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ARTICLE *in* PLANTA · MARCH 2010

Impact Factor: 3.26 · DOI: 10.1007/s00425-010-1131-z · Source: PubMed

Planta (2010) 231:1277–1289
DOI 10.1007/s00425-010-1131-z

ORIGINAL ARTICLE

The role of β-ketoacyl-acyl carrier protein synthase III in the condensation steps of fatty acid biosynthesis in sunflower

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Received: 27 December 2009 / Accepted: 19 February 2010 / Published online: 10 March 2010

Abstract The β-ketoacyl-acyl carrier protein synthase III (KAS III; EC 2.3.1.180) is a condensing enzyme catalyzing the initial step of fatty acid biosynthesis using acetyl-CoA as primer. To determine the mechanisms involved in the biosynthesis of fatty acids in sunflower (*Helianthus annuus* L.) developing seeds, a cDNA coding for *Ha*KAS III (EF514400) was isolated, cloned and sequenced. Its protein sequence is as much as 72% identical to other KAS III-like ones such as those from *Perilla frutescens*, *Jatropha curcas*, *Ricinus communis* or *Cuphea hookeriana*. Phylogenetic study of the *Ha*KAS III homologous proteins infers its origin from cyanobacterial ancestors. A genomic DNA gel blot analysis revealed that *Ha*KAS III is a single copy gene. Expression levels of this gene, examined by Q-PCR, revealed higher levels in developing seeds storing oil than in leaves, stems, roots or seedling cotyledons. Heterologous expression of *Ha*KAS III in *Escherichia coli* altered their fatty acid content and composition implying an interaction of *Ha*KAS III with the bacterial FAS complex. Testing purified *Ha*KAS III recombinant protein by adding to a reconstituted *E. coli* FAS system lacking condensation activity revealed a novel substrate specificity. In contrast to all hitherto characterized plant KAS IIIs, the activities of which are limited to the first cycles of intraplastidial fatty acid biosynthesis yielding C6 chains, *Ha*KAS III participates in at least four cycles resulting in C10 chains.

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Keywords β-Ketoacyl-ACP synthase · Fatty acid biosynthesis · *Helianthus* · KAS III · Sunflower

Abbreviations

ACP	Acyl carrier protein
DAF	Days after flowering
DH	β-Hydroxyacyl-acyl carrier protein dehydratase
ENR	Enoyl-acyl carrier protein reductase
FAS	Fatty acid synthase
KAS	β-Ketoacyl-acyl carrier protein synthase
KR	β-Ketoacyl-acyl carrier protein reductase
LB	Luria–Bertani growth media
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
MCAT	Malonyl-coenzyme A:acyl carrier protein transacylase
Ni–NTA	Nickel–nitrilotriacetic acid
Q-PCR	Quantitative PCR
U	Units

Introduction

The fatty acid biosynthetic pathway is very similar in prokaryotes and eukaryotes. In animals and yeast, this process is catalyzed by a type I fatty acid synthase (FAS), consisting of one or two large multifunctional polypeptides, while in plants and bacteria each step of the biosynthesis is catalyzed by individual polypeptides (type II). The β-ketoacyl-ACP synthase (KAS) components of the type II FAS enzyme complex carry out the condensation steps of fatty acid synthesis initiating each reiterative cycle (White et al. 2005). The first condensation is carried out by KAS III (EC 2.3.1.180), using acetyl-CoA as the acceptor substrate and malonyl-acyl carrier protein (ACP) as the donor substrate (Jackowski et al. 1989; Jaworski et al. 1989). The cycle is completed by the

sequential actions of β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydratase and enoyl-ACP reductase. The subsequent two carbon condensation steps are accomplished by KAS I and KAS II, producing as final products 16- and 18-carbon acyl-ACPs in plants and bacteria. Plant *KAS III* genes have been cloned from different sources, including *Spinacia oleracea* (Tai and Jaworski 1993), *Arabidopsis thaliana* (Tai et al. 1994), *Cuphea wrightii* (Slabaugh et al. 1995), *Allium porrum* (Chen and Post-Beittenmiller 1996), *Pisum sativum* (Jones et al. 2003) and *Jatropha curcas* (Li et al. 2008).

The KAS III condensation follows a ping-pong mechanism in which after binding the acceptor substrate, acetyl-CoA, and releasing reduced CoA, the donor substrate, malonyl-ACP, is decarboxylated to give a C2 unit that is condensed with the acetyl unit. Some KAS III enzymes use other acceptor substrates resulting in different end products of fatty acid synthesis, revealing that KAS III is a primary factor in determining the structure of the finished acyl chain. For example, branched chain CoA substrates give iso- and ante-iso-fatty acids in *Bacillus subtilis* (Choi et al. 2000a), while 8–16 carbon CoAs synthesized by a type I FAS are preferred by *Mycobacterium tuberculosis* FabH (Choi et al. 2000b). Experiments with purified recombinant *S. oleracea* KAS III revealed that only C2-CoA served as an acceptor substrate (Jaworski et al. 1994). Similar studies with *C. wrightii* KAS III, however, showed that in addition to C2-CoA, C4-CoA as well as C2- and C4-ACPs also functioned (Abbadi et al. 2000a). This substrate range is similar to that of *E. coli* KAS III, FabH, which is highly selective for C2-CoA, but can also use C4 thioesters (Heath and Rock 1996). The basis for the *E. coli* versus *M. tuberculosis* FabH acceptor substrate differences is the structure of their substrate-binding pockets, which was deduced from crystallographic studies (Qiu et al. 1999; Davies et al. 2000; Scarsdale et al. 2001). In *E. coli* FabH, the active site is formed by the convergence of two α-helices and accessed by a narrow hydrophobic channel at the bottom of which are the cysteine to which the acceptor substrate is attached, as well as the histidine and asparagine residues required for the decarboxylation step of malonyl-ACP to give the C2 donor unit. Moreover, Phe87 is believed to restrict the use of acceptor substrates with more than four carbons (Qiu et al. 1999; Scarsdale et al. 2001). In *M. tuberculosis*, the analogous residue is a threonine that allows longer acyl chains to bind in combination with other subtle changes in a larger pocket (Scarsdale et al. 2001).

A second role for KAS III as the initial enzyme of fatty acid biosynthesis is in determining the number of acyl chains that can be elongated. Thus, *E. coli* KAS III is a target for feedback regulation by long-chain acyl-ACPs, the end products of the FAS reaction (Heath and Rock 1996), and in seed extracts of *Cuphea lanceolata*, a crop producing mainly decanoic acid, decanoyl-ACP inhibits *Cl*KAS III (Brück et al. 1996).

During investigations of the potential target site of decanoyl-ACP on *Cl*KAS III (Abbadi et al. 2000b), a sequence motif GNTSAAS was identified. Purified recombinant, mutant enzymes N291D, A294S and A295P had slightly reduced KAS III activity compared to wild-type KAS III, but were not sensitive to acyl-ACPs. Experiments designed to modify fatty acid profiles of oil seeds by transforming them with *KAS III* genes have yielded only modest results; see, for example, Dehesh et al. (2001) and Stoll et al. (2006), showing the participation of other regulatory factors.

Total oil content in seeds is a character influenced not only by the photosynthate from the mother plant, but also by the action of enzymes from different pathways, from glycolysis to fatty acid assembly in triacylglycerols by acyltransferases, as well as from "de novo" fatty acid biosynthesis. For example, in the *KAS III* transformation experiments referred to above, oil content sometimes remained unchanged and other times was increased or decreased. To optimize the total oil content in developing seeds, we looked for the best alleles of relevant genes from different sunflower (*Helianthus annuus*) genetic backgrounds. As an initial target, we isolated, cloned and sequenced an *HaKAS III* cDNA. The expression levels of this gene in seeds and vegetative tissues were examined and genome copy number estimated by DNA gel blot analysis. Homology modeling of the mature protein resulted in a structural model of the catalytic residues in this enzyme. Given the diversity of known KAS III acceptor substrates, the sunflower protein was heterologously expressed in *E. coli* and its activity tested in vivo, by studying the fatty acid profile. The substrate specificity of purified, recombinant enzyme was assayed in vitro by adding to a reconstituted *E. coli* FAS system lacking condensation activity.

