et al. 2007). These entire alignments were used to generate a phylogenetic tree based on the neighbour-joining algorithm (Saitou and Nei 1987), and the resulting 'phenogram' was drawn using the MEGA program 4.0.2 (Tamura et al. 2007). Transit peptides were identified through alignment with known plastid HAD sequences and using the network-based program TargetP V1.1 (Emanuelsson et al. 2007).

Modelling of the three-dimensional structures of HaHAD1 and HaHAD2

Homology modelling studies were performed as detailed in González-Mellado et al. (2010). The structure of the *P. falciparum* β-hydroxyacyl-ACP dehydratase, *FabZ*, sequence with accession number UniProtKB Q965D7 (Kostrewa et al. 2005) was used as a model for both sunflower sequences, being the most homologous HAD for which X-ray structure information was available in the database RCSB PDB (Berman et al. 2000) (PDB Entry: 1Z6B).

'In vitro' site-directed mutagenesis

To induce a transversional substitution, T→G, at position 496 in the *HaHAD1* gene, the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) was used together with (a) the primers named t496 g_antisense and t496g (Table 1), designed via the Agilent Technologies web page (www.agilent.com/genomics/qcpd), (b) the Kit's High Fidelity DNA polymerase and (c) the *HaHAD1* gene cloned into pQE80L. Following the manufacturer's protocol, PCR products were transformed into XL1-Blue supercompetent cells and several of the resulting clones subjected to sequencing to

Table 1 Sequence of PCR primers used in this work

Primer name	Sequence[a]
F-HAD1	5′-CAATTGAACTCCGGTTTCCTGC-3′
R-HAD1	5′-AAGTCAAAATGGTCCTGTTTGGAGG-3′
F-HAD1.2	5′-GGTGTGCTCATGGTTGAGGCAATGGCA-3′
R-HAD1.2	5′-AAGTCAAAATGGTCCTGTTTGGAGG-3′
R-HAD1.3	5′-GCTGGCCGCAATATCACTCACTATTACC-3′
R-HAD1.4	5′-TCACCGGCTTTCTAAACCTCACCTTG-3′
F-HAD1SacI	5′-A**GAGCT**CTGCTCTTCACCTGCAAGTGA-3′
R-HAD1XmaI	5′-T**CCCGGG**TCACTCACTATTACCCATAG-3′
F-HAD1qpcr	5′-ATCTCTCTGCTCATTCAACCC-3′
R-HAD1qpcr	5′-TTGGGGTTTCATCTTTTGAGC-3′
F-atg_HAD1	5′-ATGTCTTCCAACACTTTCTCT-3′
R-stop_HAD1	5′-CTATGGGTAATAGTGAGTGA-3′
F-HAD2	5′-TCCGCGATTGATACCATCTCAAC-3′
R-HAD2	5′-TCACAAACGACCTCGCCTCC-3′
F-HAD2.2	5′-GGTGGGTCCCGTGAAAATTTCGTCT-3′
R-HAD2.2	5′-TCACAAACGACCTCGCCTCC-3′
R-HAD2.3	5′-ACTTCTGGTTGCAGCATTACCACACC-3′
R-HAD2.4	5′-TGCCATTGCCTCAACCATAAGAACACC-3′
F-HAD2BamHI	5′-A**GGATCC**TCATCCGCGATTGATACCATC-3′
R-HAD2PstI	5′-CTG**CTGCAG**TTAGTTTACTGTGTTACCCA-3′
F-HAD2qpcr	5′-ACCCCCTCAATTAATCAACTC-3′
R-HAD2qpcr	5′-TCAGTTGAGATGGTATCAATCG-3′
F-atg_HAD2	5′-ATGGCGTCTTCATCTCTCACTC-3′
R-stop_HAD2	5′-TTAGTTTACTGTGTTACCCATAGC-3′
t496 g_antisense	5′-AATTCCGGCGAAGACGAAATTGTCACGTGACCCACC-3′
t496g	5′-GGTGGGTCACGTGACAATTTCGTCTTCGCCGGAATT-3′
QHaActin-F4	5′-GCTAACAGGGAAAAGATGACT-3′
QHaActin-R4	5′-ACTGGCATAAAGAGAAAGCACG-3′
FA2Z	5′-AACTGGAAGAATTCGCGG-3′

[a] Restriction sites are indicated in bold

confirm the desired mutation, that is, the mutant gene, *HaHAD1* F166V.

Genomic DNA Southern blot analysis

Genomic DNA from 2.5 g of sunflower leaves was isolated as described previously (González-Mellado et al. 2010). DNA samples were digested with different restriction enzymes (all from New England Biolabs, Hitchin, UK) and electrophoresed in a 0.8 % agarose gel. The gel was soaked in 250 mM HCl for 30 min, then washed three times in distilled water and finally blotted onto a Hybond-N + transfer membrane (GE Healthcare). The filter was probed with [α-^{32}P]dCTP-labelled *HaHAD1* and *HaHAD2* gene-specific DNA probes, 684 bp and 666 bp, respectively, obtained by PCR amplification with the following pairs of primers: F-atg_HAD1/R-stop_HAD1 for *HaHAD1* and F-atg_HAD2/R-stop_HAD2 for *HaHAD2* (Table 1). Hybridization was performed as detailed previously (González-Thuillier et al. 2015). Images of radioactive filters were obtained as described by González-Mellado et al. (2010).

Real time quantitative PCR (RT-qPCR)

The cDNAs obtained from developing seeds and different vegetative tissues as described in González-Thuillier et al. (2015) were subjected to RT-qPCR with the following gene-specific pair of primers: F-HAD1qpcr/R-HAD1qpcr for *HaHAD1* and F-HAD2qpcr/R-HAD2qpcr for *HaHAD2* (Table 1) using SYBR Green (QuantiteTect™ SYBR® Green PCR Kit, Qiagen, Hilden, DE) in an MiniOpticon system (Bio-Rad, Hercules, CA, USA). The reaction was run, a calibration curve constructed and the sunflower actin

gene *HaACT1* (GenBank Accession number FJ487620) used as the internal reference as detailed previously (González-Thuillier et al. 2015). Three biological and two technical replicates were carried out on each sample.

Expression of sunflower recombinant HAD proteins in *E. coli*

The expression vector pQE-80L contains an N-terminal 6× histidine affinity tag to facilitate protein purification. The genes *HaHAD1* and *HaHAD2* were amplified using the primer pairs F-HAD1SacI/R-HAD1XmaI and F-HAD2BamHI/R-HAD2PstI (Table 1), respectively. The PCR product, corresponding to each gene missing the signal peptide contained the desired restriction sites at each end, *Sac*I/*Xma*I (*HaHAD1*) and *Bam*HI/*Pst*I (*HaHAD2*) for directional cloning into pQE80L after restriction with such enzymes. Constructions pQE-80L::*HaHAD1* and pQE-80L::*HaHAD2* were transformed into competent *E. coli* XL1-Blue strain (Stratagene, La Jolla, CA, USA), respectively.

