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Abstract: Acyl-acyl carrier protein (ACP) desaturases (EC 1.14.19.2) are soluble enzymes that catalyse the insertion of a double bond into saturated fatty acid bound in saturated acyl chains bound to ACP in higher plants, producing cis-monounsaturated fatty acids. Three types of soluble acyl-ACP desaturases have been described: Δ9-acyl-ACP, Δ6-acyl-ACP and Δ4-acyl-ACP desaturases, which differ in the substrate specificity and the position in which the double bond is introduced. In the present work, Camelina sativa (CsSAD), Macadamia tetraphylla (MtSAD) and Dolichandra unguis-cati (DuSAD) desaturases were cloned, sequenced and characterized. Single copies of CsSAD, MtSAD and DuSAD with three, one and two different alleles respectively, were found. The corresponding mature proteins were heterologously expressed in Escherichia coli for biochemical characterization in protein extracts. The recombinant CsSAD enzyme showed 300-fold higher specificity towards 18:0-ACP than 16:0-ACP. Similar profile exhibited MtSAD although the differences in the specificity were lower, around 170-fold higher for 18:0-ACP than 16:0-ACP. Furthermore, DuSAD presented a profile showing preference towards 16:0-ACP against 18:0-ACP, around twice more, being so a Δ9 palmitoyl-ACP desaturase. Also, we reported the expression profile of CsSAD, which showed the highest levels of expression in expanding tissues that typically are very active in lipid biosynthesis such as developing seed endosperm. Moreover, the possibility to express a new desaturase in Camelina sativa (oilseed crop that store high levels of oil and is easy to transform) to create a new line rich in short monounsaturated fatty acid is discussed.

Cover Letter

Sevilla, 8th October 2014

Dear sir or madam,

We are sending with this letter the manuscript "*Characterization of soluble acyl-ACP desaturases from Camelina sativa, Macadamia tetraphylla and Dolichandra unguis-cati*" for publication in Journal of plant Physiology. Our group has been working on biochemistry of sunflower and other oil seeds. In the present work we focused acyl-ACP desaturasas of *Camelina sativa*, an oil crop of interest for plant biotechnology. Developing oils with new composition and properties has been one of the main objectives of oilseed biotechnology. The production of new mutant lines should involve the knowledge on how these plants metabolism works and how they assimilate the changes in their synthetic metabolism. Acyl-CoA desaturases are important in fatty acid synthesis metabolism due they determine the level of desaturation of the acyl chains synthetized *de novo*. The information resulting from the characterization of this enzyme could be useful for better redesigning Camelina lipid metabolism for the production of new oils with industrial interest. Moreover, acyl-ACP desaturases from other sources with biotechnological interest like *Macadamia tetraphylla* and *Dolichandra unguis-cati* were also characterized. So, I expect you consider this work for publication in your journal.

Sincerely,

Dr. Joaquín J. Salas

Instituto de la Grasa (CSIC)

*Summary

Characterization of soluble acyl-ACP desaturases from *Camelina sativa, Macadamia tetraphylla* and *Dolichandra unguis-cati*.

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Acyl-acyl carrier protein (ACP) desaturases (EC 1.14.19.2) are soluble enzymes that catalyse the insertion of a double bond into saturated fatty acid bound in saturated acyl chains bound to ACP in higher plants, producing *cis*-monounsaturated fatty acids. Three types of soluble acyl-ACP desaturases have been described: Δ^9-acyl-ACP, Δ^6-acyl-ACP and Δ^4-acyl-ACP desaturases, which differ in the substrate specificity and the position in which the double bond is introduced. In the present work, *Camelina sativa* (*CsSAD)*, *Macadamia tetraphylla* (*MtSAD)* and *Dolichandra unguis-cati* (*DuSAD)* desaturases were cloned, sequenced and characterized. Single copies of *CsSAD*, *MtSAD* and *DuSAD* with three, one and two different alleles respectively, were found. The corresponding mature proteins were heterologously expressed in *Escherichia coli* for biochemical characterization in protein extracts. The recombinant *Cs*SAD enzyme showed 300-fold higher specificity towards 18:0-ACP than 16:0-ACP. Similar profile exhibited *Mt*SAD although the differences in the specificity were lower, around 170-fold higher for 18:0-ACP than 16:0-ACP. Furthermore, *Du*SAD presented a profile showing preference towards 16:0-ACP against 18:0-ACP, around twice more, being so a Δ^9 palmitoyl-ACP desaturase. Also, we reported the expression profile of *CsSAD*, which showed the highest levels of expression in expanding tissues that typically are very active in lipid biosynthesis such as developing seed endosperm. Moreover, the possibility to express a new desaturase in *Camelina sativa* (oilseed crop that store high levels of oil and is easy to transform) to create a new line rich in short monounsaturated fatty acid is discussed.