Materials and methods

Biological material

Sunflower (*Helianthus annuus* L.) wild-type line CAS-6 (Sunflower Collection of Instituto de la Grasa, CSIC, Sevilla, Spain) was grown as described by Álvarez-Ortega et al. (1997). Plants were cultivated in growth chambers at 25/15°C (day/night cycles), with a 16 h photoperiod and a photon flux density of 200 μmol $m^{-2}s^{-1}$. Seeds used for the synthesis of cDNA were collected at 15 days after flowering (DAF). For the quantitative expression studies, Q-PCR, seed samples were collected from 12 to 22 DAF every other day. Samples of vegetative tissues (stem, leaf, root and seedling cotyledons) were collected from plants 20 days after germination. All the samples were frozen and stored at −80°C.

The *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA) was used as the plasmid host for *HaKAS III* cloning,

protein expression and production. The bacteria were grown in LB medium [1% bacto tryptone, 0.5% bacto yeast extract, 1% NaCl (pH 7)] at 37°C with shaking, and plasmid selection was performed in the presence of ampicillin (50 μg mL^{-1}). The specific growth rate was calculated from a plot of the increase in log OD_{600} with time. Each value for growth rate was the mean of three separate experiments.

mRNA preparation and cDNA synthesis

Approximately 0.1 g of each tissue was ground in liquid N_2 with a precooled sterile mortar and pestle. Total RNA was extracted using a Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA), and mRNA was isolated from total RNA using the GenElute mRNA Miniprep kit (Sigma-Aldrich). The mRNA pellet was resuspended in 33 mL of TE buffer, RNAase free (10 mM Tris–HCl, 1 mM EDTA, pH 8) and the corresponding cDNA was synthesized using a Ready-To-Go T-Primed First Strand Kit (GE Healthcare Life Science, Buckinghamshire, UK).

Cloning of the sunflower *KAS III* gene

An inner PCR fragment of *HaKAS III* was amplified using the following primers (Fig. 1): KASIIIF1 5′-TWYGAYATYACNGCHGC-3′ (192 times degenerate) and KASIIIR1 5′-ARHCCRGCDCCRAANCC-3′ (288 times degenerate). Both were designed through homologous regions in an alignment of plant KAS III protein sequences using the ClustalX 2.0 program (Thompson et al. 1997). The full sequence was obtained in the 3′-end using the primer FA2Z 5′-AACTGGAAGAATTCGCGG-3′, sequence added by the cDNA synthesis kit, and the specific primer KASIIIF2: 5′-CACTTCTCCAAAATCTGCAGCGCC-3′; the 5′-end was amplified using the Smart™-RACE cDNA amplification kit (BD Bioscience Clontech Company, Palo Alto, CA, USA) and the specific primer KASIIIR2: CTCTGGTTAGCCTGATGG-3′. All PCR fragments were cloned in pGEM-T-Easy® vector (Promega, Madison, WI, USA), transformed into XL1-Blue, sequenced by Secugen SL (Madrid, Spain), and their identities confirmed using the BLAST software (Altschul et al. 1990).

cDNA and protein sequence analyses

Sequences homologous to the predicted sequence of *Ha*KAS III were retrieved using the BLASTP program (http://www.ncbi.nlm.nih.gov). Alignment of the amino acid sequences, including the transit peptides, for KAS III proteins deposited at GENBANK was performed using the ClustalX v.2.0 program with the default settings (Thompson et al. 1997). These entire alignments were used to generate a phylogenetic tree based on the neighbor-joining algorithm (Saitou and Nei 1987), and the resulting 'phenogram' was drawn using the TreeView program (Page 1996). The chloroplast transit peptides were identified through alignment with known KAS III sequences and using the network-based method TargetP V1.0 (Emanuelsson et al. 2000).

Modeling of the three-dimensional structure of *Ha*KAS III

Homology modeling studies were performed using Swiss-Model server (http://swissmodel.expasy.org/) (Schwede et al. 2003) and JPred prediction server (Cuff and Barton 2000). The *Ec*FabH sequence used was taken from Tsay et al. (1992), accession number P0A6R0. The chosen template was the most homologous KAS III for which X-ray structure information was available (PDB Entry: 1EBL), showing 42% sequence identity with 317 residues of *Ha*KAS III. SWISS-MODEL was used in first approach and project (optimize) modes using default parameters. Structures were visualized using Swiss-PDBViewer (Guex and Peitsch 1997).

Genomic DNA gel blot analysis

Genomic DNA was isolated from sunflower leaf tissue by the CTAB method (Murray and Thompson 1980). Samples of sunflower DNA (15 μg) were digested with restriction enzymes and resolved on 0.8% agarose gel. The gel was soaked in 250 mM HCl for 30 min, washed three times in distilled water and finally the gel was blotted onto a Hybond-N+ nylon transfer membrane (GE Healthcare Life Science). The filter was probed with a 1,001-bp [α-^{32}P] dCTP-labeled *HaKAS III* gene-specific DNA fragment obtained by PCR amplification using oligos KASIIIS1 (5′-GCATCTAGGGTAGTTGGGAAGG-3′) and KASIIIS2 (5′-TTAGTTCCATCTGATAATAGC-3′), and labeled with a "Ready-to go" labeling kit (GE Healthcare Life Science) and [α-^{32}P]dCTP. Hybridization was performed in 0.2 mM of potassium buffer, 250 mM of SDS and 1 mM of EDTA overnight at 65°C and the filter was then washed twice in 2× SSC buffer, 0.1% SDS for 20 min at the same temperature. Images of radioactive filters were obtained and quantified using a Cyclone TM Storage Phosphor System (PerkinElmer, Boston, MA, USA) and the Optiquant TM image analysis software (Packard Co., Meriden, CT, USA).

Determination of mRNA levels by quantitative real-time PCR

The cDNAs obtained as described above from developing seeds and vegetative tissues were subjected to quantitative real-time PCR with primer pairs specific to the *HaKAS III* gene (KASIIIF3, 5′-TTTGGGTCTAGTCTCAGC-3′, and