E. coli cells harboring recombinant plasmids were grown under continuous shaking at 37 °C in SB medium (2.5 % tryptone, 1.5 % yeast extract, 0.5 % NaCl, pH 7.5) containing ampicillin (100 μg ml^{-1}). Heterologous expression of these two sunflower genes, as well as the mutant *Ha*HAD1 F166V, was induced at OD 0.8 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown for an additional 2 h. The cells were collected by centrifugation at 4000*g* for 15 min. The cell pellets were resuspended in 10 ml of 50 mM Tris (pH 8.0), 1 mg ml^{-1} lysozyme, 0.1 mg ml^{-1} DNAse, 5 mM DTT and lysed by sonication at 0 °C using a Branson sonicator (Model SLPe; Thomas Scientific, Swedesboro, NJ, USA), applying 10 pulses of 20 s. The lysed bacterial suspension was centrifuged for 40 min at 20,000*g*. The protein was purified from the resulting supernatant by immobilized metal affinity chromatography (IMAC) and submitted to kinetic characterization as previously described (Moreno-Perez et al. 2011). Protein concentration was measured using a BCA Protein Assay Kit Pierce from Thermo Scientific (Waltham, MA, USA).

Protein gel electrophoresis

Recombinant proteins were submitted to SDS–polyacrylamide gel electrophoresis (PAGE) and the gels stained with Coomassie blue as detailed previously (González-Thuillier et al. 2015).

Growth-rate and fatty acids analysis in *E. coli*

50 ml cultures of *E. coli* carrying either pQE80L::*HaHAD1* or pQE80L::*HaHAD2* were grown for the sunflower proteins' expression. The OD of the bacterial culture (three replicates per construct) was measured in a spectrophotometer Ultrospec 3300pro (GE Healthcare) at 30 min intervals, as an index of increasing cellular content. Based on this information, the specific growth rates were calculated from the plots generated from the increase in log OD_{600} with time using the mean of three independent experiments to calculate each value in the growth curves.

Culture cells were harvested by centrifugation (15 min at 3200*g*), washed and resuspended in water (1/10 from the initial volume) for fatty acid methyl ester analysis. 150 μg of heptadecanoic acid was added to the samples to serve as an internal standard for quantification. Extraction of the lipids, preparation of the methyl esters and their analysis by gas chromatography is described in González-Mellado et al. (2010). Statistical significance of the results was estimated using Student's t test (SigmaPlot 8.0, SPSS, Chicago, IL, USA), with $P < 0.05$ considered significant.

In vitro characterization of *Ha*HAD1, *Ha*HAD2 and *Ha*HAD1 F166V

To study their activity, the reverse reaction of these enzymes, which is favored in vitro, dehydration was measured using crotonyl-CoA (Sigma-Aldrich) as substrate and monitoring the decrease in absorbance at 260 nm, similar to the procedure described in Shimakata and Stumpf (1982b) and Dautu et al. (2008) with some modifications. The purified proteins *Ha*HAD1, *Ha*HAD2 or *Ha*HAD1 F166V were added at 5.0 μg ml^{-1} to reaction buffer (17.9 mM potassium phosphate, 1.6 mM DTT pH 6.8) to give a final volume of 600 μl. The reaction was started by adding the crotonyl-CoA substrate at different concentrations between 20 and 100 μM. The background at 260 nm, decreasing due either to substrate instability or to the *E. coli* extract's endogenous activity was also monitored using purified protein extract from induced cultures containing empty expression vector. This background activity was subtracted from sample values. The reaction was carried out at 25 °C and monitored each minute using the spectrophotometer Ultrospec 3300pro (GE Healthcare) and the software Datrys Life Science (2.2.0.0 Version). The extinction coefficient of crotonyl-CoA $\varepsilon_{260} = 22.6 \times 10^3$ M^{-1} cm^{-1} (Dautu et al. 2008; Shimakata and Stumpf 1982b) was used to convert absorbance measurements to concentration of β-hydroxyacyl-CoA produced from crotonyl-CoA. For each enzymatic assay three independent experiments were done with each of the three purified proteins. Kinetic parameter analysis was carried out as described in González-Thuillier et al. (2015).

Results

Cloning and sequence analysis of two sunflower β-hydroxyacyl-[ACP]-dehydratase cDNAs

Two groups of ESTs for plastid HAD enzymes in sunflower were identified on the basis of their homology to the Arabidopsis *HAD* gene (At2g22230). Two cDNAs, consisting of 684 bp for *HaHAD1* and 666 bp for *HaHAD2*, were amplified from 15 days after flowering (DAF) developing seed mRNA (see Materials and methods). The amino acid sequences, deduced from the cDNA of *HaHAD1* and *HaHAD2* genes using bioinformatics tools, consisted of 227 and 221 residues, respectively. Through alignment with known HAD sequences and using the network-based method TargetP V1.1 to identify chloroplast transit peptides (Emanuelsson et al. 2007), Asn56 and Ala51 of the sunflower HAD1 and HAD2 sequences, respectively, were the best candidates for the N-termini of the mature proteins (Fig. 1). Both transit peptides contained phosphorylation motifs specific for binding the 14-3-3 protein that together with HSP70 can form a cytosolic guidance complex towards the chloroplast (May and Soll 2000) (Fig. 1). These motives were predicted between the Pro35 and Ser42 in *Ha*HAD1 and between the Pro33 and Ser39 in *Ha*HAD2. In the case of *Ha*HAD1 proteolytic processing of the transit peptide would produce a 171 residues protein with a putative molecular mass of 18.9 kDa and a pI of 5.74, while *Ha*HAD2 would have 170 residues with a mass of 18.6 kDa and a pI of 5.67. These two sunflower amino acid sequences with 75 % identity were compared with homologous proteins from different phylogenetic groups such as *A. thaliana*, *Oryza sativa*, *A. hypogea* or *P. aeruginosa* (Fig. 1).

