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Molecular cloning and characterization of the genes encoding a microsomal oleate Δ^{12} desaturase (CsFAD2) and linoleate Δ^{15} desaturase (CsFAD3) from *Camelina sativa*

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Abstract

Camelina sativa produces oil that is rich in polyunsaturated linoleic (18:2^{Δ9,12}) and linolenic (18:3^{Δ9,12,15}) acids. These fatty acids are obtained by the successive desaturation of oleic (18:1^{Δ9}) acid, which is catalyzed in the endoplasmic reticulum by two different microsomal desaturases: oleate Δ¹² desaturase (CsFAD2) and linoleate Δ¹⁵ desaturase (CsFAD3). The objective of the present study is to investigate the contribution of these two desaturases to the composition and properties of *C. sativa* seed oil, and look over the effect that temperature exerts on their activity. Three different copies of both these genes were identified, which when analysed contained three histidine rich motifs (HXCGHX, HRXHH and HVXHH) and six highly conserved transmembrane domains. Comparing their sequences, the CsFAD2 copies accommodated four conservative changes (Glu-36-Asp, Arg-48-His, Val-97-Ala and Ala-177-Pro) and two semi-conservative ones (Val-63-Ile and Leu-249-Met), whereas only one semi-conservative change (Ala-327-Ser) was detected in CsFAD3 but with two extra amino acids (His-147 and Gly-148). The CsFAD2 and CsFAD3 cDNAs were heterologously expressed in *Saccharomyces cerevisiae* to confirm that they were active enzymes and their dependence on temperature was investigated by growing the recombinant yeast cells at low (22 °C) and optimal (30 °C) temperatures. The conversion of 18:1^{Δ9} to 18:2^{Δ9,12} by CsFAD2 was slightly better at 30 °C than at 22 °C, and it was also able to desaturate palmitoleic acid (16:1^{Δ9}) to hexadecadienoic acid (16:2^{Δ9,12}). By contrast, the conversion driven by CsFAD3 was enhanced 5-fold at 22 °C compared to 30 °C, although it was not able to desaturate 16:2^{Δ9,12}. The distribution of these microsomal desaturases was also studied in *C. sativa*, which were expressed most strongly in expanding leaves and developing seeds.

Keywords: *Camelina sativa*, microsomal desaturase, FAD2, FAD3, fatty acid biosynthesis.

Abbreviations: DAF, days after flowering.

1. Introduction

Camelina sativa (L.) Crtz. is a member of the *Brassicaceae* family that was cultivated in Europe from the Bronze Age to the twentieth century, being abandoned after World War II (Al-Shehbaz, 1987; Bouby, 1998). There is now interest in resuscitating the use of *C. sativa* as a plant in biotechnology as it is phylogenetically close to the model plant *Arabidopsis thaliana* (Collins-Silva et al., 2011) and it can be readily transformed with *Agrobacterium* by floral dip infiltration under vacuum (Lu and Kang, 2008). Moreover, *C. sativa* presents some agronomic features that give it a competitive advantage over other oilseed crops (Budin et al., 1995). Indeed, it has a rather short vegetative period of about 4 months so that it could be incorporated into double cropping systems during cooler growth periods (Putnam et al., 1993), and it displays very efficient nutrient use that allows it to be grown with reduced nitrogen fertilization in semi-arid regions (Bramm et al., 1990; Zubr, 1997). In addition, *C. sativa* is an allelopathic plant that secretes secondary metabolites and phytoalexins in order to inhibit the development of pathogens, insect pests and neighbouring plants (Lovett and Jackson, 1980), reducing the dependence on pesticides.

The oil accumulated by *C. sativa* seeds is rich in polyunsaturated fatty acids. The most abundant fatty acids in *C. sativa* oil are linoleic ($18:2^{\Delta 9,12}$) and linolenic ($18:3^{\Delta 9,12,15}$) acids, as well as oleic acid ($18:1^{\Delta 9}$), each accounting for about 22%, 29%, and 17% of the oil fatty acids composition, respectively (Rodríguez-Rodríguez et al., 2013). In seeds, *de novo* fatty acid biosynthesis takes place in the plastids and it is catalyzed by the fatty acid synthase complex (FAS), producing palmitoyl-ACP ($16:0$ -ACP) and stearoyl-ACP ($18:0$ -ACP) as the final products (Pidkowitc et al., 2007), both of which can be desaturated by the soluble Δ^9 stearoyl-ACP desaturase to yield palmitoyl-ACP ($16:1^{\Delta 9}$ -ACP) and oleoyl-ACP ($18:1^{\Delta 9}$ -ACP: Shanklin and Cahoon, 1998; Rodríguez-Rodríguez et al., 2015). This pool of acyl-ACPs are hydrolysed by acyl-ACP thioesterases (Voelker et al., 1997; Rodríguez-Rodríguez et al., 2014), and the resulting free fatty acids are exported out of the plastid and esterified with coenzyme A by long-acyl-CoA-synthetases (Aznar-Moreno et al., 2014). These acyl-CoAs can be incorporated into phosphatidylcholine, and oleic acid can be further desaturated by endoplasmic reticulum desaturases to linoleic and linolenic acids (Kang et al., 2011). These

desaturated fatty acids are mainly transferred to triacylglycerols (TAG) through different pathways and they accumulate as an energy source in the seeds (Dyer et al., 2002).

Plant fatty acid desaturases can be classified into two main subfamilies: the soluble acyl-acyl carrier protein desaturases found in the stroma of plastids that mainly produce 18:1^{Δ9}-ACP (Shanklin and Cahoon, 1998); and the acyl-lipid desaturases that desaturate fatty acids esterified to glycerolipids, membrane bound-enzymes associated with the chloroplast (Ohlrogge and Browse, 1995) and endoplasmic reticulum membranes (Totcher et al., 1998) that regulate the linoleic and linolenic acid content in plants. The microsomal Δ^{12} oleate desaturase (1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12} desaturase or FAD2) desaturates oleic to linoleic acid in the ER, while linolenic acid is produced through the activity of the microsomal Δ^{15} linoleate desaturase (1-acyl-2-linoleoyl-*sn*-glycero-3-phosphocholine Δ^{15} desaturase or FAD3) that introduces the third double bond in linoleic acid esterified at the *sn*-2 position of phosphatidylcholine (Heinz, 1993). The reactions catalysed by FAD2 and FAD3 involve the concomitant reduction of molecular oxygen to water and they require the presence of NADH, NADH-cytochrome-*b*5 reductase and cytochrome-*b*5 (Smith et al., 1990). In terms of their structure, the FAD2 and FAD3 sequences from Arabidopsis (Arondel, et al., 1992), soybean (Bilyeu et al., 2003), rapeseed (Reed et al., 2000), olive (Hernández et al., 2005) or flax (Fofana et al., 2004) have a highly conserved C-terminal amino acid sequence that is involved in sub-cellular microsomal location, and there are three conserved histidine rich motifs or histidine boxes, which coordinate two iron atoms and form the typical di-iron centre present in all desaturases (Shanklin and Cahoon, 1998). Three different *FAD2* genes being expressed during *C. sativa* seed development have been described by Hutcheon et al. (2010) when studying the polyploidy of this species. In a similar way, when doing transcriptome studies in two developmental stages of *C. sativa* seed, Wang et al. (2015) described three *FAD3* genes.