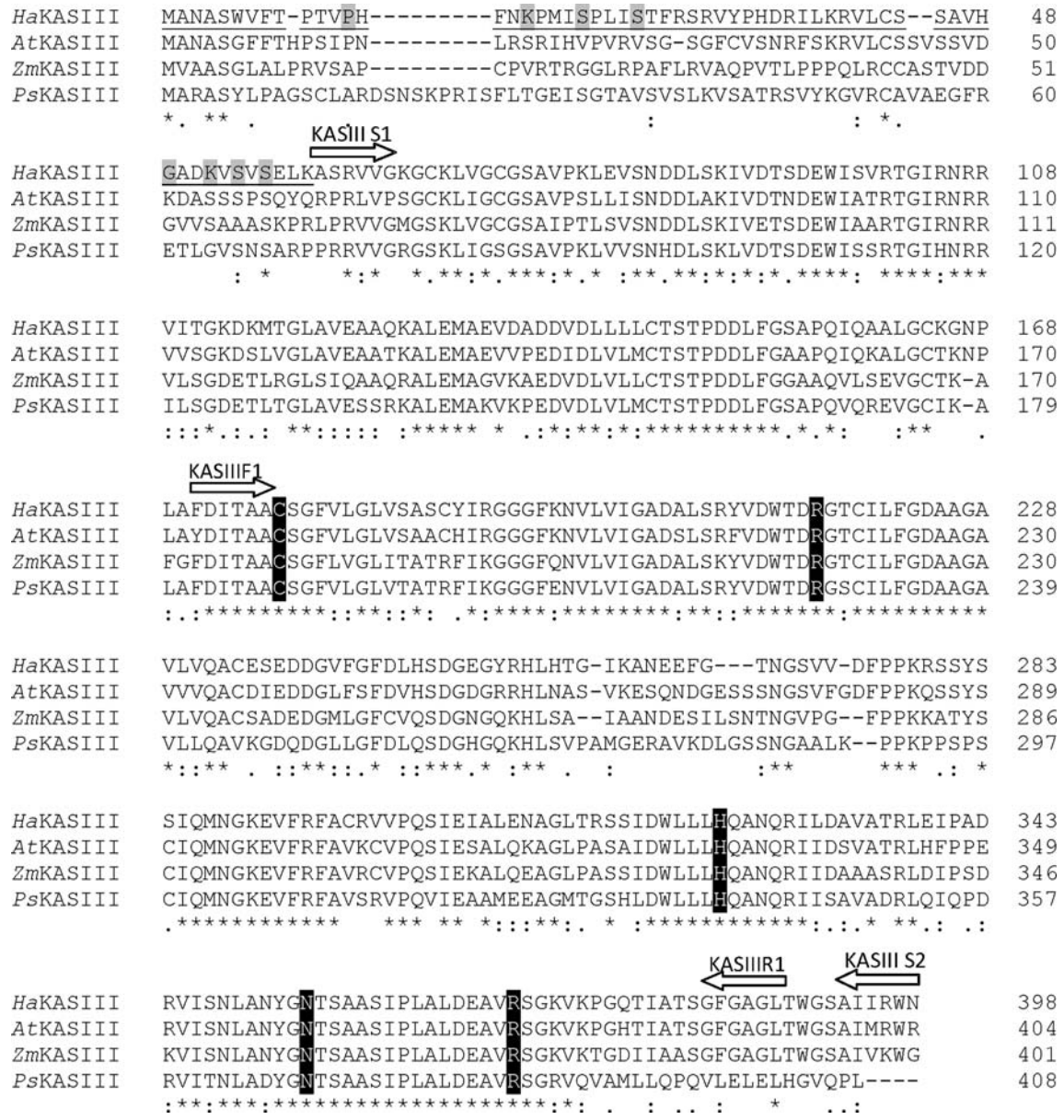

Fig. 1 Comparison of *Ha*KAS III with its homologs from different phylogenetic groups: *At A. thaliana*, *Zm Z. mays* and *Ps P. sitchensis*. The putative transit peptide corresponding to the first 59 amino acids is underlined, predicted phosphorylation sites in the putative signal peptide are shaded in *gray* and the conserved residues involved in the catalytic center are shown as *black boxes*. Additionally, the positions of the degenerated oligos KASIIIF1 and KASIIIR1 used for the internal fragment PCR cloning, and oligos KASIIIS1 and KASIIIS2 used for the DNA gel blot analysis are also shown. *Asterisk* identical residues, *colon* conservative changes, and *dot* weakly conservative changes

KASIIIR3, 5′-GCGCCAGCAGCATCCCC-3′), and SYBR Green I (QuantiteTect™ SYBR® Green PCR Kit, Qiagen, Hilden, Germany) using an Opticon system (Bio-Rad, Hercules, CA, USA). 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, 50°C for 30 s and 72°C for 15 s, while monitoring the resulting fluorescence. The calibration curve for absolute quantification was done using sequential dilutions of the pGEMT:*HaKAS III* plasmid. Triplicates of each seed stage and vegetative tissues samples were obtained and analyzed.

Expression of *Ha*KAS III recombinant protein

To get the in-frame fusion of the gene *HaKAS III* into pQE-80L (Qiagen), the expression system with N-terminal 6× histidine affinity tags, the following synthetic oligonucleotide primers were designed: *Ha*KASIIIF*Sph*I: 5′-ATCGGC ATGCGCATCTAGGGTAGTTGGGAAGG-3′ and *Ha*KASIIIR*Hin*dIII: 5′-AGCTAAGCTTTTAGTTCCATCTGATAATAGC-3′. The PCR products were cut with *Sph*I and *Hin*dIII restriction enzymes and cloned into the corresponding sites in the pQE-80L vector. Confirmation that the

correct construction had been obtained was determined by sequencing. The newly constructed vector, pQE-80L::*HaKAS III*, was used for transforming chemically competent *E. coli* XL1-Blue and the resulting colonies were screened by ampicillin resistance.

Expression and purification were carried out as previously described (McGuire et al. 2001). Overnight cultures containing recombinant *Ha*KAS III in LB were pelleted, resuspended and used to inoculate 50 mL of SB plus 100 μg mL^{-1} of ampicillin and grown to OD_{600} ~1 at 37°C. After adding IPTG to a final concentration of 1 mM to induce expression, the cultures were grown for 2 h at temperatures ranging from 10 to 37°C. Maximum soluble protein was obtained by incubating at 37°C. Cells were harvested by centrifugation (15 min at 2,500*g*) and resuspended in 1–2 mL of purification buffer plus 10 mM of DTT and 5 mM of EDTA. Recombinant protein was purified at 4°C using lysozyme and a sonication step to break the cells, followed by cycles of freezing and thawing plus sonication. After obtaining the supernatant, the recombinant protein was purified by passing the supernatant twice through a His-tag Poly-Prep Column (Bio-Rad).

E. coli fatty acid analysis

Cultures of 250 mL of *E. coli* carrying pQE80L or pQE80L::*HaKAS III* were grown to OD_{600} 0.8, induced as described for 4 h, and their total lipids extracted (Kates 1986). The isolated lipid samples were heated to 80°C for 1 h in 3 mL of methanol/toluene/H_2SO_4 (88:10:2 by vol), to which 1 mL of heptane was added after cooling and the fatty acid methyl esters were recovered in the upper phase (Garcés and Mancha 1993). Methyl esters were separated on an SP2380 fused silica capillary column (30-m long, 0.25 mm i.d., 0.20-μm film thickness: Supelco, Bellefonte, PA, USA) and quantified by hydrogen flame ionization detection using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA). Hydrogen was used as the carrier gas, at a linear gas rate of 28 cm s^{-1}. The injector and detector temperatures were 200°C, the oven temperature was 170°C and the split ratio was 1:50. Fatty acids were identified by comparison of retention times with a combination of known standards. The total fatty acid content was determined by GLC using heptadecanoic acid as the internal standard added to the sample just before lipid extraction (Cantisán et al. 2000).

In vitro *Ha*KAS III elongation assay

The ability of *Ha*KAS III to elongate acyl-ACP substrates was tested using a coupled reaction in which radiolabeled malonyl-ACP was generated using *E. coli* malonyl-CoA:ACP transacylase (MCAT) and then adding selected components of the *E. coli* fatty acid synthase complex: *β*-ketoacyl-ACP reductase (KR), *β*-hydroxyacyl-ACP dehydratase (DH) and enoyl-ACP reductase (ENR). The *E. coli* genes encoding MCAT, KR, DH and ENR were cloned into pQE-30. After expression, the proteins were purified by Ni–NTA as described (McGuire et al. 2001).

Radiolabeled malonyl-ACP was synthesized from 10 μM of *E. coli* ACP (Sigma-Aldrich) and MCAT (1.3 μg), after pre-incubation in the presence of DTT (McGuire et al. 2001), by adding a reaction mixture containing 10 μM of acetyl-CoA, 60 μM of malonyl-CoA, 0.76 μM of [2-^{14}C] malonyl-CoA (2.2 TBq mol^{-1}, PerkinElmer Life Sciences, Boston, MA, USA), 1 mM of NADPH, 1 mM of NADH and 50 mM of potassium phosphate buffer (pH 6.8) and incubating 65 μL for 5 min at 25°C. Addition of 1.6 μg of *Ha*KAS III plus 1–2 μg of *E. coli* KR, DH and ENR as required in 50 mM potassium phosphate buffer (pH 6.8) gave an assay mix of 100 μL. Reactions proceeded at 37°C for 30, 60 or 120 min, before adding cold trichloroacetic acid and centrifuging to precipitate the acyl-ACPs as described (McGuire et al. 2001). Acyl-ACPs, resolved by 2, 3 or 4 M urea-PAGE on the basis of chain length, were blotted to a PVDF membrane and detected using radioautoradiography using a PhosphorImager.