This alignment shows, excluding the transit peptide, which is the less conserved region, identical or highly conserved domains between the HADs. The prediction of the structures and the location of catalytic residues in both sunflower proteins was performed using the SWISS-MODEL server (http://swissmodel.expasy.org/) and the well-studied *P. aeruginosa* FabZ structure (Kimber et al. 2004). The comparison suggests that the residues involved in recognition and binding of ACP are Arg174, Arg176 and Gln195 in *Ha*HAD1 and Arg167, Arg169 and Gln188 in *Ha*HAD2. The predicted catalytic residues of *Ha*HAD1 are His129 and Glu143 and in *Ha*HAD2 are His122 and Glu136. While the arginine is highly conserved in different kingdoms such as plant, bacteria, and protozoa, the glutamine residue is plant exclusive. Kimber and collaborators (2004) propose that the catalytic residues localize to the

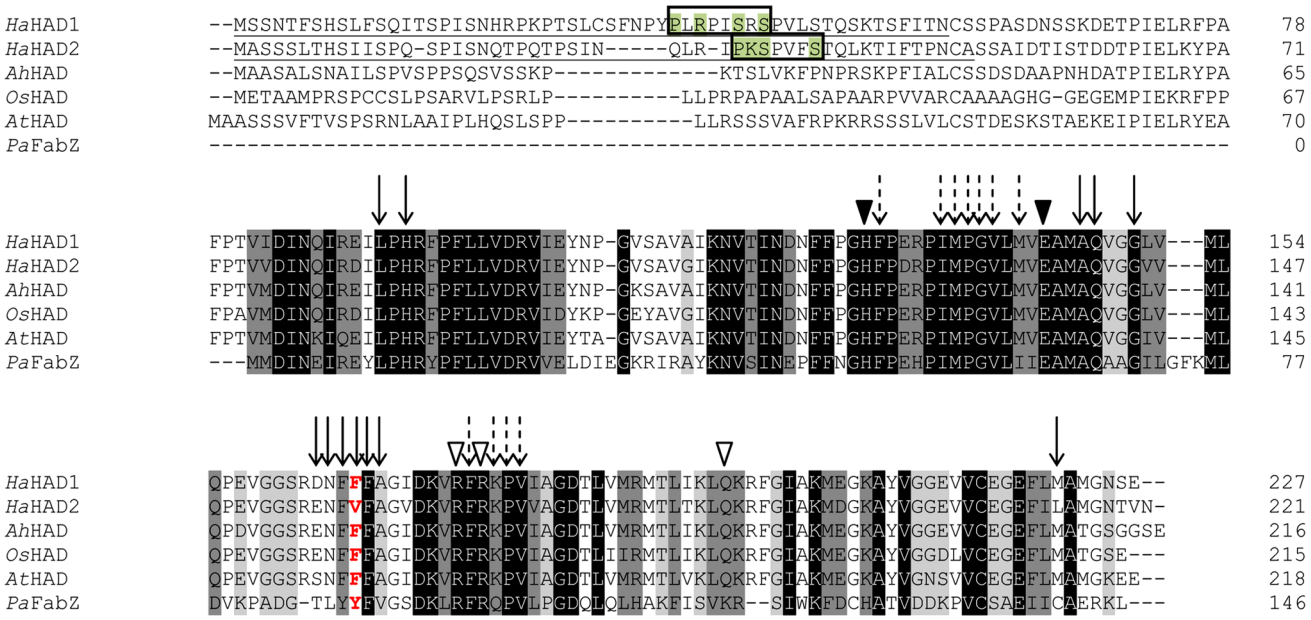

Fig. 1 Alignment of the deduced amino acid sequences of *H. annuus* β-hydroxyacyl-[ACP]-dehydratase *Ha*HAD1 and *Ha*HAD2, with the closely related sequences from *A. hypogea*, *Ah*HAD (gil268527763|), *A. thaliana*, AtHAD1 (gil18399910|), *Oryza sativa*, OsHAD (gil 115464091|) and *Pseudomonas aeruginosa*, *Pa*FabZ (gil674745580|). Identical residues are highlighted as *black boxes*, highly conserved residues as *dark grey boxes* and weakly conserved residues as *light grey boxes*. The active sites are indicated with *black triangles* and those residues participating in the ACP binding site are indicated with *white triangles*. *Bold red letters* indicate the residue involved in tunnel opening regulation, Tyr88 in *P. aeruginosa*. *Arrows* show residues located at the catalytic tunnel, from the first (*solid*) and second (*dash*) monomer of the homodimer. The putative N-terminal plastid transit peptides of *Ha*HAD1 and *Ha*HAD2, are underlined, 56 and 51 amino acids, respectively. The putative phosphorylation motives are highlighted in green and enclosed in a rectangle

hydrophobic tunnel spanning both monomers which has been identified in the FabZ crystal structure from microbes. For *Ha*HAD1 these residues for the first monomer are Leu92, His94, Ala146, Gln147, Gly150, Asp163, Asn164, Phe165, Phe166, Phe167, Ala168 and Met221, and for the second monomer Phe130, Ile135, Met136, Pro137, Gly138, Val139, Met141, Phe175, Lys177, Pro178 and Val179. In the case of *Ha*HAD2, the equivalents are Leu85, His87, Ala139, Gln140, Gly143, Glu156, Asn157, Phe158, Val159, Phe160, Ala161 plus Leu214, and Phe123, Ile128, Met129, Pro130, Gly131, Val132, Met134, Phe168, Lys170, Pro171 plus Val172. The tunnel residues in sunflower *Ha*HAD1 and *Ha*HAD2 are identical except for Asp163 to Glu156, Phe166 to Val159 and Met221 to Leu214. The homologues of Tyr88 in *P. aeruginosa* FabZ are Phe166 in *Ha*HAD1 and Val159 in *Ha*HAD2.

Whole sequenced genome availability of different organisms from many phylogenetic groups together with bioinformatic tools permits identification of groups potentially sharing a common ancestor with HAD from plants (Fig. S1). As the dendrogram shows both sunflower HADs emerge relatively close in the phylogeny. These protein sequences have ≥81 % identity with their plant homologues, such as *Vitis vinifera*, *A. thaliana* or *A. hypogea*. The dendrogram (Fig. S1) also reveals that plant HADs are relatively close to their Chlorophyta and Cyanobacteria homologues.

Tertiary structure prediction of *Ha*HAD proteins

The amino acid sequences of *Ha*HAD1 and *Ha*HAD2 were compared to the known structure of the protozooan protein from *P. falciparum* FabZ (UniProtKB Q965D7) (Berman et al. 2000). The 47 % sequence identity, of the sunflower proteins, to that of *Pf*FabZ (Fig. 2) infers that the structure of the latter could be used as model for the sunflower proteins. The distribution of the deduced α helices and β sheets of *Ha*HAD1 and *Ha*HAD2 are very similar to that of the model (Fig. 2). This suggests that the sunflower enzymes will also display an "L" shape formed by three α helices (α1-α3) surrounded by six β sheets (β1–β6) that has previously been described in other fatty acid synthesis related proteins, such as acyl-ACP thioesterases (Mayer and Shanklin 2005; Serrano-Vega et al. 2005). The deduced secondary structures of sunflower HADs differ slightly from the *Pf*FabZ structure, as β3 and β6 are longer in the former proteins.