Schulte et al. (2013) describe that fatty acid composition of *C. sativa* oil can be influenced by environment and variety, although the effects detected were smaller than those in other oil crops like sunflower, soybean or canola. Previously, Crowley and Frohlich (1998) found 2% less linolenic acid in *C. sativa* grown during a dry warm

year than in the same variety grown during a normal year. More recently, Vollmann and Eynck (2015) when reviewing the current knowledge about *C. sativa*, comment the dependence of linolenic acid content on genotype, agronomic treatment or environmental conditions, pointing the fact that, as described in other oilseeds, linolenic acid content was lower in warmer climates than in cooler ones (Seehuber 1984; Rodríguez-Rodríguez et al. 2013). These changes should be related to the temperature effect on desaturases activities, indicating that FAD2 and FAD3 proteins from *C. sativa* are more stable than the ones from other crops. To verify these hypotheses, we report here the cloning of microsomal oleate Δ^{12} (CsFAD2) and linoleate Δ^{15} (CsFAD3) desaturases from *C. sativa*, their expression in *Saccharomyces cerevisiae* and functional characterization. Furthermore, we identified the *CsFAD3* copies in the *C. sativa* genome and studied the expression of these genes in different plant tissues.

2. Materials and methods

2.1. Biological material and growth conditions

C. sativa plants of the CAS-CS0 cultivar were grown in growth chambers equipped with fertirrigation at 25/15 °C (day/night) with a 16 h photoperiod and at a light intensity of 250 $\mu\text{E m}^{-2}\text{s}^{-1}$. Pots were filled with Presstopf (tray 20/80) as substrate (Gramoflor GmbH & Co. KG, Vechta, Germany) and watered daily. Developing *C. sativa* seeds were collected during the active oil synthesis period (6-30 days after flowering, DAF), frozen in liquid nitrogen and stored at -80 °C.

The *Escherichia coli* XL1-Blue strain (Stratagen) was used as the plasmid host for the cloning of the *CsFAD2* and *CsFAD3* genes. Bacteria were grown in liquid LB media (1 % Bacto Tryptone, 0.5 % Yeast Extract and 1% NaCl [pH7]) shaken vigorously at 37 °C. Plasmid selection was performed in the presence of ampicillin (100 $\mu\text{g}/\mu\text{l}$).

Saccharomyces cerevisiae W303-1A strain (MATa, ade2-1, his3-11, leu2-3, 112 trp1-1, ura3-1, can1-100) was used as the plasmid host for the expression of microsomal *C. sativa* desaturase protein. Recombinant yeast was grown in poor, synthetic complete drop out medium (SC medium: 0.5% NH_4SO_4 , 0.5% YNB, 0.13% amino acids [pH 5.7]) supplemented with glucose (2% v/v) as the sole carbon source, and the liquid culture was shaken vigorously at low (22 °C) or optimal (30 °C) temperatures.

2.2. mRNA preparation and cDNA synthesis

Developing *C. sativa* seeds (approximately 0.25 g) were ground in liquid nitrogen using a precooled sterile mortar and pestle. Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and the mRNA was isolated from the total RNA using a GenElute mRNA Miniprep Kit (Sigma-Aldrich), resuspending the mRNA pellet in 33 μl RNAase-free TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8]). This mRNA was used as the template to synthesise cDNAs using the Ready-To-Go T- Primed First-Strand Kit (GE Healthcare Life Sciences, Uppsala, Sweden).

2.3. Cloning of genes encoding microsomal fatty acid desaturases from *C. sativa*

The FAD2 and FAD3 protein sequences from public databases were aligned using the ClustalX v1.8 program (Thompson et al., 1997) to identify highly conserved

regions that could be used to design primers for PCR amplification. RNA isolated from developing seeds of *C. sativa* was used as template and two different PCR fragments were amplified with degenerate primers designed from the highly conserved regions identified in FAD2 (Deg_FAD2_F and Deg_FAD2_R; see in Table 1) and FAD3 (Deg_FAD3_F and Deg_FAD3_R; see in Table 1). The 5'- and 3'- ends of the corresponding cDNAs were obtained using the SMART-RACE cDNA Amplification Kit (Clontech, France) and two pairs of specific primers for each gene: CsFAD2_F1, CsFAD2_R1, CsFAD2_F2 and CsFAD2_R2 (Table 1) for the *CsFAD2* gene; and CsFAD3_F1, CsFAD3_R1, CsFAD3_F2 and CsFAD3_R2 for *CsFAD3* (Table 1). The PCR fragments were cloned into the pMBL-T vector (Dominion MBL, Spain), sequenced and their identities were confirmed using the BLAST software (Altschul et al., 1990).

To identify different copies of microsomal Δ^{12} oleate desaturase and Δ^{15} linoleate desaturase in *C. sativa*, two specific pairs of primers for each microsomal desaturase were designed: CsFAD2_XmaI_F3 and CsFAD2_XmaI_R3 for the *CsFAD2* gene and CsFAD3_SpeI_F3 and CsFAD3_XmaI_R3 for the *CsFAD3* gene (Table 1). The PCR fragments amplified from the cDNA from *C. sativa* developing seeds with these pairs of primers, and that corresponded to the full open reading frames (ORF), were cloned into the pSpark vector (Canvax Biotech, Spain), sequenced and their identities were confirmed using the BLAST software (Altschul et al., 1990).

The coding regions of the mature proteins were amplified by PCR using the primers indicated above. The *CsFAD2* and *CsFAD3* complete ORFs were cloned into p416GDP(URA) at the *XmaI-XmaI* sites and p416GDP(LEU) at the *Spe-XmaI* sites vectors, respectively (Mumberg et al., 1995). Ligation into the correct reading frame (ORF) was confirmed by sequencing and the resulting constructs were named p416GDP(URA)::CsFAD2 and p416GDP(LEU)::CsFAD3.

2.4. Structure prediction of microsomal fatty acid desaturases from *C. sativa*

The structure of the *CsFAD2* and *CsFAD3* microsomal desaturases was predicted using the free bioinformatics software TopPred (Claros and von Heijne, 1994), which is based on identifying areas rich in hydrophobic residues. This, together with the comparison with previously proposed models (Hoffman et al., 1993), enabled the identification of the transmembrane domains (α -helix) and a peripheral membrane-

associated segment (von Heijne 1992). The hydrophobic value selected in TopPred was 0.5.

2.5. Cloning of microsomal CsFAD3 introns of *Camelina sativa*

Genomic DNA was isolated from *C. sativa* using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich). On the basis of the genomic structure of *FAD3* from *A. thaliana* (gene model in TAIR: AT2G29980.1), two pairs of primers were designed to amplify the intron-containing regions. The first of these, CsFAD3_F4 and CsFAD3_R4 (Table 1), amplified a DNA fragment of around 1150-1250 bp from the genomic DNA of *C. sativa*. The PCR products of the second pair of primers, CsFAD3_F5 and CsFAD3_R5 (Table 1), were around 1300-1400 bp. After amplification, the DNA fragments were cloned into the pSpark vector (Canvax) for sequencing.