Characterization of soluble acyl-ACP desaturases from *Camelina sativa, Macadamia tetraphylla* and *Dolichandra unguis-cati*.

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Abstract.

Acyl-acyl carrier protein (ACP) desaturases (EC 1.14.19.2) are soluble enzymes that catalyse the insertion of a double bond into saturated fatty acid bound in saturated acyl chains bound to ACP in higher plants, producing *cis*-monounsaturated fatty acids. Three types of soluble acyl-ACP desaturases have been described: Δ^9-acyl-ACP, Δ^6-acyl-ACP and Δ^4-acyl-ACP desaturases, which differ in the substrate specificity and the position in which the double bond is introduced. In the present work, *Camelina sativa* (*CsSAD)*, *Macadamia tetraphylla* (*MtSAD)* and *Dolichandra unguis-cati* (*DuSAD)* desaturases were cloned, sequenced and characterized. Single copies of *CsSAD*, *MtSAD* and *DuSAD* with three, one and two different alleles respectively, were found. The corresponding mature proteins were heterologously expressed in *Escherichia coli* for biochemical characterization in protein extracts. The recombinant *Cs*SAD enzyme showed 300-fold higher specificity towards 18:0-ACP than 16:0-ACP. Similar profile exhibited *Mt*SAD although the differences in the specificity were lower, around 170-fold higher for 18:0-ACP than 16:0-ACP. Furthermore, *Du*SAD presented a profile showing preference towards 16:0-ACP against 18:0-ACP, around twice more, being so a Δ^9 palmitoyl-ACP desaturase. Also, we reported the expression profile of *CsSAD*, which showed the highest levels of expression in expanding tissues that typically are very active in lipid biosynthesis such as developing seed endosperm. Moreover, the possibility to express a new desaturase in *Camelina sativa* (oilseed crop that store high levels of oil and is easy to transform) to create a new line rich in short monounsaturated fatty acid is discussed.

Keywords.

Lipid biosynthesis, acil-ACP desaturase, Camelina sativa, Macadamia tetraphylla, Dolichandra unguis-cati.

Abbreviations.

ACP, acyl carrier protein; FAS, fatty acid synthase complex; SAD, Δ^9 acyl-acyl carrier protein desarurase.

Introduction.

In higher plants, the first step of the *de novo* fatty acids biosynthesis is the conversion of acetyl-CoA into malonyl-CoA by the acetyl-CoA carboxylase and then to malonyl-ACP by a transacylase. Malonyl-ACP is then elongated to palmitoyl-ACP (16:0-ACP) or stearoyl-ACP (18-ACP) after eight or nine cycles of condensation-reduction-dehydration-reduction by the Fatty Acid Synthase (FAS) complex (Ohlrogge and Jaworski 1997). The products of plastidial FAS complexes are 16:0:ACP and 18:0-ACP that are the source of most of the fatty acids present in plant lipids. The proportion of saturated fatty acids in plant tissues is usually low, due they are desaturated in different cellular compartments. The first desaturation introduced in the aliphatic chains from plant fatty acids takes place in the plastids or chloroplasts by action of soluble Δ^9 acyl-acyl carrier protein desaturases (SAD) (Ohlrogge et al. 2000). These enzymes produce oleoyl-ACP ($18:1^{\Delta 9}$-ACP) from 18:0-ACP, but in some cases can desaturate other precursors like 16:0-ACP to produce palmitoleoyl-ACP ($16:1^{\Delta 9}$-ACP).