The sunflower HAD 3D structures generated by the program Swiss-PdbViewer show very similar assemblies. A minor difference is shown when the monomers of the sunflower proteins are represented; an extra residue in the loop between α3 and β3 in *Ha*HAD1 compared to *Ha*HAD2 (Fig. 3). When the molecular surfaces of the homodimers were represented, however, more differences between the two deduced protein structures occur (Fig. 3c, d, e, f). In *Ha*HAD1, as in most of the available plant HAD sequences, the tunnel is completely closed by phenylalanine 166 whereas in *Ha*HAD2 with valine 159 in the analogous position the tunnel is always open. Valine is smaller and lacks the aromatic ring present in phenylalanine acting as a "lid" (Fig. 3c d, e, f).

Southern blot analysis of *HaHAD1* and *HaHAD2*

Two DNA gel blot analyses were performed on genomic DNA extracted from sunflower leaves to evaluate the number of copies of *HaHAD1* and *HaHAD2* in the genome. An aliquot of the gDNA was digested with restriction enzymes having one (*Ale*I) or no (*Hin*dIII) cutting sites within the cDNA of *Ha*HAD1. Figure 4a shows a single band obtained with *Hin*dIII, indicating that *HaHAD1* is a single copy gene. With *Ale*I three bands (circa 1.5, 8 and 10 kb) are seen instead of the expected two. To understand these results a new search in the TAIR database (http://www.arabidopsis.org/index.jsp) was carried out revealing that *AtHAD* (At2g22230) has three introns within the gene sequence. Nevertheless, given the length of the gene (smaller than 1.5 kb) and the band sizes obtained, an *Ale*I restriction site was not expected in the introns. Incomplete digestions of the gDNA could explain the release of three fragments containing sequences of the gene, however. Therefore, assuming an *Ale*I restriction site localized upstream or downstream of the gene, complete digestion would generate, 1.5 and 8 kb bands, but an incomplete digestion a circa 10 kb fragment. This hypothesis supports the idea of a single copy of *HaHAD1gene* in the sunflower genome. Similarly, the number of copies of *HaHAD2* gene in the genome was studied. An aliquot of genomic DNA was digested with *Ale*I, having one target site within the *HaHAD2* cDNA, and others lacking targets sites in the cDNA (*Sph*I, *Xba*I, *Sac*I and *Nco*I,). Only one band was detected when a target site was lacking and two bands when a target site was present, inferring that *HaHAD2* is also a single copy gene (Fig. 4b). This concurs with the observation that a blast search of available *H. annuus* ESTs performed with the *HaHAD1* and *HaHAD2* sequences retrieved only nucleotide fragments corresponding to the latter.

Tissue expression profiles of sunflower β-hydroxyacyl-[ACP]-dehydratases

The expression of the *HaHAD1* and *HaHAD2* genes was analysed by RT-qPCR in roots, stems, 1–7 days after germination (DAG) cotyledons, leaves of 20-day-old seedlings and 12–30 DAF seeds in different developmental

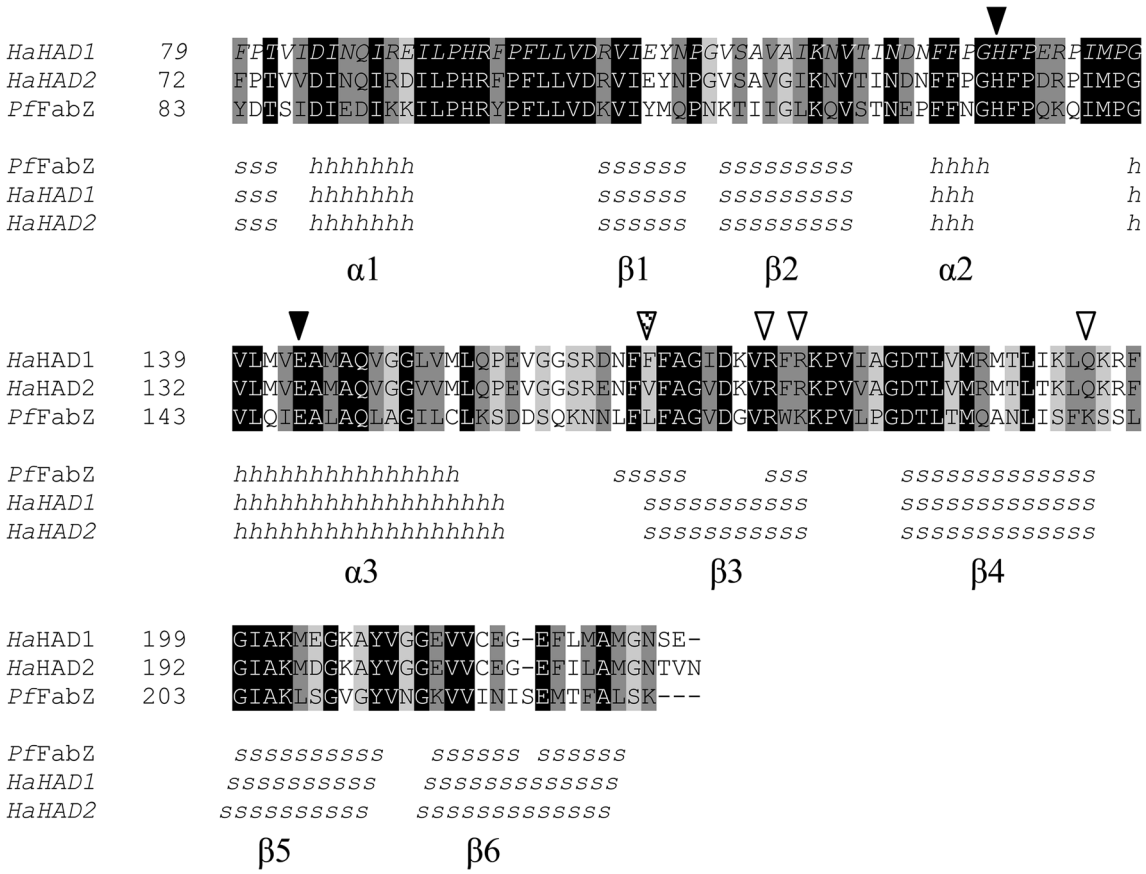

Fig. 2 Comparison between the deduced amino acid sequences of *H. annuus* β-hydroxyacyl-[ACP]-dehydratase, *Ha*HAD1 and *Ha*HAD2, and *P. falciparum*, *Pf*FabZ (Kostrewa et al. 2005). Identical residues are highlighted as *black boxes*, highly conserved residues as *dark grey boxes* and weakly conserved residues as *light grey boxes*. Residues in the active sites (*black triangles*), ACP binding residues (*white triangles*) and residues involved in the tunnel opening (*black dotted triangle*) are also shown. *Pf*FabZ structural elements are named as in Kimber et al. (2004); *h* α-helix, *s* β-sheet