2.6. Quantitative real time PCR of *CsFAD2* and *CsFAD3*

The cDNAs developed from different tissues were subjected to quantitative real time PCR (qRT-PCR) on a MiniOpticon system (Bio-Rad, CA, USA) using SYBR Green I (QuantiTect_ SYBR_ Green PCR Kit, Quiagen, Crawley, UK) and specific primer pairs: CsFAD2_qPCR_F and CsFAD2_qPCR_R for the *CsFAD2* gene (Table 1); and CsFAD3_qPCR_F and CsFAD3_qPCR_R for *CsFAD3* (Table 1). The lengths of the PCR products obtained were 166 bp and 122 bp, respectively. PCR reactions were heated to 50 °C for 2 min and then heated to 95 °C for 15 min before performing 40 PCR cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 15 s in which the resulting fluorescence was monitored. Calibration curves were drawn up using sequential dilutions of the cDNA from 15 DAF and these were used to estimate the transcript content of the calibrator gene. The Livak method (Livak and Schmittgen, 2001) was applied to calculate comparative expression between tissues. The *C. sativa* actin gene was used as an internal reference to normalize the relative amount of cDNA in each sample, amplified with the specific CsActin_qPCR_F and CsActin_qPCR_R primers (Table 1).

2.7. Functional Expression of *CsFAD2* and *CsFAD3* in yeast and fatty acid analysis

The W303-1A strain of *S. cerevisiae* was transformed using the lithium acetate method with the empty vectors, p416GDP(URA) and p416GDP(LEU),

p416GDP(URA)::CsFAD2 or p416GDP(LEU)::CsFAD3 (Elble, 1992). In addition, *S. cerevisiae* W303-1A was co-transformed using the same protocol with both plasmids, p416GDP(URA)::CsFAD2 and p416GDP(LEU)::CsFAD3. The strains containing the plasmids were selected on minimal agar plates lacking uracil or leucine, and isolated colonies were used to inoculate in 10 ml of minimal medium drop-out uracil (SC-URA) for p416GDP(URA) and p416GDP(URA)::CsFAD2; drop-out leucine (SC-LEU) for p416GDP(LEU) and p416GDP(LEU)::CsFAD3; and drop-out uracil and leucine for strain that incorporated p416GDP(URA)::CsFAD2 and p416GDP(LEU)::CsFAD3.

When the cultures reached an OD₆₀₀ of 0.8-1.2 absorption units (exponential growth phase), they were diluted to 0.1 with 50 ml of fresh medium. All the cultures were supplemented with 2% (v/v) glucose, and the recombinant yeast cells that harboured p416GDP(LEU) and p416GDP(LEU)::CsFAD3 were also complemented with 0.5 mM of the linoleic acid and 1% (w/v) tergitol-Nonidet P-40 (Sigma-Aldrich) as described previously (Venegas-Calderón et al., 2009). Growth was maintained until an OD₆₀₀ around 1.5 – 2.0 was reached at 30 °C or 22 °C.

The yeast cells were harvested by centrifugation at 1500 g for 5 min at 4 °C and they were washed with distilled water before determining the total fatty acid composition using the one-step method proposed previously (Garcés and Mancha, 1993). A volume of 3.3 ml methanol/toluene/dimethoxypropane/sulphuric acid (39:20:5:2) and 1.7 ml heptane was added to the cell pellet, and the mixture was heated at 80 °C for 1 h. After cooling, the upper phase containing the fatty acid methyl esters was transferred to a fresh tube, washed with 6.7% sodium sulphate and evaporated to dryness with nitrogen. The methyl esters were dissolved in an appropriate volume of heptane and analyzed by GLC. The different methyl esters were identified by comparing their retention times with those of known standards.

3. Results and discussion

3.1. Isolation and sequence analysis of *CsFAD2* and *CsFAD3* desaturases

Internal fragments of *CsFAD2* (610 bp) and *CsFAD3* (421 bp) genes were amplified from *C. sativa* cDNA using degenerate primer pairs (see Materials and Methods). These internal sequences allowed us to design two pairs of specific primers for each gene and through RACE, to obtain the full-length *CsFAD2* and *CsFAD3* cDNA clones of 1155 and 1164 bp, respectively. Alignment of the deduced amino acid sequences showed strong identity with the internal coding regions of known FAD2 and FAD3 microsomal desaturase proteins. Subsequently, specific primer pairs were designed to amplify all possible copies of the *CsFAD2* and *CsFAD3* genes, identifying three different copies for each microsomal desaturase: *CsFAD21*, 1155bp (GenBank accession number JN831155); *CsFAD22*, 1155bp (JN831156); *CsFAD23*, 1155bp (JN831157); *CsFAD31*, 1170bp (KJ541074); *CsFAD32*, 1164 bp (KJ541075) and *CsFAD33*, 1164 bp (KJ541076). The *CsFAD21*, *CsFAD22* and *CsFAD23* open reading frames (ORFs) identified encode predicted proteins of 384 amino acid residues (Figure 1), corresponding to molecular masses of 44.1 kDa and pI values of 8.1, 8.1 and 8.0, respectively. The ORFs corresponding to *CsFAD31*, *CsFAD32* and *CsFAD33*, each encode predicted proteins of 389 or 387 amino acid residues (Figure 2), and with an estimated molecular mass of 44.3, 44.5 and 44.4 kDa, respectively. All these proteins had pI values of 8.4.

Like other plant microsomal desaturases, the putative proteins encoded by the three *CsFAD2* and *CsFAD3* genes had three highly conserved histidine rich motifs or histidine boxes. These motifs are essential for desaturase activity and they are thought to form part of the di-iron centre where oxygen activation and hydrogen subtraction takes place in the desaturases, hydroxylases and epoxxygenases found in bacteria, fungi, plants and animals (Shanklin et al., 1997). The position of the eight histidine residues in *C. sativa* desaturases were: His-106, 110, 142, 145, 149, 316, 319 and 320 for *CsFAD2* (Figure 1); and His-98, 103, 133, 138, 139, 303, 306 and 307 for *CsFAD3* (Figure 2). Moreover, the spacing between them was also conserved. The first histidine box is named HXCGHX, the non-conserved positions corresponding to glutamic acid (Glu-107) and another histidine residues (His-111) that are probably involved in

desaturase activity in CsFAD2 (Shanklin et al., 1994). In CsFAD3, these residues were aspartic acid (Asp-103) and glycine (Gly-108). The last amino acid position of the first histidine box in *Carthamus tinctorius* and *Lesquerella lindheimeri* was glutamine, a polar amino acid with similar physicochemical properties to glycine and related to its hydroxylase activity (Dauk et al., 2007; Cao et al., 2013). The second and third histidine rich motifs were HRXHH and HVXHH. The non-conserved amino acids found in these CsFAD2 domains were arginine (Arg-144) and alanine (Ala-318), and in CsFAD3 they were threonine (Thr-137) and isoleucine (Ile-309).