Acyl-ACP desaturases are soluble enzymes with a di-iron center that catalyse the insertion of a double bond into saturated fatty acids bound to ACP in higher plants, producing cis-monounsaturated derivatives, in an oxygen-dependent reaction (Guy et al. 2007). This activity requires reducing equivalents from NADPH that are transferred by ferredoxin reductase (FdR) to ferredoxin (Fd) and then to the enzyme. In presence of oxygen, two hydrogen atoms of the acyl-ACP are removed to give the equivalent cis-monounsaturated acyl-ACP and H_2O. The reaction involves activation of molecular oxygen by a two electron reduced di-iron center. The crystal structure of Δ^9-stearoyl-ACP desaturase from *Ricinus communis* (castor) showed that the two irons are separated by a distance of 4.2 Å (Lindqvist et al. 1996). Acyl-ACP desaturases are homodimeric proteins, each monomer is formed by 11 α-helices. Nine of these form an antiparallel helix bundle. The di-iron active site of these enzymes is buried by four α-helix leaving inside a hydrophobic pocket where substrate is bound during catalysis (Shanklin and Somerville 1991). The amino acid residues that formed this hydrophobic pocket are determinant for the specificity of these enzymes.

The most common members of this family are the Δ^9-stearoyl-ACP desaturases, identified and characterized from several different species like castor bean (Shanklin and Somerville 1991) or soybean (Zhang et al. 2008). However, there are acyl-ACP desaturases with an unusual specificity profile like the Δ^6-palmitoyl-ACP desaturase from blacked-eyed Susan vine (*Thumbergia alata*) seed (Cahoon et al. 1994a) and Δ^9-palmityl-ACP desaturase from cat's claw (*Dolichandra unguis-cati* L.) seed (Cahoon et al. 1998). These enzymes are the responsible of the production of some unusual monoenes, which are of a big interest for the production of oils with new properties and applications (Damude and Kinney 2008, Salas et al.

2004). Amongst these enzymes, desaturases that introduce a double bond in the Δ^9 position of a fatty acid different to stearate have been reported, as it is the case of myristoyl-ACP (14:0-ACP) and palmitoyl-ACP desaturases (Schultz et al. 1996, Cahoon et al. 1998). Moreover, there are acyl-ACP desaturases that insert the double bond in a different position of the fatty acid acyl chain like the Δ^6 acyl-ACP and Δ^4 acyl-ACP desaturases from *Thunbergia alata* and *Hedera helix* (Whittle et al. 2005) that act on palmityl-ACP to give place to interesting monoenoic fatty acids like $16{:}1^{\Delta 6}$ or petroselinic acid ($18{:}1\Delta^6$) after further elongation of each reaction product (Cahoon et al. 1992; Whittle et al. 2005).

In the present work we studied acyl-ACP desaturases present in *Camelina sativa*, *Macadamia tetraphylla* and *Dolichandra unguis-cati*. The first one was a regular Δ^9 desaturase present in a species with importance in biotechnology. *M. tetraphylla* and *D. unguis-cati* accumulate palmitoleic acid in their oils, a monoene produced by unusual acyl-ACP desaturases. These desaturases were cloned and expressed in *E. coli* for biochemical characterization and their impact of their specificities on their oil composition was discussed at the view of the results. Furthermore, the work was completed with the identification of alleles of *CsSAD* in the *C. sativa* genome and studies of their levels of expression in different tissues from that plant.

Material and methods.