stages (Fig. 5). Both sunflower genes showed the highest levels of expression in seeds, particularly *HaHAD2*, as has been described previously for *AtHAD* [At2g22230; Schmid et al. 2005)]. *HaHAD1* and *HaHAD2* presented different expression patterns. *HaHAD2* showed the highest level of expression in 12DAF seeds, decreasing gradually as the seed developed similar to the profile found for *AtHAD* (Schmid et al. 2005). *HaHAD1*, by contrast, showed a constant level of expression during seed development. *HaHAD2* was much more highly expressed than *HaHAD1* in 12 and 20 DAF seeds, whereas *HaHAD1* exhibited slightly more expression in the rest of tissues compared to *HaHAD2*, especially in the roots where only traces of expression were detected.

Fatty acid modifications in *E. coli* cells expressing recombinant *HaHAD1* and *HaHAD2*

The constructions pQE80L::*HaHAD1* and pQE80L::*HaHAD2*, missing the deduced plastid targeting sequences, respectively, were transformed into XL1-Blue cells. The growth rate of the cells expressing *HaHAD*1, *HaHAD*2 or the empty vector as background control was monitored at 37 °C as explained in "Materials and methods". Lower growth rates were observed when expression of either *HaHAD*1 or *HaHAD*2 was induced compared to the control. The cells expressing the first gene showed half of the growth rate compared to the cells without induction or the control (0.33/0.74/0.76 h^{-1}, respectively). A similar decrease, albeit of a lower magnitude (0.57/0.8/0.76 h^{-1}, respectively) was found when the second gene was induced. Induced expression of both genes resulted in cessation of growth after 2–3 h.

A small increase, but non-statistically significant, in the bacteria fatty acid content occured when the *HaHAD*1 or *HaHAD*2 gene was present, but was not induced. Induction resulted in further increases, especially significant for *Ha*HAD1. This accumulation was accompanied by modification of the fatty acid profiles (Table 2). Namely, expression resulted in a circa 2-fold increase of the saturated:unsaturated fatty acids which was accompanied by a circa 33–50 % reduction in the C16:0/C18:0 ratio. The C16:1/C18:1 ratios by comparison showed little change.

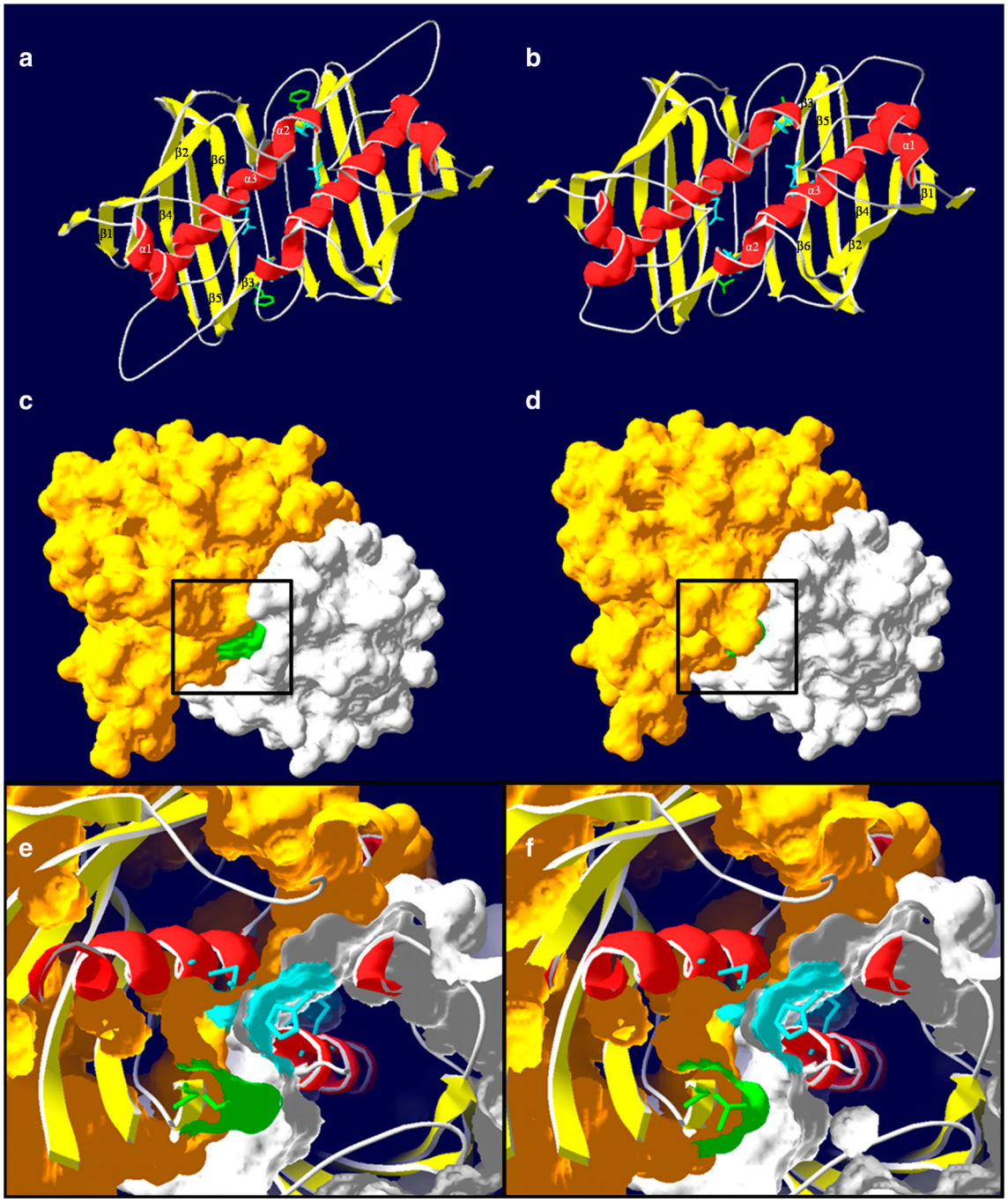

Fig. 3 Proposed structural models for sunflower β-hydroxyacyl-[ACP]-dehydratase homodimers, *Ha*HAD1 (**a**, **c**, **e**) and *Ha*HAD2 (**b**, **d**, **f**), modelled from that known for *Pf*FabZ (1z6b) (Kostrewa et al. 2005). **a** and **b** Ribbon diagrams. **c** and **d** Views of the molecular surfaces. **e** and **f** Internal views of the active sites and catalytic tunnel. Residues in α-helices are *red*, in β-sheets *yellow*, in the tunnel opening *green*, and in the catalytic site *blue*. For *Ha*HAD1/*Ha*HAD2: in α3, Glu143/Glu136; in loop following α2, His129/His122; in β3, Phe166/Val159