Radiolabeled acetyl- and malonyl-ACP standards were prepared by carrying out decarboxylase assays in the presence and absence of the *E. coli* KAS I mutant protein bearing an Ala in place of Cys163 that efficiently decarboxylates malonyl-ACP to acetyl-ACP, but does not carry out the condensation (McGuire et al. 2001). The preparation and purification of the radiolabeled C16-ACP standard has been described previously. To obtain a series of acyl-ACPs with intermediate chain lengths, a soluble protein extract was prepared from CY244 cells expressing the *Arabidopsis* mitochondrial condensing enzyme, mtKAS, under conditions in which both the FabB and FabF defects in CY244 are complemented by mtKAS (Yasuno et al. 2004). In vitro assays with the soluble protein extract as detailed in Yasuno et al. (2004) result in a homologous series of acyl-ACPs, among which C8 is prominent.

Results

Isolation and sequence analysis of a sunflower *β*-ketoacyl-ACP synthase III cDNA

Conserved regions from known plant KAS III protein sequences were used to design degenerated oligonucleotide primers: KASIIIF1, corresponding to a conserved cluster near the cysteine residue involved in the active site, and KASIIIR1, corresponding to a region close to the carboxylic

end (Fig. 1). Using these primers, we amplified by PCR a 650-bp fragment from developing sunflower seeds cDNA, corresponding to an internal region of a *KASIII* mRNA. Subsequently, we obtained a full-length 1,197-bp cDNA clone of *HaKAS III* by RACE using the primers shown in "Materials and methods". That this cDNA codes for a KAS III was suggested using the Blast software (Altschul et al. 1990). The full-length cDNA encodes a preprotein of 398 amino acids, with an expected molecular mass of 42.48 kDa and a p*I* of 6.60 (Fig. 1).

Through alignment with known KAS III sequences and using the network-based method TargetP V1.1 to identify chloroplast transit peptides (Nielsen et al. 1997; Emanuelsson et al. 2000), Ala60 of the sunflower KAS III sequence was the best candidate for the N-terminus of the mature protein (Fig. 1). The putative transit peptide includes two consensus phosphorylation motifs [(P/G)X(n)(R/K)X(n)(S/T)X(n)(S*/T*), where n = a 0 to 3 amino acid spacer and S*/T* represents the phosphate acceptor] as described by Waegemann and Soll (1996) (Fig. 1). Proteolytic processing of the prepeptide would produce a 339 amino acid protein with a molecular mass of around 35.87 kDa and a p*I* of 5.01.

The *Ha*KAS III amino acid sequence was compared with homologous proteins from different phylogenetic groups such as *A. thaliana*, *Zea mays* or *Picea sitchensis* (Fig. 1). This alignment shows, following the transit peptide region, two highly conserved domains, residues 62–229 and 285–373, separated by a low conserved region. Both conserved regions include residues involved in the condensation activity as described for homologous proteins in the genus *E. coli* (Davies et al. 2000; Qiu et al. 2001) and *C. wrightii* (Abbadi et al. 2000a): the catalytic triad, Cys177, His324 and Asn354 and arginine residues, 216 and 369. The first is involved in CoA-binding and the second, as revealed by alanine scanning, in KAS III catalysis (Abbadi et al. 2000a).

The availability of the complete sequence of prokaryotic genomes from different phylogenetic groups enabled us to look for distantly related proteins that may share a common ancestry with plant KAS III (Fig. 2). To simplify the phylogenetic tree, only one sequence from each prokaryotic order was included except for the cyanobacteria. These prokaryotic sequences lacking the signal peptide conserve the catalytic residues as well as the surrounding amino acids. As expected, the *Ha*KAS III protein sequence is as much as 70% identical to other KAS III-like ones, such as those from *S. oleracea*, *A. thaliana* or *P. sativum*, appearing closely related to cyanobacterial homologs.

Proposed structural model for *Ha*KAS III

*Ha*KAS III protein was modeled after *Ec*FabH (PDB 1EBL), using Swiss-Model server (http://swissmodel.expasy.org/) (Schwede et al. 2003) and JPred prediction server (Cuff and Barton 2000), to which mature *Ha*KAS III is identical by 42% (Fig. 3). The alignment in Fig. 3 reveals that the conserved residues are distributed relatively uniformly, while Fig. 4 compares two views of FabH (a and c) with the *Ha*KAS III models (b and d). The conserved residues include those participating in catalysis (Cys177, His324 and Asn354), those containing the CoA-binding residues, that is 91–105 (L1α2) and 214–228 (L7α2 and start of Nβ5), plus 353–369 (Cα3) contributing to the active site configuration (Fig. 3) (Davies et al. 2000; Qiu et al. 2001). When the molecular surface is computed for *Ha*KAS III (Fig. 4f), Arg216 involved in CoA-binding at the entrance to the active site and the catalytic triad at the bottom of the tunnel are located similarly to their counterparts in *Ec*FabH (Fig. 4e). On the other hand, the region encompassing residues 251–286 in *Ha*KAS III (in green in Fig. 4b, d) differs from the corresponding region in *Ec*KAS III.

DNA gel blot analysis of *HaKAS III*

A DNA gel blot analysis was performed on genomic DNA extracted from wild-type sunflower leaves to evaluate the number of copies of *KAS III* in the genome. Sunflower genomic DNA was digested with restriction enzymes having one (*Eco*RI, *Mfe*I and *Bam*HI) or no (*Hin*dIII and *Sac*I) cutting sites within the cDNA, and a 1,001-bp PCR probe was used for hybridization. The results (Fig. 5) obtained with *Eco*RI, *Sac*I and *Bam*HI (2, 1 and 2 hybridization bands, respectively) indicate that *HaKAS III* is a single copy gene. The more complex pattern obtained with *Mfe*I, three bands when two were expected, and *Hin*dIII, two bands when one was expected, point to the existence of introns in the gene. This prediction was confirmed by carrying out a search in the WGS (Whole-genome Shotgun Reads) database using TBLASTN 2.2.21 program (Altschul et al. 1997) with the deduced protein sequence of *Ha*KAS III. Not only could the introns have restriction sites for the enzymes *Mfe*I and *Hin*dIII, but also fragment sizes after the pertinent digests could increase by at least 2 kb.

Tissue expression levels of *HaKAS III*

The expression of *HaKAS III* was analyzed by Q-PCR in roots, stems, cotyledons and leaves of 20-day-old seedlings and 12–20 DAF seeds in different developmental stages of sunflower (Fig. 6a). Our results are compared with the ones obtained from an *A. thaliana* microarray (Fig. 6b; data from Schmid et al. 2005) available through the AtGenExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp) developed by Christian K. Widmer. We observed circa ten times more expression of *HaKAS III* in seeds than in the other tissues.