Earlier studies on oleate desaturase enzymes from soybean showed that the serine located 36 amino acids after the second histidine-box (Ser-185) can be phosphorylated during seed development, predicting a post-translational regulatory mechanism of FAD2 activity during safflower seed development and oil accumulation (Tang et al., 2005). The RcFAD2, HaFAD2 and OsFAD2 desaturases have residues susceptible to be phosphorylated at the same position (Thr-181, Ser-179 and Tyr-162, respectively), whereas the Met-185 residue in oleate desaturase from *C. sativa* would not normally be phosphorylated and thus, it is probable that this enzyme could be subjected to some other post-translational regulatory mechanism that is currently unknown (Figure 1).

A phylogenetic tree based on the deduced amino acid sequences and their relationships to other known plant microsomal desaturases is shown in Figure 3. Each set of three microsomal desaturases identified here were situated in the ω -3 or ω -6 desaturase group, very close to the *A. thaliana* and *Arabidopsis lyrata* enzymes. This relationship has been shown previously when characterizing other *C. sativa* proteins involved in fatty acid biosynthesis (Rodríguez-Rodríguez et al., 2014, 2015) and corroborated by whole-genome sequencing (Kagale et al., 2014). In both cases, CsFAD2 and CsFAD3 proteins are phylogenetically closer to microsomal desaturases from dicotyledonous species like *Glycine max*, *Ricinus communis* or *Populus trichocarpa*, than those from monocotyledonous species, *Sorghum bicolor* or *Zea mays* (Figure 3).

3.2. Structure of microsomal desaturases from *C. sativa*

The structural models of CsFAD2 and CsFAD3 desaturases are comprised of nine consecutive domains, numbered I to IX (Figure 4): (I) N-terminal cytosolic region; (II)

two transmembrane α -helices (TM1 and TM2) connected by a short loop in the ER lumen; (III) cytosolic region containing the first histidine box (HXCGHX); (IV) first peripheral membrane-associated segment (PMS1); (V) second histidine box (HRXHH); (VI) second peripheral membrane-associated segment (PMS2); (VII) cytosolic loop connecting PMS2 and TM3; (VIII) second set of transmembrane α -helices (TM3 and TM4) connected by a short loop in the reticular lumen; and (IX) C-terminal cytosolic region of the protein with the third histidine box (HVXHH). The amino acid residues that formed the transmembrane domains and peripheral membrane-associated segments are also shown in Figures 1 and 2. Moreover, after the third histidine rich motif in the C-terminal region (domain IX), the putative CsFAD2 and CsFAD3 polypeptides contain sequences necessary and sufficient for ER localization: the CsFAD3 enzyme contains a functional prototypic dilysine ER retrieval motif (-KSKIN: Figure 1), while the CsFAD2 enzyme contains an atypical C-terminal aromatic acid sequence (-YNNKL: Figure 2; McCartney et al., 2004).

3.3. Genomic organization of *C. sativa* microsomal Δ^{15} linoleate desaturase

C. sativa is a hexaploid organism with twenty chromosomes ($n=3x=20$; Gehringer et al., 2006), which could be the result of the combination of two or more genomes, or the triplication of an original genome in an autopolyploidized (6x) organism (Hutcheon et al., 2010). The alignment of the different CsFAD2 and CsFAD3 genes found here, along with the sequences published by the *C. sativa* Genome Project (part of The Prairie Gold Project), allow these genes to be located within the *C. sativa* genome. Thus, the *CsFAD21*, *CsFAD22* and *CsFAD23* genes are located on chromosomes 1, 15 and 19, respectively, whereas *CsFAD31*, *CsFAD32* and *CsFAD33* are on chromosomes 5, 16 and 7, respectively.

The *CsFAD2* genes have one intron in the 5'UTR, whereas the *CsFAD3* genes have 8 exons and 7 introns, with a similar genomic structure to *A. thaliana* and *Oryza sativa* FAD3 genes (Kodama et al., 1997). Two genomic DNA fragments from the *CsFAD3* loci were amplified to analyse their genomic organization, producing two PCR products. After the assembly of the two genomic fragments, three clones of 3297, 3296 and 3277 bp were found for *CsFAD31*, *CsFAD32* and *CsFAD33*, respectively. The intron/exon organization of the three *CsFAD3* genes was revealed by aligning the cDNA

and genomic DNA sequences (Figure 5), which finally revealed a structure with 8 exons and 7 introns. The length of the *CsFAD3* exons was identical, except for exon E3 of *CsFAD31* that has six additional nucleotides, coding for the two extra amino acid residues that are present in the *CsFAD31* enzyme (His-144 and Gly-145: Figure 5).

The lengths and sequences of introns 2, 4, 5 and 6 are highly conserved, differing in only two or three nucleotides. By contrast, there is high genetic variability in intron 1. This intron has two insertions at the beginning of the *CsFAD32* gene, causing the difference in length between this and the other genes (*i1CsFAD31*, 609 bp; *i1CsFAD32*, 631 bp; *i1CsFAD33*, 609 bp: Figure 5). It was also remarkable that the first histidine rich motif (HDCGHG) is interrupted by intron 1, such that this histidine box is encoded by six nucleotides in exon 1 and the first nine nucleotides of the exon 2. Moreover, there are many single nucleotide polymorphisms in intron 3 and some deletions in the *CsFAD32* gene (*i3CsFAD3*, 532 bp; *i3CsFAD3*, 522 bp; *i3CsFAD3*, 527 bp: Figure 5).

There is strong sequence identity between the *CsFAD31* and *CsFAD33* genes when compared to *CsFAD32*, although the latter contains more insertions and deletions that affect the length of introns 1 and 3. These results argue against the hypothesis of the *C. sativa* genome resulting from two allopolyploidy events and suggest that it derives from the combination of an autotetraploid (4x) and diploid (2x) species, in an autopolyploidized (6x) genome. The chromosome combination could be 7+7+6 or 6+6+8, producing the total chromosome number of 20 (Hutcheon et al., 2010).

3.4. *CsFAD2* and *CsFAD3* expression in different tissues

CsFAD2 and *CsFAD3* fulfil important cellular functions related to plant viability and in defining the fatty acid profile of the accumulated seed oil (Browse and Xin, 2001). In this regard, the expression profile of microsomal desaturases from *C. sativa*, was investigated by qRT-PCR analysis in cotyledons, hypocotyls, leaves, roots and developing seeds.

These genes were expressed weakly in hypocotyls, cotyledons and roots (Figure 6). As expected, stronger expression was evident in leaf tissue due to the fact that polyunsaturated fatty acids are the most abundant in thylakoid lipids, such as

monogalactosyldiacylglycerols and digalactosyldiacylglycerols (Li-Beisson et al. 2013). The *CsFAD3* gene was expressed 24-fold more strongly than the *CsFAD2* gene in this tissue. An important part of the polyunsaturated fatty acids present in leaves is produced by plastidial omega-3 desaturase. However, studies on *A. thaliana* revealed that the desaturation of 18:2^{Δ9,12} to 18:3^{Δ9,12,15} catalysed by microsomal Δ¹⁵ desaturase was responsible for around 40% of the activity in leaves, 50% of that in the stem and 60 % of that in the roots (data estimated from the results of Schmid et al., 2005).