Purification of the recombinant *Ha*HAD1, *Ha*HAD2 and *Ha*HAD1 F166V proteins and their biochemical characterization

Given that the deduced tertiary structures revealed differences at the catalytic tunnel between the two sunflower proteins; the differing amino acid, phenylalanine 166, was mutagenized to a valine in *Ha*HAD1. The two wild-type constructs as well as the pQE80L::*Ha*HAD1 F166V gene were expressed in XL1-Blue cells and the resulting proteins purified (Fig. S2).

'In vivo' β-hydroxyacyl-(ACP]-dehydratase eliminates a H_2O molecule from β-hydroxyacyl-ACP to generate an

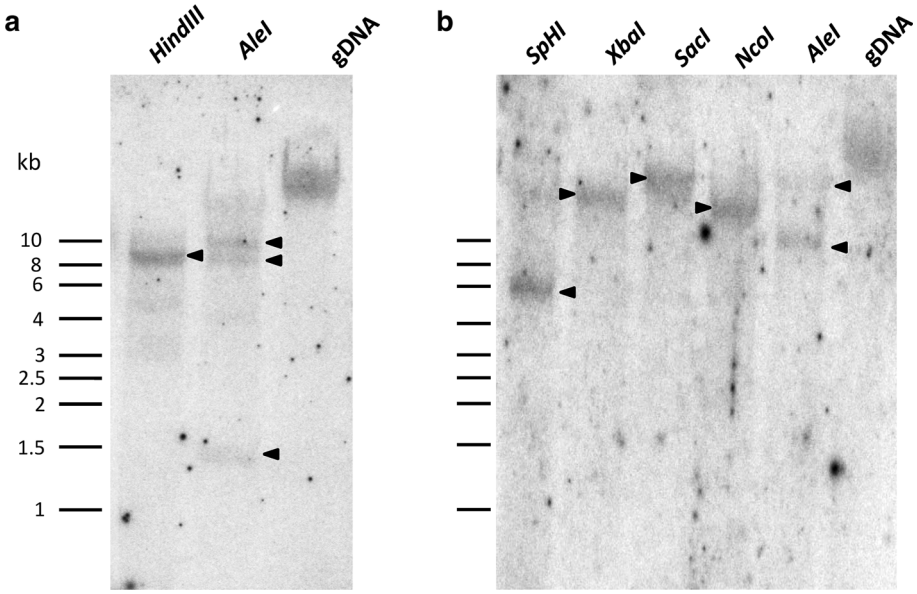

Fig. 4 Southern blot analysis of *H. annuus* genomic DNA digested with the indicated restriction enzymes and hybridized with *HaHAD1* (**a**) and *HaHAD2* (**b**) gene specific probes. Hybridizing fragments are marked with arrows, and the position of DNA molecular weight standards in kb are shown on the *left*

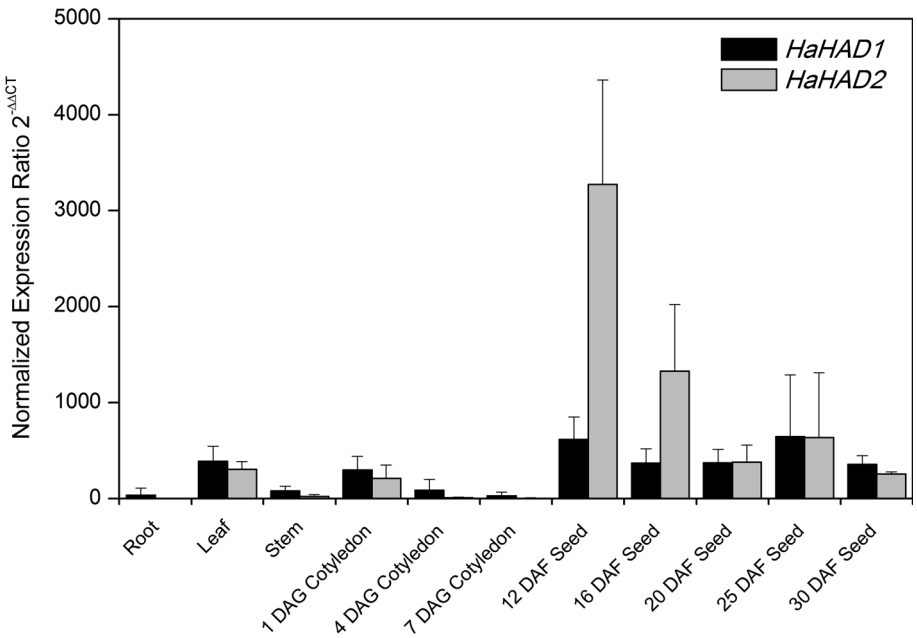

Fig. 5 Expression levels of β-hydroxyacyl-[ACP]-dehydratase in developing seeds and vegetative tissues from *H. annuus*, *HaHAD1* and *HaHAD2*, determined by RT-qPCR from sunflower line CAS-6. Stages in cotyledon development are designated days after germination (DAG) and in seed development days after flowering (DAF). Data are mean values ± SD of three independent biological samples

enoyl-ACP. This reversible reaction, hydration, is favored under '*in vitro*' conditions; hence crotonyl-ACP or crotonyl-CoA are used as substrates (Shimakata and Stumpf 1982a, b; Sharma et al. 2003; Liu et al. 2005; Dautu et al. 2008). Crotonyl-CoA was used in the present experiments. All three heterologously expressed proteins, *Ha*HAD1, *Ha*HAD2 and *Ha*HAD1 F166V were active (Fig. S3) with the production of β-hydroxyacyl-CoA dependent on concentration and time of the reaction. The slopes generated from the graphs and the Hill equations were used to calculate K_m and V_{max} values. This resulted in a K_m for crotonyl-CoA of 68.49 ± 6.15 μM and V_{max} of 13.04 ± 1.22 μmol min^{-1} mg^{-1} for *Ha*HAD1 protein and a K_m of 97.73 ± 8.67 μM and V_{max} of 29.16 ± 2.07 μmol min^{-1} mg^{-1} for *Ha*HAD2 protein. These results showed that *Ha*HAD2 presented higher activity and lower substrate affinity than *Ha*HAD1 for crotonyl-CoA. The *Ha*HAD1 F166V protein had a K_m for crotonyl-CoA of 82.30 ± 6.01 μM and the V_{max} 21.61 ± 1.80 μmol min^{-1} mg^{-1}. These results indicate that the mutant protein was more active with crotonyl-CoA than *Ha*HAD1 but less so than *Ha*HAD2.