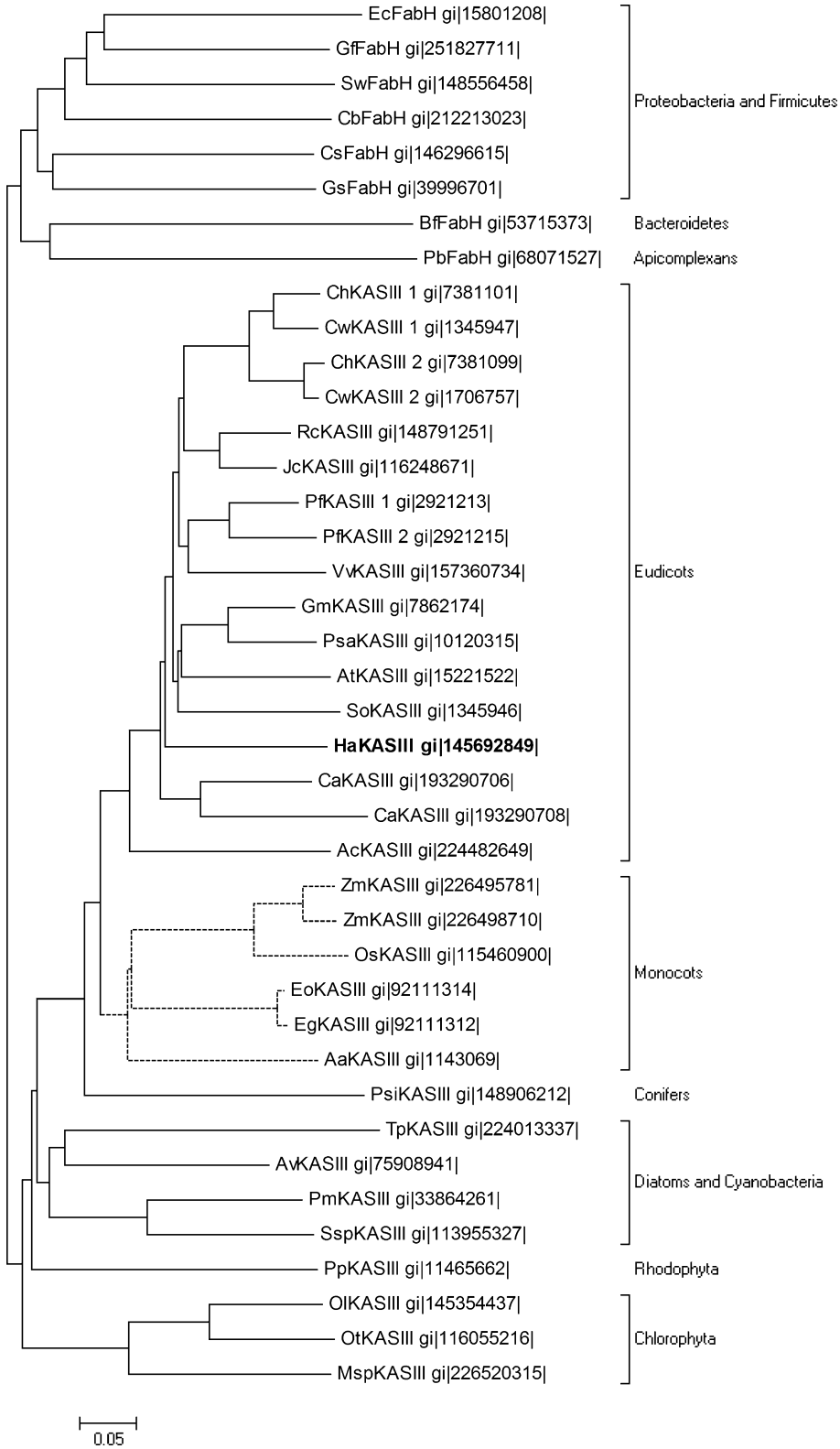

Fig. 2 Phylogenetic comparison of KAS III proteins from plants, algae and bacteria rooted in the bacteria FabH protein sequences. The groups and species include: Eudicotyledoneae (*Ac Annona cherimola*, *At A. thaliana*, *Ca Capsicum annum*, *Ch Cuphea hookeriana*, *Cw C. wrightii*, *Gm Glycine max*, *Ha H. annuus*, *Jc J. curcas*, *Pf Perilla frutescens*, *Psa Pisum sativum*, *Rc Ricinus communis*, *So S. oleracea*, *Vv Vitis vinifera*), Monocotyledoneae (*Zm Z. mays*, *Os Oryza sativa*, *Eo Elaeis oleifera*, *Eg Elaeis guineensis*, *Aa Allium ampeloprasum)*, conifers (*Psi P. sitchensis*), diatoms (*Tp Thalassiosira pseudonana*), Cyanobacteria (*Av Anabaena variabilis*, *Pm Prochlorococcus marinus*, *Ssp Synechococcus* sp.), Rhodophyta (*Pp Porphyra purpurea*), Chlorophyta (*Ol Ostrecococcus lucimarinus*, *Ot Ostreococcus tauri*, *Msp Micromonas* sp.), Apicomplexans (*Pb Plasmodium berghei*), Bacteroidetes (*Bf Bacteroides fragilis*), Firmicutes (*Cs Caldicellulosiruptor saccharolyticus*) and Proteobacteria (*Cb Coxiella burnetti*, *Ec E. coli*, *Gf Gallinonella ferruginea*, *Gs Geobacter sulfurreducens*, *Sw Sphingomonas wittichii*)

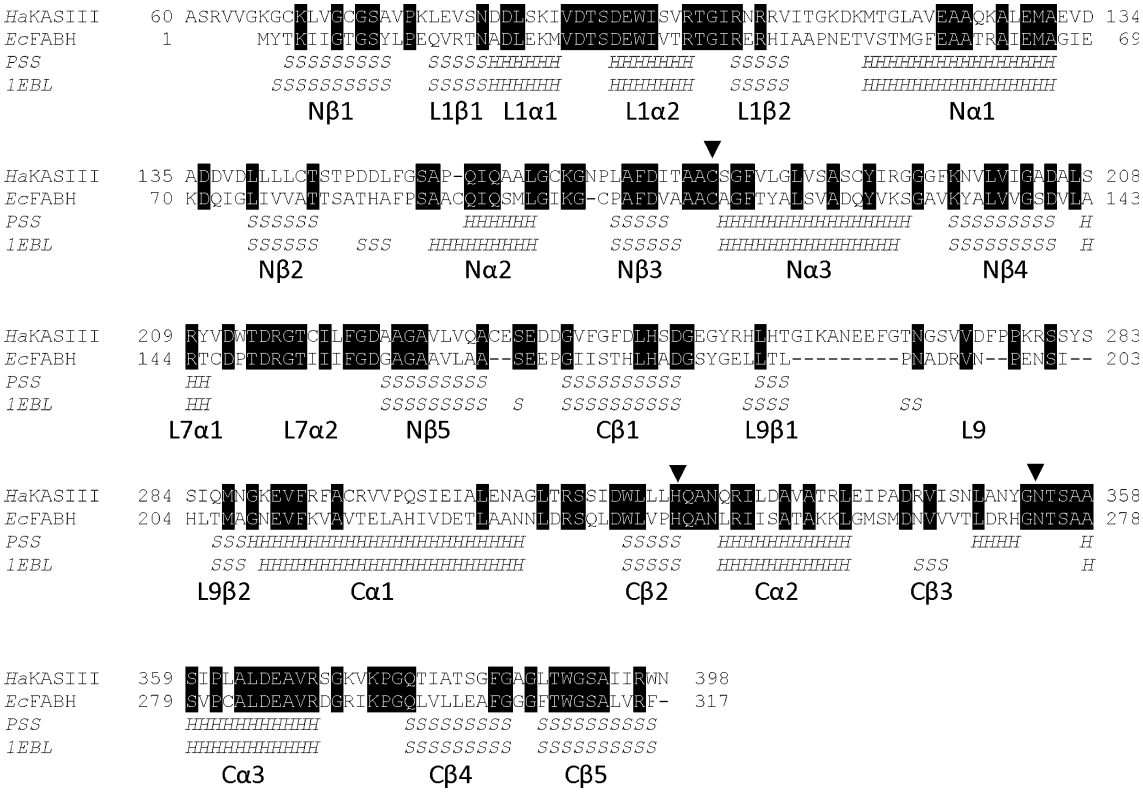

Fig. 3 Comparison between the deduced amino acid sequence and predicted secondary structure (PSS) for the sunflower *HaKAS III* gene and the sequence and structure (*1EBL*) for the *EcFabH* gene (Davies et al. 2000). Identical residues are shown as *black boxes*, and the catalytical triad formed by residues Cys177, His324 and Asn354 are marked with inverted back triangles. *H* α-helix, *S* β-sheet. FabH structural elements are named as in Davies et al. (2000)

Heterologous expression of sunflower KAS III in *E. coli* and purification

The plastidial *KAS III* in construct pQE-80L:*HaKAS III*, in which the first 59 residues constituting the plastid targeting sequence were replaced by a His_6-tag, expressed well in *E. coli*. As growth temperature increased, the amount of soluble recombinant protein increased (Fig. 7a). Above 20°C, appreciable amounts of the protein were sequestered in inclusion bodies. The expected His-tagged protein with a molecular weight of 37.5 kDa was purified from the total extract using a Ni–Sepharose affinity column. Figure 7b reveals that after the initial Ni-affinity column step, the protein was ≥98% pure.