The *CsFAD2* and *CsFAD3* genes are expressed strongly in developing seeds, particularly from 6 to 12 DAF and from 18 to 24 DAF, corresponding with the phases of seed expansion and oil accumulation respectively. Finally, the expression of these genes fell during the desiccation period (Figure 6; Rodríguez-Rodríguez et al., 2013). *CsFAD2* expression was about 7-fold stronger than that of *CsFAD3* genes at the beginning of development (6 DAF), adjusting to the composition of *C. sativa* oil in that period and with the pathway of fatty acids biosynthesis itself. This is particularly relevant given that the product of the reaction catalysed by *CsFAD2* is the substrate of *CsFAD3*, and the linoleic and linolenic acid content at 6 DAF is 21.3% and 4.3%, respectively (Rodríguez-Rodríguez et al., 2013). At later stages of development *CsFAD2* and *CsFAD3* were expressed similarly (Figure 6).

3.5. Functional characterization of *CsFAD2* and *CsFAD3* desaturases in *S. cerevisiae*

S. cerevisiae is a good model for the functional characterization of microsomal desaturases (Los and Murata, 1998). In order to confirm the functional identity of the *FAD2* genes found in *C. sativa* and explore the substrate-product relationships, we cloned the corresponding ORF of one of the three *CsFAD2* genes into the p416GDP(URA) expression vector under the control of the constitutive GDP promoter and we used it to transform *S. cerevisiae* cells. As indicated above, the differences between the three genes of *CsFAD2* do not appear to be functionally significant, involving four conservative changes (Glu-36-Asp, Arg-48-His, Val-97-Ala and Ala-177-Pro) and only two semi-conservative changes (Val-63-Ile and Leu-249-Met) located in non-relevant areas (Figure 1).

While polyunsaturated fatty acids, including hexadecadienoic (16:2^{Δ9,12}) and linoleic acids, were not produced in the control yeast cells transformed with the empty

p416GDP(URA) vector, these two fatty acids were produced by yeast cells transformed with the plasmid harbouring *CsFAD2*, p416GDP(URA)::*CsFAD2*, with linoleic acid being more abundant than hexadecadienoic acid (Figure 7). In transformed yeast cells grown at 30 °C, the 16:2^{Δ9,12} and linoleic acid content reached around 2.6% and 21.0%, respectively (Table 2). When the influence of temperature on *CsFAD2* activity was studied the conversion from 18:1^{Δ9} to 18:2^{Δ9,12} at 30 °C was similar to that seen at 22 °C (40.5% and 38.9%, respectively: Table 3). The 16:0 content at both temperatures increased to 15.5%, 3-fold higher than that found in control cells, which probably compensated for the accumulation of unsaturated fatty acids (Table 2). These data indicated that *CsFAD2* encodes a functional microsomal Δ^{12} oleate desaturase enzyme that can recognize two substrates, palmitoleic and oleic acid, of which the latter is the preferred substrate in yeast cells. This result was similar to that obtained for *FAD2* from *Xanthoceras sorbifolia* (Guo et al., 2013), although it differed from the majority of the *FAD2* desaturases expressed in yeast that only desaturated 18:1 to 18:2^{Δ9,12}, like olive *FAD2* (Hernández et al., 2005), soybean *FAD2* (Li et al., 2007) and peanut *FAD2*, (Mietkiewska et al., 2006).

To obtain a recombinant yeast expressing *FAD3*, one of three genes described above for *CsFAD3*, *CsFAD31*, was cloned into p416GDP(LEU). There are only small amino acid differences between this and the other two genes, with only one semi-conservative change (Ala-327-Ser) and two extra amino acids (His-147 and Gly-148 in *CsFAD31*). The fatty acid profile of yeasts cells transformed with p416GDP(URA) or the empty p416GDP(LEU) vector was similar, and the main difference between control yeast cells and those harbouring p416GDP(LEU) was related to the high 18:2^{Δ9,12} content in transformants as they were grown in the presence of linoleic acid. Here, the content of palmitic acid also increased, as in yeast cell expressing *CsFAD2* (Figure 7).

Yeast cells expressing *CsFAD3* could produce linolenic acid, which was not present in yeast containing the empty control vector (Table 3). The rate of linoleic to linolenic acid conversion in these recombinant yeast cells depended on the temperature and like the omega-3 fatty acid desaturase from soybean (Román et al., 2012), it was higher at 22 °C (46%) than at 30 °C (9.3%). Moreover, the linolenic acid content was five times higher in cells grown at low temperature than in cells grown at the optimal temperature (25.2% against 4.7%: Table 2), an effect that could be due to

the lower levels of β -oxidation displayed by *S. cerevisiae* cells (Veenhuis and Goodman, 1990).

Yeast cells co-transformed with p416GDP(URA)::CsFAD2 and p416GDP(LEU)::CsFAD3 display an interesting fatty acid profile, containing palmitolinoleic, linoleic and linolenic acid (Figure 7). Hence, CsFAD2 and CsFAD3 microsomal desaturases appear to have been well integrated into yeast metabolism and they can act sequentially to produce linolenic acid from oleic acid. The linoleic content was slightly lower at 22 °C (27.4 %) than at 30 °C (34.0%: Table 2), although the linolenic acid content was higher at the lower temperature (12.1%) than at the optimal growth temperature (3.1%: Table 2). These results suggest that microsomal Δ^{12} oleate desaturase is slightly more active at the optimal growth temperature and indeed, the conversion rate of 18:1 ^{Δ^9} to 18:2 ^{$\Delta^{9,12}$} at 30 °C was 52.1% as opposed to 43.7% at 22 °C (Table 3). This difference in conversion was not patent when the yeast cells expressed CsFAD2 alone, probably because this desaturase is inhibited by its product in such conditions.

Both effects, slightly increase of FAD2 activity at higher temperature and the 5-fold increase of FAD3 activity at lower temperature, explain the correlation observed by Schulte et al. (2013) in *C. sativa* plants between linoleic and linolenic acids contents with the mean high temperature during the grain fill, the increase of linoleic acid and the reduction on linolenic acid content at higher growth temperatures.

Conclusions

The cloning and sequencing of CsFAD2 and CsFAD3 from *C. sativa* seeds identified three different genes of these enzymes that bear no relevant changes in their amino acids sequences. Both families of desaturases have six transmembrane domains and three highly conserved histidine rich motifs (HXCGHX, HRXHH and HVXHH) involved in the coordination of the two iron atoms essential for their desaturase activity. Moreover, the genomic organization of CsFAD3 is similar to that reported in *A. thaliana* and *Oryza sativa*, with 8 exons and 7 introns. An analysis of the genomic sequences of CsFAD3 support the hypothesis that *C. sativa* probably

originated through the combination of an autotetraploid (4x) and a diploid (2x) species into an allopolyploidized (6x) genome.