Table 2 Lipid content and fatty acid composition of *E. coli* XLB1 strain bearing the vector pQE80L, or either one of the recombinant plasmids pQE80L::*HaHAD1* and pQE80L::*HaHAD2* growing at 37 °C on rich medium supplemented with ampicillin in two expression conditions: (1) non-induction and (2) induction with IPTG

Vectors	Conditions	Genes expressed	Total (μg)	Fatty acids (mol %)						SAT/UNSAT	16:1/18:1[c]	16:0/18:0
				16:0	16:1[a]	17[b]	18:0	18:1[a]	19[b]			
pQE80L	No IPTG	*FabZ*	636 ± 52[i]	39.4 ± 0.5	22.5 ± 0.8	10.4 ± 0.5	1.4 ± 0.0	25.8 ± 0.6	0.5 ± 0.1	0.7 ± 0.0	1.3 ± 0.0	28.5 ± 0.6
pQE80L::*Ha HAD1*	No IPTG	*FabZ*	655 ± 32[i]	56.7 ± 0.2	17.2 ± 0.2	9.0 ± 0.3	1.4 ± 0.2	15.3 ± 0.3	0.4 ± 0.1	1.4 ± 0.0	1.7 ± 0.1	41.7 ± 5.2
pQE80L::*Ha HAD1*	IPTG	*HaHAD1*+*FabZ*	818 ± 48[ii]	71.1 ± 0.3	9.9 ± 0.2	3.8 ± 0.3	5.3 ± 0.0	9.7 ± 0.2	0.2 ± 0.0	3.2 ± 0.0	1.4 ± 0.0	13.5 ± 0.1
pQE80L::*Ha HAD2*	No IPTG	*FabZ*	609 ± 33[i]	41.5 ± 0.3	22.3 ± 1.8	10.5 ± 1.1	1.5 ± 0.1	23.6 ± 0.4	0.5 ± 0.1	0.8 ± 0.0	1.4 ± 0.1	26.9 ± 1.0
pQE80L::*Ha HAD2*	IPTG	*HaHAD2*+*FabZ*	662 ± 59[i]	65.1 ± 0.9	12.2 ± 0.7	4.0 ± 0.4	4.6 ± 0.4	13.9 ± 0.4	0.3 ± 0.1	2.3 ± 0.1	1.1 ± 0.1	14.4 ± 1.4

Data are the average ± SD of three independent samples

[a] In *E. coli* the monoene fatty acids are C16:1cisΔ9 and 18:1cisΔ11

[b] C17 and 19 cyclopropanes derived from C16:1 and 18:1, respectively

[c] (16:1 + 17)/(18:1 + 19)

[i,ii] Values statistically different ($P < 0.05$)

Discussion

Sunflower seeds are one of the most important sources of oil. To provide a basic groundwork for improving and modifying the quality of its oil we are characterizing the structural genes and their encoded proteins taking part in its synthesis. Below we discuss the peculiarities of the β-hydroxyacyl-[ACP]-dehydratases, the third of four components of the FAS enzyme complex. Previously studied are the enoyl-[ACP]-reductase and β-ketoacyl-[ACP]-synthase III (González-Mellado et al. 2010; González-Thuillier et al. 2015). Remaining are the β-ketoacyl-[ACP]-reductase and the other two β-ketoacyl-[ACP]-synthases, I and II.

Two β-hydroxyacyl-[ACP]-dehydratase genes, *HaHAD1* and *HaHAD2,* were isolated and cloned from sunflower developing seed cDNA. The deduced proteins had transit peptides targeting the mature protein to the plastid or chloroplast. Both transit peptides showed a phosphorylation motif, as was the case for most of the previously studied sunflower proteins involved in fatty acid biosynthesis, such as FatA-type thioesterases (Serrano-Vega et al. 2005), the ω3-desaturase *Ha*FAD7 (Venegas-Calerón et al. 2006) and the condensing enzyme *Ha*KASIII (González-Mellado et al. 2010). Southern blot analysis of *HaHAD1* and *HaHAD2* indicated that both are most likely single copy genes as was observed for *H. annuus* enoyl-[ACP]-reductases (González-Thuillier et al. 2015).

The phylogenetic analysis of the sunflower HADs with related proteins from different taxonomic groups pointed to a common origin for these two proteins. According to the location of both proteins in the phylogenetic tree the duplication of HAD occurred recently. The existence of a common ancestor with cyanobacterial hydroxyacyl-[ACP]-dehydratase concurs with the cyanobacterial origin of the chloroplast via endosymbiosis (Goksøyr 1967), as was observed for the KASIII and ENR proteins from the sunflower FAS complex (González-Mellado et al. 2010; González-Thuillier et al. 2015). During evolution the plastid/chloroplast HAD gene sequences have acquired transit peptides, the major difference to the cyanobacterial HAD genes.

According to the RT-qPCR analysis both sunflower β-hydroxyacyl-[ACP]- dehydratases had higher levels of expression in seeds than other tissues. The profiles were not identical, however. *HaHAD2*, expressed throughout seed development, had markedly higher levels of mRNA in 12 and 20 DAF seeds. *HaHAD1* was expressed at a relatively low, constant level throughout seed development, and remarkably, also in all tested plant tissues. Such results imply that *HaHAD1* functions as a housekeeping gene, ensuring maintenance of FAS activity for polar membrane lipid synthesis and repair. *HaHAD2*, by comparison, is the

gene primarily responsible for synthesis of storage lipids. This concurs with the fact that the maximum rate of lipid accumulation in developing sunflower seeds takes place 18–19 DAF (Martínez-Force et al. 2000). The expression changes of *HaHAD2* during seed development mimic that of the previously analyzed *Arabidopsis* At2g22230 gene (Schmid et al. 2005) and of the sunflower *HaENR2* gene (González-Thuillier et al. 2015). The low level of mRNA for both *HaHAD* genes in young cotyledons was unexpected given the elevated levels of mRNA found for both sunflower *HaENR* genes in this tissue. During the change from dark/reserve to photosynthetically active cotyledons and the phase of rapid expansion of young leaves higher quantities of polar membrane lipids are required.