Fatty acid alterations in *E. coli* expressing recombinant *Ha*KAS III

Nonsignificant changes in cell growth rates were observed in *E. coli* bearing pQE80L and pQE-80L:*HaKAS III* minus/plus induction (0.47/0.55 h^{-1}, respectively). Nevertheless, the expression of *Ha*KAS III reduced the total bacterial fatty acid content to circa 47% of that in other cells (Table 1). The mol% of saturated fatty acids was also slightly reduced. In terms of total μg saturated fatty acids, however, the decrease amounted to 59% (262 to 108), which was primarily caused by a reduction in C16:0. The mol% of C17 cyclopropanes was also reduced, while their C16:1 precursor increased. The mol% of C18:1 also increased, but the C19 cyclopropanes remained unchanged. Despite the increases in mol%, the induced cells had circa 20% less microgram of C16:1 and C18:1 than the uninduced cells.

Characterization of *Ha*KAS III

To determine whether *Ha*KAS III carries out only the initial condensation giving a C4 acyl chain or additional condensations, purified recombinant protein was tested in an in vitro assay by adding it to a reconstituted *E. coli* FAS system lacking condensation activity (Heath and Rock 1995; Yasuno et al. 2004). In the course of the assay, malonyl-ACP is generated from radiolabeled malonyl-CoA by MCAT. The KAS III enzyme then carries out a tripartite reaction; initially, the C2 acceptor unit is transferred from CoA to the active site cysteine, then malonyl-ACP is decarboxylated to give the C2 donor unit and finally condensation takes place to give β-ketoacyl C4-ACP. If KR is also

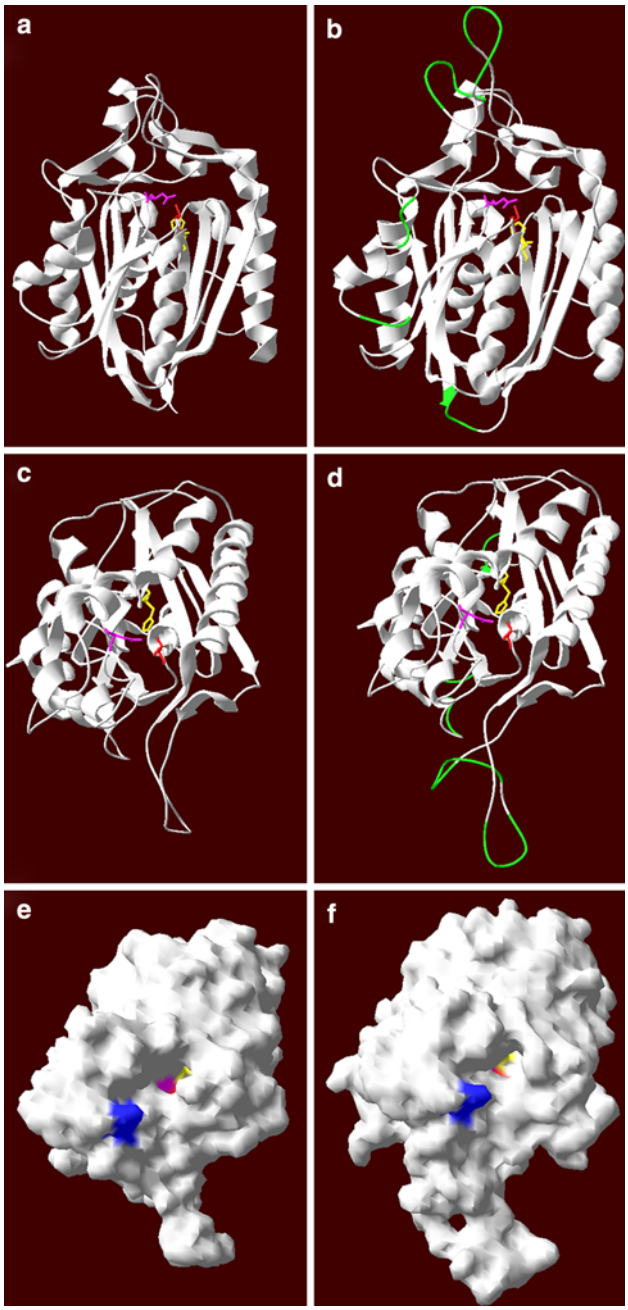

Fig. 4 Proposed structural models for *Ha*KAS III compared with those known for the *Ec*FabH. **a** Ribbon diagram of the *Ec*FabH structure with the active site residues (Cys112 in red, His244 in yellow and Asn274 in pink) in ball and stick. **b** Analogous ribbon diagram of *Ha*KAS III modeled region from residue 68–397 showing in *green* nonconserved structural regions. **c**, **d** *Top* views of ribbon diagrams from **a** and **b**, respectively. **e** View of the *Ec*FabH molecular surface showing in blue Arg151 near the active site tunnel entrance and the active site residues at the bottom of the tunnel. **f** A similar view for the *Ha*KAS III protein showing residues Cys177, His324, Asn354 and Arg216

present, then the latter is converted by DH to β-hydroxyacyl C4-ACP, which in turn in the presence of ENR becomes C4-ACP that serves as primer for another extension. The results of the assays are shown in Fig. 8. Simultaneously with running the assays in Fig. 8a, two additional sets of assays were run, which are shown in the lower two panels (b and c) of Fig. 8. As expected β-hydroxy C4-ACP accumulates when KR is present (lane 4), which is converted in the presence of DH and ENR (lanes 5–7) to C4-ACP and subsequently to C6- and C8-ACPs (Fig. 8a). The amount of the latter relative to C4-ACPs increases as the assay time increases from 30 to 120 min. Figure 8b, which is of 3 M urea gel resolving ACPs from C6 to C12, reveals that some C10-ACP is also synthesized with time. The 4 M urea gel, resolving longer acyl-ACPs, in Fig. 8c intimates that at 120 min C12-ACP may also be synthesized.