Recombinant *S. cerevisiae* expressing CsFAD2 can desaturate 16:1^{Δ9} to 16:2^{Δ9,12} and 18:1^{Δ9} to 18:2^{Δ9,12}, displaying stronger preference towards oleic acid. CsFAD3 expressed in yeast cells can desaturate 18:2^{Δ9,12} to 18:3^{Δ9,12,15} being more active at lower temperatures correlating with the temperature effect observed on linolenic acid content. CsFAD2 and CsFAD3 were expressed strongly in developing *C. sativa* seeds, reaching a maximum at 18 DAF. They were also expressed strongly in leaf tissue, indicating that they contribute to the synthesis of linolenic acid present in photosynthetic lipids.

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FIGURES CAPTIONS.

Figure 1. Alignment of the deduced amino acid sequences of the microsomal Δ^{12} oleate desaturase enzyme from *C. sativa* (CsFAD21, AFQ60940; CsFAD22, AFQ60941; CsFAD23, AFQ60942), *A. thaliana* (AtFAD2, NP187819), *Ricinus communis* (RcFAD2, XP002530704), *Helianthus annuus* (HaFAD2, AF251842), *Oryza sativa* (OsFAD2, BAC45173). The histidine rich motifs are in boxes, while the transmembrane α -helices (TM), peripheral membrane-associated segment (PMS) and endoplasmic reticulum localization signal are underlined. The conservative and semi-conservative changes in the amino acid sequence between three CsFAD2 proteins are marked with a black and white dot, respectively.

Figure 2. Alignment of the deduced amino acid sequences of the microsomal Δ^{15} linoleate desaturase enzyme from *C. sativa* (CsFAD31, KJ541074; CsFAD32, KJ541075; CsFAD33, KJ541076), *A. thaliana* (AtFAD3, NP180559), *Ricinus communis* (RcFAD3, XP002525657), *Helianthus annuus* (HaFAD3, AAP78965), *Oryza sativa* (OsFAD2, NP001060733). The histidine rich motifs are in boxes, while the transmembrane α -helices (TM), peripheral membrane-associated segment (PMS) and endoplasmic reticulum localization signal are underlined. The semi-conservative change in the amino acid sequence between three CsFAD3 proteins is marked with white black dot and the two extra amino acids of CsFAD31 are indicated with a black square.

Figure 3. Phylogenetic comparison of plant microsomal Δ^{12} oleate and Δ^{15} linoleate desaturases. Plant species included in the phylogenetic tree are: *A. thaliana*, *Arabidopsis lyrata*, *C. sativa*, *Medicago truncatula*, *Glycine max*, *Physcomistrella patens*, *Populus trichocarpa*, *Ricinus communis*, *Sorghum bicolor*, *Zea mays*. The phylogenetic tree was rooted using the sequence of microsomal desaturase from Cyanobacteria of the genus *Anabaena* and *Pseudanabaena*.

Figure 4. Structural prediction of the microsomal desaturase from *C. sativa*: PM, transmembrane domain (α -helices); PMS, peripheral membrane-associated segment; His, histidine box; Fe, ferrous ion (Fe^{2+}).

Figure 5: Microsomal Δ^{15} linoleate desaturase gene of *C. sativa*. Diagram showing the three genes of the microsomal Δ^{15} linoleate desaturase genes from *C. sativa*. The boxes represent the exons while introns are represented by lines and numbered in Roman numerals.

Figure 6. Expression of *CsFAD2* (black columns) and *CsFAD3* (white columns) genes in the developing seeds and vegetative tissues of *C. sativa*. The values represent the mean values $\pm$ SD of three independent samples.

Figure 7. The fatty acid composition of the total lipid extracted from yeast cells grown at 22 °C and transformed with p416GDP(URA), p416GDP(URA)::*CsFAD2*, p416GDP(LEU), p416GDP(LEU)::*CsFAD3*, and co-transformed with p416GDP(URA)::*CsFAD2* and p416GDP(LEU)::*CsFAD3*: 16:0, palmitic acid; 16:1 $^{\Delta 9}$, palmitoleic acid; 16:1 $^{\Delta 9,12}$, hexadecadienoic acid; 18:0, stearic acid; 18:1 $^{\Delta 9}$, oleic acid; 18:2 $^{\Delta 9,12}$, linoleic acid; 18:3 $^{\Delta 9,12,15}$, linolenic acid.

713 **TABLES.**
714

Table 1
Degenerate and non-degenerate oligonucleotide primers used in this work

Primer name	Sequence 5' - 3'
CsFAD2	
Deg_FAD2_F	DCCVTGYGAGAMRCCDCC
Deg_FAD2_R	DATYARAAGHGGDACYCC
CsFAD2_F1	ACTGACATCATTGTTGCCTCCTGC
CsFAD2_F2	TCTGGAGGCGTTACGGTCG
CsFAD2_R1	CCGTCACCATCCAACACAGG
CsFAD2_R2	GGAGCGTTGGGGAAGAAATGGC
CsFAD2_XmaI_F3 ^a	TAT <u>CCCGGG</u> ATGGGTGCAGGTGG
CsFAD2_XmaI_R3	AT <u>ACCCGGG</u> TCATACTATTGTTGTACC
CsFAD2_qPCR_F	CACAACATCACGGACACACA
CsFAD2_qPCR_R	TAGATACACTCCTTTGCCCC
CsFAD3	
Deg_FAD3_F	RGTYCTHAAAGTYTAYGG
Deg_FAD3_R	RTRWMCGTWGAKATYTGG
CsFAD3_F1	TGGACGCTGTCACTTATTTACATCACC
CsFAD3_F2	AATACTTGCGACCAAACCTCTCCACC
CsFAD3_R1	ATTCATCACGACATTGGAACACG
CsFAD3_R2	CGTTTGTGGTTCTCTGTAGTATCTTCC
CsFAD3_SpeI_F3	ATC <u>ACTAGT</u> ACCATGGTTGTTGCTATGGACAAACG
CsFAD3_XmaI_R3	TAG <u>CCCGGG</u> ATTTTAGACTTGTCAGAAGCGTAAACG
CsFAD3_F4	TGGGCCATCTTCGTCC
CsFAD3_R4	TTTTCAACATGGCCATGG
CsFAD3_F5	ATGGCCATGTTGAAAACG
CsFAD3_R5	GCTCCTGACGTTTGTGG
CsFAD3_qPCR_F	ACTTGGTGGAGAGTTTGGTCG
CsFAD3_qPCR_R	GATTTTAGACTTGTCAGAAGCG
CsActin	
CsActin_qPCR_F	TTGGAAGGATCTGTACGGTAAC
CsActin_qPCR_R	TGTGAACGATTCTGGACC

^a The restriction sites introduced are underlined.

H: A or C or T; K: G or T; N: A or C or G or T; R: A or G; Y: C or T.

All primers were synthesized by Eurofins MWG Operon (Germany).

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Table 2

Fatty acid composition of *S. cerevisiae* cells over expressing CsFAD2 and CsFAD3 microsomal desaturase and grown at two different temperatures.