Biological material and growth conditions

C. sativa CAS-CS0 cultivar, used to clone acyl-ACP desaturase genes (*CsSAD*), was cultivated in growth chambers at 25/15 °C (day/night) with 16 h photoperiod and a light intensity of 250 µE m^{-2} s^{-1}. The seeds were collected during a period between 15 and 18 days after synthesis (Rodríguez-Rodríguez et al. 2013) and were frozen in liquid nitrogen and stored at -80 °C until use. *M. tetraphylla* and *D. unguis-cati* plants were cultivated in the same conditions but this time young leaf tissue was used for cloning .

E. coli strain XL1-Blue (Stratagen) was used as the plasmid host for their production and heterologous protein expression of all cloned acyl-ACP desaturases. *E. coli* XL1-Blue was grown in LB media (1 % Bacto Tryptone, 0.5 % yeast extract, 1 % NaCl and pH 7) and the liquid culture was shaken vigorously at 37 °C. For plasmid selection, 100 µg $µl^{-1}$ ampicillin was used.

mRNA preparation and cDNA synthesis

Approximately 0.25 g of tissue (seeds from *C. sativa* and leaves from *M. tetraphylla* and *D. unguis-cati*) was ground in liquid nitrogen using a precooled sterile mortar and pestle. Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma) and subsequently, the mRNA was isolated from the total RNA using a GenElute mRNA Miniprep Kit (Sigma). The mRNA pellet was resuspended in 33 µl RNAase-free TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8) and cDNAs were synthesized using a Ready-To-Go T-Primed First-Strand Kit (Amersham Bioscience).

Cloning of genes coding for acyl-ACP desaturases from C. sativa, M. tetraphylla and D. unguis-cati

Acyl-ACP desaturase protein sequences from public databases were aligned to identify high homologous regions using the ClustalX v1.8 program (Thompson et al. 1997) to design a pair of degenerated primers: Deg_SAD_F1 and Deg_SAD_R1 (Table 1). Internal fragments of the putative genes were cloned from the cDNA templates described above. The PCR fragments were cloned into the pMBL-T vector (Dominion MBL), sequenced, and their identities confirmed using the BLAST software (Altschul et al. 1990). Subsequently, the 5' and 3' ends of the corresponding genes were obtained using the SMART-RACE cDNA Amplification Kit (Clontech) and the following specific primers: CsSAD_F1, CsSAD_F2, CsSAD_R1 and CsSAD_R2 for *CsSAD* gene; and MtSAD_F1, MtSAD_F2, MtSAD_R1 and MtSAD_R2 for *MtSAD* gene (Table 1). The sequences from products obtained from those PCR reactions were used to design primers to amplify full open reading frames (ORF): CsSAD_SphI_F3 and CsSAD_XmaI_R3 for *CsSAD* gene, MtSAD_SphI_F3 and MtSAD_PstI_R3 for *MtSAD* gene. However, to clone the completed ORF of *DuSAD* gene was designed one pair of primers using the sequence published by Professor Cahoon in 1998 (DuSAD*) (GenBank accession number AF051134). The primers were: DuSAD_XmaI_F1 and DuSAD_KpnI_R1.

Using this approach, three different alleles for acyl-ACP desaturases from *C. sativa,* two from *D. unguis-cati* and only one from *M. tetraphylla* were cloned into the pSpark vector (Canvax), sequenced, and their identities were confirmed using the BLAST software (Altschul et al. 1990). Primers with external endonuclease (*Sph*I, *Xma*I, *Pst*I or *Hind*III) restriction sites for *CsSAD* (CsSAD_SphI_F4 and CsSAD_XmaI_R3), *MtSAD* (MtSAD_SphI_F4 and MtSAD_PstI_R3) and *DuSAD* (DuSAD_SphI_F2 and DuSAD_HindIII_R2) were designed to amplify by PCR the coding regions of the mature proteins (without signal peptides). These PCR products corrsponding to *CsSAD*, *MtSAD,* and *DuSAD* were subcloned into the *SphI-XmaI* sites, *SphI-PstI* and *SphI-HindIII* of the pQE-80L expression vector (Quiagen) respectively, to

produce the corresponding fusion proteins with a 6xHis tag at the protein N-terminus. Ligation into the correct reading frame without signal peptide was confirmed by sequencing. The resulting constructs were named pQE80L::*Cs*SAD, pQE80L::*Mt*SAD and pQE80L::*Du*SAD.