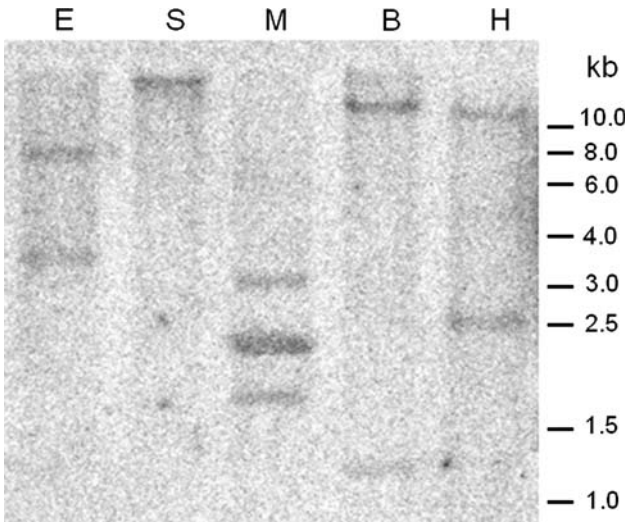

Fig. 5 Genomic DNA gel blot analysis of *Ha*KAS III under high stringency conditions. The restriction enzymes were: *Eco*RI (*lane E*), *Sac*I (*lane S*), *Mfe*I (*lane M*), *Bam*HI (*lane B*) and *Hind*III (*lane H*). Molecular weight markers (*kb*) are indicated on the *right*

Discussion

Using the available sequences for plant KAS III proteins, we were able to identify the gene responsible for this activity in sunflower. The deduced protein sequence for *Ha*KAS III contained a putative signal peptide for targeting to the plastid/chloroplast, similarly to all the plant KAS IIIs described to date. In many plastid/chloroplast enzymes, phosphorylation of the transit peptide leads to the binding of the 14-3-3 protein that together with HSP70 can form a cytosolic guidance complex (May and Soll 2000). Preproteins bound thereto are more rapidly imported into chloroplasts than monomeric preproteins, suggesting that *Ha*KAS III could be involved in fast regulatory responses. The presence of these phosphorylation motifs has been described previously in other sunflower chloroplast proteins involved in fatty acid biosynthesis, such as FatA-type thioesterases

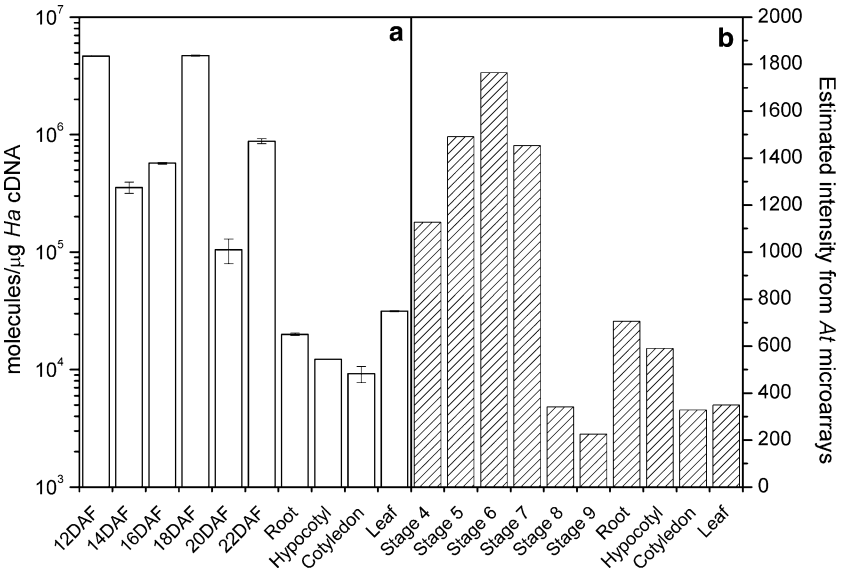

Fig. 6 Expression of *KAS III* genes in developing seeds and vegetative tissues of *H. annuus* and *A. thaliana*. **a** *HaKAS III* expression determined by real-time Q-PCR. **b** *AtKAS III* expression estimated from microarrays of Schmid et al. (2005). *DAF* days after flowering, *Stage 4* early to late heart embryos, *Stage 5* late heart to mid-torpedo embryos, *Stage 6* mid to late torpedo embryos, *Stage 7* late torpedo to early walking-stick embryos, *Stage 8* walking-stick to early curled cotyledons embryos, *Stage 9* curled cotyledons to early green cotyledons embryos. Data in panel **a** are mean values ± SD of three independent seedling samples

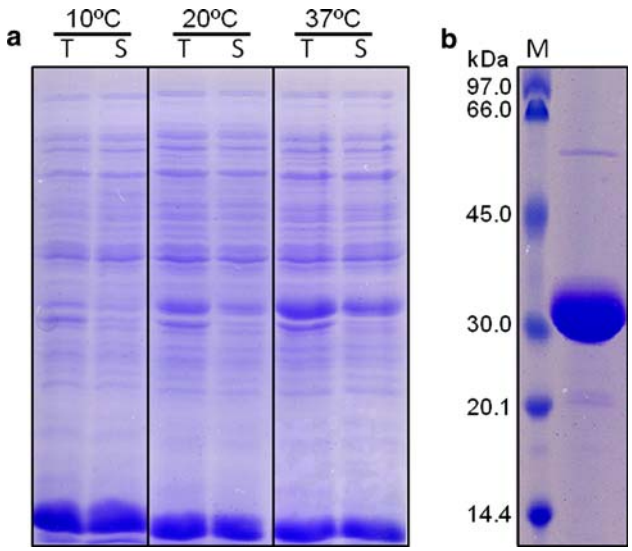

Fig. 7 Coomassie blue-stained SDS-PAGE of recombinant *Ha*KAS III protein. **a** Effect of growth temperature on the intracellular level of recombinant *Ha*KAS III protein after 2 h of induction. **b** Purification of recombinant *Ha*KAS III using Ni^{2+}-charged HisTrap FF columns. *T* total *E. coli* extract, *S* soluble fraction, *M* size markers, *B* His-tagged purified *Ha*KAS III first round

(Serrano-Vega et al. 2005) or the ω3-desaturase *Ha*FAD7 (Venegas-Calerón et al. 2006).

The phylogenetic tree of KAS III related proteins from different taxonomic groups revealed the high level of identity of the *Ha*KAS III protein sequence with other plant KAS III-like ones, such as those from *S. oleracea*, *A. thaliana* or *P. sativum* (Fig. 2), and the existence of common ancestors with cyanobacterial β-ketoacyl-ACP synthases III. This concurs with the cyanobacterial origin of chloroplasts via endosymbiosis (Goksøyr 1967), as is true for the other FAS components. This is in contrast to acyl-ACP thioesterases and stearoyl-ACP desaturases also functioning in plastidial fatty acid metabolism, the origins of which are instead attributed to a guest cell from the δ-subdivision of the proteobacteria (Serrano-Vega et al. 2005).

Gel blot analysis of *HaKASIII* (Fig. 5) indicates that it is a single gene copy, as are the *KASIII* genes in *S. oleracea* (Tai and Jaworski 1993), *P. frutescens* (Hwang and Hwang 2000) and *J. curcas* (Li et al. 2008). The same analyses pointed to the existence of introns in the *HaKASIII* gene. This was supported by the identification of seven, very well conserved introns in *A. thaliana*, *Sorghum bicolor*, *O. sativa*, *Populus trichocarpa*, *R. communis*, *Carica papaya*, *V. vinífera*, *Physcomitrella patens*, *Z. mays*, *Medicago truncatula* and *Lotus japonicas* among others. Thus, *HaKASIII* is likely to also have seven introns.