Construct	°C	Fatty acid composition (mol%)							
		16:0	16:1 ^{Δ9}	16:2 ^{Δ9,12}	18:0	18:1 ^{Δ9}	18:2 ^{Δ9,12}	18:3 ^{Δ9,12,15}	Otros
p416GDP(URA)	22	5.0 ± 0.0	25.4 ± 0.1	0.0 ± 0.0	5.1 ± 0.1	60.6 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	3.9 ± 0.1
	30	8.1 ± 0.4	27.2 ± 3.0	0.0 ± 0.0	4.8 ± 1.1	54.5 ± 2.1	0.0 ± 0.0	0.0 ± 0.0	5.5 ± 0.3
p416GDP(URA)::CsFAD2	22	15.5 ± 2.0	25.6 ± 0.8	2.6 ± 0.2	7.7 ± 0.4	20.4 ± 1.8	26.7 ± 0.8	0.0 ± 0.0	1.6 ± 0.0
	30	15.4 ± 1.6	21.0 ± 0.4	2.6 ± 0.1	9.3 ± 0.3	22.1 ± 0.7	26.7 ± 1.1	0.0 ± 0.0	2.9 ± 0.1
p416GDP(LEU)	22	15.9 ± 0.8	20.0 ± 2.6	0.0 ± 0.0	6.1 ± 0.6	21.6 ± 2.8	35.6 ± 4.4	0.0 ± 0.0	0.9 ± 0.2
	30	20.0 ± 0.3	9.8 ± 1.4	0.0 ± 0.0	9.2 ± 0.4	15.8 ± 1.8	44.4 ± 3.3	0.0 ± 0.0	0.7 ± 0.2
p416GDP(LEU)::CsFAD3	22	14.6 ± 0.3	8.2 ± 0.8	0.0 ± 0.0	8.3 ± 0.2	13.3 ± 1.1	29.7 ± 2.3	25.2 ± 0.1	0.7 ± 0.0
	30	16.5 ± 0.9	6.3 ± 0.4	0.0 ± 0.0	11.4 ± 0.2	14.2 ± 1.2	46.1 ± 1.2	4.7 ± 0.7	0.9 ± 0.0
p416GDP(URA)::CsFAD2	22	7.7 ± 0.0	18.7 ± 0.2	2.6 ± 0.1	8.7 ± 0.4	20.0 ± 0.2	27.4 ± 0.2	12.1 ± 0.4	2.8 ± 0.0
p416GDP(LEU)::CsFAD3	30	10.8 ± 1.5	18.3 ± 2.4	4.1 ± 0.3	9.6 ± 0.6	16.7 ± 0.6	34.0 ± 2.4	3.1 ± 1.1	3.3 ± 0.1

16:0, palmitic acid; 16:1^{Δ9}, palmitoleic acid; 16:2^{Δ9,12}, hexadecadienoic acid; 18:0, stearic acid; 18:1^{Δ9}, oleic acid; 18:2^{Δ9,12}, linoleic acid; 18:3^{Δ9,12,15}, linolenic acid; Others: 20:0 eicosanoic acid; 22:0, behenic acid; 24:0 lignoceric acid.

The data are the mean ± SD of three independent experiments.

Table 3

Conversion of palmitic, oleic and linoleic acid in *S. cerevisiae* cells overexpressing CsFAD2 and CsFAD3 microsomal desaturases, and grown at two different temperatures.

Construct	°C	Conversion (%mol)		
		16:1 ^{Δ9} to 16:2 ^{Δ9,12}	18:1 ^{Δ9} to 18:2 ^{Δ9,12}	18:2 ^{Δ9,12} to 18:3 ^{Δ9,12,15}
p416GDP(URA)	22	0.0 ± 0.0	1.1 ± 0.1	0.0 ± 0.0
	30	0.0 ± 0.0	1.1 ± 0.2	0.0 ± 0.0
p416GDP(URA)::CsFAD2	22	9.1 ± 0.2	38.9 ± 0.0	0.0 ± 0.0
	30	11.1 ± 0.6	40.5 ± 0.6	0.4 ± 0.1
p416GDP(LEU)	22	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
	30	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
p416GDP(LEU)::CsFAD3	22	0.0 ± 0.0	0.0 ± 0.0	46.0 ± 2.0
	30	0.0 ± 0.0	0.0 ± 0.0	9.3 ± 1.5
p416GDP(URA)::CsFAD2	22	12.1 ± 0.4	43.7 ± 0.2	30.7 ± 0.8
p416GDP(LEU)::CsFAD3	30	18.4 ± 0.3	52.1 ± 3.9	8.3 ± 2.4

16:1^{Δ9} to 16:2^{Δ9,12}: $([16:2^{\Delta9,12}] / [16:1^{\Delta9}] + [16:2^{\Delta9,12}]) * 100$.

18:1^{Δ9} to 18:2^{Δ9,12}: $([16:2^{\Delta9,12}] + [18:2^{\Delta9,12}] / [16:1^{\Delta9}] + [16:2^{\Delta9,12}] + [16:2^{\Delta9,12}] + [18:2^{\Delta9,12}]) * 100$.

18:2^{Δ9,12} to 18:3^{Δ9,12,15}: $([18:3^{\Delta9,12,15}] / [18:2^{\Delta9,12}] + [18:3^{\Delta9,12,15}]) * 100$.

16:1^{Δ9}, palmitoleic acid; 16:2^{Δ9,12}, hexadecadienoic acid; 18:1^{Δ9}, oleic acid; 18:2^{Δ9,12}, linoleic acid; 18:3^{Δ9,12,15}, linolenic acid;

The data are the mean ± SD from three independent experiments.

Figure 1

<i>CsFAD31</i>	-----MDKRSNVNGD-----PGAG-----DQKKKG-----	20
<i>CsFAD32</i>	-----MDKRSNVNGD-----PGAG-----DQKKKG-----	20
<i>CsFAD33</i>	-----MDKRSNVNGD-----PGAG-----DQKKKG-----	20
<i>AtFAD3</i>	-MVMAMDQRTNVNGD-----PGAG-----DRKKE-----	23
<i>RcFAD3</i>	MSAVMLARATNVAANNVTSRTGSHERPHTPAAAAHFNLSLCLSPFKGSLPLQLEGILLMKKADSVNAFDALDGD-----	75
<i>HaFAD3</i>	MAGLVISGCIAIKPFSG---SLPIPTKRFTITNPNSINLLHPKDPIFSNFHGFGRSAWKVSAP-LRIPSIDQQDLDDLDER	76
<i>OsFAD3</i>	MARLLISGVAPLP-----LPCRR----RAIAFALP-----LGNVRLRLRVAAPTSRSVATVEEDNNENNAPP	58