Expression of the sunflower β-hydroxyacyl-[ACP]-dehydratses in *E. coli* resulted in a decreased growth rate, especially for *HaHAD1* containing cells. Concomitant with these changes an increase of total fatty acids occurred. Presumably this results from an increase in fatty acid synthesis that the bacteria initiate in an attempt to produce enough unsaturated fatty acids to maintain membrane fluidity. This will be unsuccessful due to the massive expression of the plant enzyme and its incapability to isomerize and as a consequence the bacteria will die (see below). The reduction by 9 and 5 % of the C18 fatty acids in the bacteria heterologously overexpressing either *Ha*HAD1 or *Ha*HAD2, respectively, is puzzling as sunflower enzymes presumably have a higher affinity for a C16 substrate than the bacterial enzyme.

In bacteria two β-hydroxyacyl-[ACP]-dehydratase enzymes, FabA and FabZ, take part in fatty acid biosynthesis. FabA can isomerize as well as dehydrate. The β-hydroxyacyl-[ACP] dehydratases from plants are more similar to FabZ. In *E. coli* the separation between unsaturated and saturated fatty acid biosynthesis occurs during chain elongation at 10 carbons atoms when isomerization is carried out by FabA. The production of unsaturated versus saturated fatty acids relies, therefore at least in part, on the FabA/FabZ ratio (Fig. S4) (Brock et al. 1967). The normal result is circa 50 % each of saturated and monoenoic fatty acids (Garwin et al. 1980) which is close to the 0.8 ratio found for the vector control cells (Fig. S4). Heterologously expressing the sunflower genes lacking isomerase activity increased the amount of saturated fatty acids to 76 and 70 % for *HaHAD1* and *HaHAD2*, respectively (Fig. S4). Early work has shown that in *E. coli* below 20 % unsaturated fatty acids is insufficient to maintain membrane fluidity at 35 °C, and hence leads to cell death (Cronan and Gelmann 1973).

Using the *P. aeruginosa* FabZ crystal structure as a model, monomer structures were predicted for *Ha*HAD1 and *Ha*HAD2 with α helices and β strands forming the "hot dog" structure analogous to those described previously (Kimber et al. 2004; Kostrewa et al. 2005). The functional residues, catalytic site as well as recognition and ACP binding sites were localized in conserved regions of these polypeptides. A minor difference between the sunflower structures was a one residue longer loop between α3 and β3 in *Ha*HAD1 than *Ha*HAD2. As the dehydratases function as dimers (Sharma et al. 2003; Kimber et al. 2004; White et al. 2005; Swarnamukhi et al. 2006) dimeric structures were modelled for *Ha*HAD1 and *Ha*HAD2. This revealed an interesting structural difference resulting from the presence of a phenylalanine in position 166 in *Ha*HAD1 versus a valine in position 159 in *Ha*HAD2. Only in the Nostoc genera of the Cyanobacteria is a valine also found. The specified residue is located at the entrance of the catalytic tunnel formed between the monomers upon dimerization, thereby potentially regulating substrate access to the catalytic site. These two residues present very different structures at the tunnel entrance. In *Ha*HAD1 Phe166 totally blocks the entrance while in *Ha*HAD2 Val159 does not. To probe whether this residue is important for enzyme activity, its mutation to a valine in *Ha*HAD1 giving *Ha*HAD1 F166V was accomplished. All three proteins were heterologously expressed and purified.

The activity of the proteins to carry out the reverse reaction, hydration of crotonyl-CoA to β-hydroxyacyl-CoA was tested in vitro. All three were active. The K_m for crotonyl-CoA of the *Ha*HAD1 enzyme was lower than for *Ha*HAD2 (68.49 and 97.73 μM, respectively), indicating a higher substrate affinity of the first enzyme for crotonyl-CoA. K_m values between 82.6 and 86 μM, within the range of the ones observed for the sunflower proteins, were previously described for these proteins in different microorganisms, such as *H. pylori*, *T. gondii* and *P. falciparum* (Sharma et al. 2003; Liu et al. 2005; Dautu et al. 2008). The V_{max} values were contrary, however, as *Ha*HAD2 exhibited a greater reaction velocity than *Ha*HAD1, 29 and 13 μmol min^{-1} mg^{-1}, respectively. Together these results suggest that although *Ha*HAD2 needs a higher concentration of crotonyl-CoA than *Ha*HAD1, the former is more efficient in generating the product than *Ha*HAD1. For the *Ha*HAD1 F166V protein the V_{max} and K_m were 82.30 μM and 21.61 μmol min^{-1} mg^{-1}, respectively, that is, intermediate to the values obtained for the two wild-type enzymes. Combined these observations intimate that the residue at the tunnel opening may have little influence of the entrance of the substrate to the active site. Additional subtle structural differences must exist that contribute to the different K_m and V_{max} values found for these enzymes.

An interesting correlation occurs between the enzyme kinetic and RT-qPCR results. During early seed development high *HaHAD2* gene expression levels occur when 'de novo' fatty acid synthesis is highly active, and therefore

larger amounts of substrate are available for dehydration. This concurs with the enzymatic analyses revealing that the *Ha*HAD2 enzyme requires higher levels of substrate than *Ha*HAD1. This characteristic makes *Ha*HAD2 the more suitable enzyme to carry out the production of large amounts of fatty acid during, for instance, lipid accumulation in the seed even though it carries out the dehydration more slowly than *Ha*HAD1 does. The appearance of this isoform, *Ha*HAD2, with the valine substitution and the accompanying mutations resulting in a higher substrate concentration requirement and lower reaction rate, should be a recent evolutionary event due to the predominance of Phe166 in the plant kingdom HAD sequences. In any case, the real relevance in oil biosynthesis of this isoform and its changes respect to the most phylogenetically conserved during sunflower seed development must be stated taking in account that these proteins form part of the FAS complex interacting with other subunits for which other isoforms and temporal expression windows have been described (González-Mellado et al. 2010; González-Thuillier et al. 2015), in such a way that coevolution cannot be discarded.

Author contribution statement IGT, MVC and EMF conceived and designed research. IGT conducted experiments and contributed to data elaboration. RS contributed to the RT-qPCR and biochemical analyses. IGT, MVC, PWK and EMF analyzed data. IGT, MVC, RG, PWK and EMF wrote the manuscript. All authors read and approved the manuscript.

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