The tissue expression of *HaKAS III* is nearly ten times higher in seeds than in other tissues, analogous to that observed in *A. thaliana* microarrays. In vegetative tissues, as expected for a unique copy gene, expression was constitutive. A clear pattern for *KAS III* expression during seed formation was not observed in sunflower, although it was in *A. thaliana, P. frutescens* (Hwang and Hwang 2000) and *J. curcas* (Li et al. 2008). These results are in accordance with the facts that fatty acid biosynthesis is an essential process in cells and that *HaKAS III* is a single copy gene, being expressed in all tissues as a housekeeping gene and

Table 1 Lipid content and fatty acid composition of *E. coli* strains bearing the vector pQE80L or the recombinant plasmid pQE80L::*HaKAS III* growing on rich medium supplemented with ampicillin plus/minus induction with IPTG

	Total (μg)	Fatty acids (mol%)								18/16[c]
		14:0	16:0	16:1[a]	17[b]	18:0	18:1[a]	19[b]	ΣSat	
pQE80L	474	5.5	47.5	14.4	17.0	0.9	13.2	1.5	53.8	0.19
pQE80L induced	475	6.2	47.2	13.8	18.2	0.9	11.9	1.8	54.3	0.18
pQE80L::*HaKAS III*	463	7.7	47.9	12.8	18.5	0.8	10.8	1.5	56.4	0.17
pQE80L::*HaKAS III* induced	220	7.2	40.3	21.1	9.3	1.5	18.6	2.0	49.0	0.31

Data are the average of three independent samples. SD <5% of mean value

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

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

[c] (18:0 + 18:1 + 19)/(16:0 + 16:1 + 17)

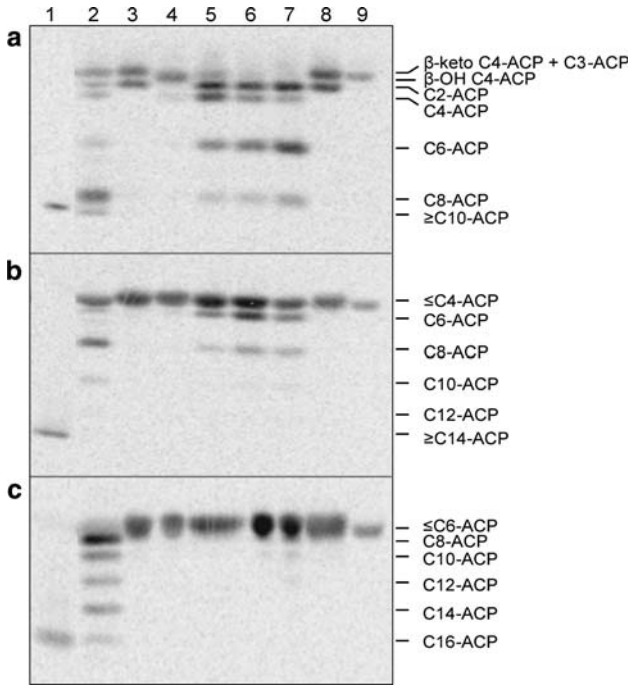

Fig. 8 PhosphorImager scans of the acyl-ACPs, as separated by 2 M (**a**), 3 M (**b**) and 4 M (**c**) urea-PAGE, after in vitro assays with the reconstituted *E. coli* FAS system lacking condensation activity. *Lane 1* C16-ACP, *lane 2* acyl-ACPs synthesized by *At*mtKAS, as detailed in "Materials and methods", are dominated by C8, *lane 3* C_2-ACP + C_3-ACP, *lane 4* *Ha*KAS III + KR 30 min, *lane 5* *Ha*KAS III + KR + ENR + DH 30 min, *lane 6* *Ha*KAS III + KR + ENR + DH 60 min, *lane 7* *Ha*KAS III + KR + ENR + DH 120 min, *lane 8* C_2-ACP + C_3-ACP, and *lane 9* C_3-ACP. *KR* β-Ketoacyl-ACP reductase, *ENR* enoyl-ACP reductase, *DH* β-hydroxyacyl-ACP dehydratase

increasing its expression in those tissues accumulating oil as a storage product, such as seeds. In contrast, higher expression levels were found in roots than in the high oil-containing seeds of *J. curcas* (Li et al. 2008).

Although nonsignificant changes in growth rate were observed, *E. coli* expressing *Ha*KAS III had circa 47% of the fatty acid content of the controls, indicating that the recombinant protein was not only active, but also interfered with normal fatty acid biosynthesis. Potentially, these changes in fatty acid biosynthesis reflect the necessity of the cells to maintain a given level of unsaturated fatty acids. When the mol% ratio of 18/16 acyl carbon chains is compared among the cells, induction resulted in almost a doubling of the ratio showing that relatively more elongations of existing chains took place compared to initiations of new chains. These results were unexpected since earlier work in which cloned *EcFabH* expressed in *E. coli* produced an elevation in saturated fatty acid content, a reduction in the levels of cis-vaccenic and sometimes an arrest of cell growth (Tsay et al. 1992; Verwoert et al. 1995). The only report existing in which the fatty acid composition of *E. coli* expressing a plant KAS III (Hwang and Hwang 2000) revealed that the expression of one of the isoenzymes found in *P. frutescens*, *Pf*KAS IIIa, produced an increase of saturated fatty acids, mainly myristic, and a reduction in palmitoleic acid. But, under the same conditions, the other isoenzyme, *Pf*KAS IIIb, did not produce any change in the fatty acid composition of the bacteria. All these observations point to the fact that (1) *Ha*KAS III and *Ec*FabH activities are not comparable, and (2) plant KAS III proteins show enough catalytic variability to be used in the transgenic manipulation of seed oil content and composition, with respect to the proportions of given chain lengths, as shown and suggested previously (Dehesh et al. 2001; Stoll et al. 2006).

The known *Ec*FabH crystal structure allowed us to model a structure for *Ha*KAS III. With the exception of the region corresponding to residues 251–286 in *Ha*KAS III, the structures are quite similar. The *Ec*FabH region corresponding to 251–286 is shorter and encompasses the secondary structure elements L9β1 and L9. The latter is one of the four elements that compose the monomer–monomer interface (Davies et al. 2000). The noted region is not well conserved among bacterial KAS IIIs (Qiu et al. 2001) or plant KAS IIIs. Combined, the observations of the structural model of the

*Ha*KAS III protein show that greater variability is possible in this region for monomer binding to produce the functional dimer than for the core structure of the monomers.

Initial experiments with purified recombinant spinach KAS III (Jaworski et al. 1994) revealed that C2-CoA, but not C2-, C4- or C6-ACP functioned as primers. On the other hand, results from experiments with spinach extracts (Jaworski et al. 1994) intimated that KAS III could also elongate C4- and C6-ACPs. Purified recombinant *C. wrightii* KAS III was shown to use C2-, C3- and C4-CoAs plus C2- and C4-ACPs (Abbadi et al. 2000a). *Ec*FabH protein, which prefers C2-CoA, is also capable of using C2 to C4 thioesters (Heath and Rock 1996). In vitro assays in which purified recombinant *Ha*KAS III was added to a reconstituted *E. coli* FAS system lacking condensation activity (Heath and Rock 1995; Yasuno et al. 2004) clearly revealed that *Ha*KAS III can use ACP acceptor substrates with eight carbons and potentially also with ten carbons, and is not limited to using C2-CoA in the initial condensation step. Combined, the above observations imply that substrate specificity of plant KAS IIIs are variable. More extreme examples include the ability of *B. subtilis*, FabH1 and FabH2, to use branched chain and C6-, C7- and C8-CoAs as primer substrates that neither the third *B. subtilis* FabH nor *Ec*FabH can (Choi et al. 2000a). Moreover, the *M. tuberculosis* FabH does not use short or branched chain acyl primers, but instead C8- to C20-CoAs with a preference for C12-CoA (Choi et al. 2000b). The capability of *Ha*KAS III to use medium chain acyl-ACP substrates is of interest with respect to the known inhibition by C12- to C20-acyl-ACPs of purified recombinant *Ec*FabH (the effect of shorter acyl-ACPs was not studied; Heath and Rock 1996), and of C10- and C12-ACPs to inhibit KAS IIIs in *Cuphea* seed extracts, which produce mainly medium chain length acyl-ACPs (Brück et al. 1996; Abbadi et al. 2000b). In the future, it will be interesting to study the effect of long-chain acyl-ACPs on *Ha*KAS III, and to produce a crystal structure to confirm the required modification of the acceptor binding site deduced from our results. This information will be essential if the goal of rationally modifying the chain length composition of oil seeds is to be attained.

Acknowledgments Our thanks are due to Marianne Mortensen for her skillful technical assistance. This work was supported by the Ministerio de Ciencia e Innovación and FEDER, project AGL2008-01086/ALI and the Danish Natural Sciences Research Council Grant Number 272-05-0401.

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