<i>CsFAD31</i>	-----EGFDPSAQPPPKIGDIRAAIPKHCHWKSILRSMYSYVRDICAAVALAVAAYVFNSWFLNPLYWAAQGTL	89
<i>CsFAD32</i>	-----EGFDPSAQPPPKIGDIRAAIPKHCHWKSILRSMYSYVRDICAAVALAVAAYVFNSWFLNPLYWAAQGTL	89
<i>CsFAD33</i>	-----EGFDPSAQPPPKIGDIRAAIPKHCHWKSILRSMYSYVRDICAAVALAVAAYVFNSWFLNPLYWAAQGTL	89
<i>AtFAD3</i>	-----ERFDPSAQPPPKIGDIRAAIPKHCHWKSILRSMYSYVRDIIVAALATAAAYVDSWFLNPLYWAAQGTL	92
<i>RcFAD3</i>	-----ADFDPSEFPFKIABIRAAIPKHCHWNKRWRSLSYVRDAFVVLAALAAAFLDLSCWCPFWLYWAAQGTLM	144
<i>HaFAD3</i>	ERISSLDVQEEEIFDAGAFPPEKLADIRAAIPKRCWVKDEWRSMYSYVRDVAIVLGAAAAHLNNWLWPLYWAAQGTLM	156
<i>OsFAD3</i>	-----PPCEDFDPGAAPPGLADIRAAIPKHCHWVKDEWRSMGYVRDVVVVFALAAAAARLHSCLAWPLYWAAQGTLM	130

<i>CsFAD31</i>	FWAEFVLGHDCGHGGSFSDIPLLNSVVGHLHLSFILVPYHGWRISRTHHQNHHGCHVENDESWEVFFPERVYKNLPHSTRM	169
<i>CsFAD32</i>	FWAEFVLGHDCGHGGSFSDIPLLNSVVGHLHLSFILVPYHGWRISRTHHQNHG--HVENDESWEVFFPERVYKNLPHSTRM	167
<i>CsFAD33</i>	FWAEFVLGHDCGHGGSFSDIPLLNSVVGHLHLSFILVPYHGWRISRTHHQNHHG--HVENDESWEVFFPERVYKNLPHSTRM	167
<i>AtFAD3</i>	FWAEFVLGHDCGHGGSFSDIPLLNSVVGHLHLSFILVPYHGWRISRTHHQNHHG--HVENDESWEVFFPERVYKLLPHSTRM	170
<i>RcFAD3</i>	FWAEFVLGHDCGHGGSFSDTLLNNVVGHLHLSFILVPYHGWRISRTHHQNHHG--NQDKDESWEVFLPEKIYNLSDVSTRI	222
<i>HaFAD3</i>	FWAEFVLGHDCGHGGSFNNAKLNSVVGHLHLSFILVPYHGWRISRTHHQNHHG--HVENDESWEHFLTEKTEKSLDWITRT	234
<i>OsFAD3</i>	FWAEFVLGHDCGHGGSFNSNRSLNSVVGHLHLSFILVPYHGWRISRTHHQNHHG--HVQKDESWEHFLPERLYRSLNRATRIM	208

<i>CsFAD31</i>	LRYTVPLEMLAYPLYL CYRSPGKESGSHFNYSLEFAEERKLIATSTTCWSIMFVTLLIALSIFFGPLSVLKVYGVPYMIIF	249
<i>CsFAD32</i>	LRYTVPLEMLAYPLYL CYRSPGKESGSHFNYSLEFAEERKLIATSTTCWSIMFVTLLIALSIFFGPLSVLKVYGVPYMIIF	247
<i>CsFAD33</i>	LRYTVPLEMLAYPLYL CYRSPGKESGSHFNYSLEFAEERKLIATSTTCWSIMFVTLLIALSIFFGPLSVLKVYGVPYMIIF	247
<i>AtFAD3</i>	LRYTVPLEMLAYPLYL CYRSPGKESGSHFNYSLEFAEERKLIATSTTCWSIMFVSLIALSYFGPLSVLKVYGVPYIIIF	250
<i>RcFAD3</i>	MRTVPLEBLLAYPYLWTRGPCKDGSHSFBSYSLFAENERRAVLTSTVCWIMILLILLSYCVFSGVQVQLKVYGVPYIIIF	302
<i>HaFAD3</i>	LRFTLPEMLAYPFYLNWRSPGKSGSHFDSSELVFAEQKDVITSTICWTMTALLFGLNSVVGVPVQMLKVYGVPYILIN	314
<i>OsFAD3</i>	LRESIFPEMLAYPFYLSRSPGKSGSHFHSSALCFENERNVLTSTACWVAMAALLAGLTFLMGPLLLNLVFPYNTIF	288

<i>CsFAD31</i>	VMWLDAVTYLHHHGHDEKLPWYRGKEWSYLRGLTTIDRDYGI FNNIHHDIGTHVIHHLFPQIPHYYHLVDATKAARKHVLG	329
<i>CsFAD32</i>	VMWLDAVTYLHHHGHDEKLPWYRGKEWSYLRGLTTIDRDYGI FNNIHHDIGTHVIHHLFPQIPHYYHLVDATKAARKHVLG	327
<i>CsFAD33</i>	VMWLDAVTYLHHHGHDEKLPWYRGKEWSYLRGLTTIDRDYGI FNNIHHDIGTHVIHHLFPQIPHYYHLVDATKAARKHVLG	327
<i>AtFAD3</i>	VMWLDAVTYLHHHGHDEKLPWYRGKEWSYLRGLTTIDRDYGI FNNIHHDIGTHVIHHLFPQIPHYYHLVDATKAARKHVLG	330
<i>RcFAD3</i>	IMWLDFVITYLHHHGHDEKLPWYRGKEWSYLRGLTTIDRDYGI WNNIHHDIGTHVIHHLFPQIPHYYHLVDATKAARKHVLG	382
<i>HaFAD3</i>	VMWLDFVITYLHHHGHDEKLPWYRGKEWSYLRGLTTIDRDYGI WNNIHHDIGTHVIHHLFPQIPHYYHLVDATKAARKHVLG	394
<i>OsFAD3</i>	VMWLDFVITYLHHHGHNDKLPWYRGKEWSYLRGLTTIDRDYGI WNNIHHDIGTHVIHHLFPQIPHYYHLVDATKAARKHVGM	368

<i>CsFAD31</i>	YYYREPQTSGAIPHILVESLVASIKKHQHYVSDGGDIVFYETDPDLYVYASDKSKIN	385
<i>CsFAD32</i>	YYYREPQTSGAIPHILVESLVASIKKHQHYVSDGGDIVFYETDPDLYVYASDKSKIN	383
<i>CsFAD33</i>	YYYREPQTSGAIPHILVESLVASIKKHQHYVSDGGDIVFYETDPDLYVYASDKSKIN	383
<i>AtFAD3</i>	YYYREPQTSGAIPHILVESLVASIKKHQHYVSDGGDIVFYETDPDLYVYASDKSKIN	386
<i>RcFAD3</i>	KYYREPRKSGPFPFHLFENLVKSIQDHVYVSDGGDVVYYCTDPLEYKCLKAKTKLI-	437
<i>HaFAD3</i>	KYYREPKKSSPIFPFHLGELVRSLLKHQHYVSDGGDVLYYCDDKLSKEK-----	443
<i>OsFAD3</i>	KYYREPDKSGPFPFHLFGLSRSLKRDHYVSDGGDVVYYCTDPAN-----	413

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7