Cloning of acyl-ACP desaturase introns of Camelina sativa alleles.

Genomic DNA of *C. sativa* was isolated using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Missouri). On the base of genomic structure of SAD from *Arabidopsis thaliana* (gene model in TAIR: AT2G43710), one pair of primers were designed to amplify the intron-containing regions CsSAD_INTR_F1 and CsSAD_INTR_R1 (Table 1). DNA fragments of around 1600-1700 bp were amplified from genomic DNA of *C. sativa* and cloned into the pSpark vector (Canvax) for sequencing.

Protein expression.

Five hundred millilitres cultures of *E. coli* XL1-Blue cells harbouring pQE80L::*Cs*SAD or pQE80L::MtSAD or pQE80L::DuSAD were grown as described above. The expression of all acyl-ACP desaturases was induced at an OD_{600} value of 0.5 by adding 0.5 mM IPTG and then incubated for additional 4 h. Afterwards, cells were harvested by centrifugation for 10 min at 2500 × g, washed with distilled water, and resuspended in 10 ml of Tris-HCl 50 mM, pH 8. Cells were pre-lysed adding 0.5 % Triton X-100, 5 mM DTT, 1 mM $MgCl_2$, 1 mM PMSF, 20 µg ml^{-1} DNase I (EC 3.1.21.1; Roche) and 0.2 mg ml^{-1} lysozyme (EC 3.2.1.17; Roche) during 30 min with slight agitation at room temperature. Then, cells were disrupted by sonication using Digital SLPe Ultrasonic System (Branson, model 4C15) that was equipped with a micro tip. The cells were intermittently sonicated on ice for 30 s at 70 % of amplitude with 30 s cooling intervals. The resulting disrupted cell suspension was centrifuged at 25000 g for 30 min. The supernatant was filtered through 0.22 µm filters and conserved at 4 °C, these soluble protein extracts were used to assay desaturase activity.

Gel electrophoresis of protein and immunoblot analysis

Amounts of about 15 µg of protein were combined with SDS-PAGE loading Buffer 2x (62.5 mM Tris-HCL pH 6.8, 2 % SDS, 25 % glycerol, 0.01 % bromophenol blue, 5 % 2-mercaptoethanol) and heated at 98 °C for 5 min. Proteins were separated by electrophoresis on 4-15 % Mini-Protean TGX Gels (BIO-RAD) in SDS-PAGE Running Buffer (25 mM Tris-Base, 192 mM glycine, 0.1 % SDS), and the gels were fixed and stained with 0.1 % Coomassie R-250

in 40 % ethanol and 10 % acetic acid. Molecular weight standard used was Precision Plus Protein Standard (BIO-RAD). Recombinant acyl-ACP desaturases were quantified optimally from Coomassie gel using ImageJ Software (Schneider et al. 2012).

For immunodetection, 2 µg protein extract were separated as describe above and then transferred to a polyvinylidene difluoride membrane 0.2 µm (PVDF) using Trans-Blot Turbo Blotting System (BIORAD). *Cs*SAD, *Mt*SAD and *Du*SAD expressed in *E. coli* XL1-Blue were detected using a monoclonal anti-polyHistidine with a peroxidase enzyme conjugated (Clone HIS-1, Sigma-Aldrich, Missouri). The working dilution was 1:2000 in phosphate buffered saline 1x (PBS) with 0.05 % Tween-20. Finally, the membrane was treated with peroxidase substrate for visualization of the protein bands reacting with the antibody.

Holo-ACP activation and preparation of acyl-ACP substrates.

Labelled acyl-ACP substrates were prepared using *E. coli* acyl-ACP synthetase provided by Dr. John Shanklin (Brookhaven National Laboratory, Upton, NY, USA) and recombinant histidine-tagged *E. coli* ACP provided by Dr. Penny Von Wettstein-Knowles (Department of Biology, University of Copenhagen, Copenhagen, Denmark). The production of holo-ACP was carried out according the method reported by Haas et al. (2000). The reaction media contained 50 mM Tris-HCl pH 8.8, 1 mM coenzyme A, 5 mM DTT, 10 mM $MgCl_2$, and 207 µM apo-ACP was preincubated at 37°C. The reaction was started by addition of 200 nmol of *E. coli* holo-ACP synthase (EC 2.7.8.7), being run for 4 hours at 37 °C (Lambalot and Walsh 1997). Acylation reactions consisted of 50 µg of recombinant *E. coli* holo-ACP, 660 MBq (approximant 0.1 µmol) of [1-^{14}C] fatty acid ammonium salt, 5 mM ATP, 2 mM DTT, 4 mM LiCl2, 10 mM MgCl2, 100 mM Tris pH 8 and 10 µg acyl-ACP synthetase (EC 6.2.1.20), all in a final volume of 0.5 ml. Reactions were run at room temperature for 3-4 h. Acyl-ACPs were purified and concentrated by ion exchange chromatography on DEAE-sepharose as described by Rock and Garwin (1979).

In vitro desaturase activity assay

Acyl-ACP desaturase activity was measured by the method described by Cahoon et al. (1994b) with minor modifications. The activity assay consisted of 0.05 M Pipes pH6, 5 mM ascorbate, 5 µg bovine serum albumin, 2.5 mM β-nicotinamide adenine dinucleotide phosphate, reduced tetra cyclohexylammonium salt, 3000 U of catalase (EC 1.11.1.6) from bovine liver, 20 µg of ferredoxin from spinach, 25 mU of ferredoxin-$NADP^+$ reductase (EC 1.18.1.7) from spinach, 1 mM dithiothreitol and between 50 and 840 Bq of [1-^{14}C]acyl-ACP substrate in a final

volume of 0.1 ml reactions. Reactions were started by addition of an amount from 0.1 to 20 µg of protein depending on the extract activity. The assay was run at room temperature during 15 min, and the reaction stopped by the addition of 0.5 ml of 2.35 M NaOH. Then acyl-ACPs were hydrolysed for 30 min at 40 °C and acidified with 0.35 mL of 4 M H_2SO_4. The free fatty acids were extracted with hexane and methylated using methanolic HCl. The resulting methyl esters were separated in argentated silica gel plates and radioactive bands were detected and quantified in an Instant Imager (Packard). The data from desaturase assays were fitted to the Hill equation by nonlinear least-squares regression analysis using OriginPro 8 software, and correlated at $P < 0.005$ as determined by Student's t test. Both the Vmax and Km were derived from these curves.

Fatty acid analysis.

Twenty-five millilitre cultures of *E. coli* XL1-Blue cells harbouring empty pQE80L or pQE80L::*Cs*SAD or pQE80L::*Mt*SAD or pQE80L::*Du*SAD were grown and the expression of the corresponding desaturases was induced as described above. The cells were harvested by centrifugation for 10 min at 2500 g, washed with distilled water and the total fatty acid content was determined using the one-step method proposed by Garcés and Mancha (1993). Volumes of 3.3 ml of methanol/toluene/dimethoxipropane/sulphuric acid (39:20:5:2) and 1.7 ml heptane were added to the pellet of bacterial cells, 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 analysed by GLC. The different methyl esters were identified by comparing their retention times with those from known standards.

Quantitative real time PCR

The levels of gene expression of *CsSAD* were determined by quantitative real time PCR (QRT-PCR) with a specific pair of primers, CsSAD_qPCR_F and CsSAD_qPCR_R (Table 1), and SYBR Green I (QuantiTect_SYBR_Green PCR Kit, Quiagen, Crawley, UK) using a MiniOpticon system (Bio-Rad). The reaction mixture was heated to 50 °C for 2 min and then heated to 95 °C for 15 min before subjecting it to 40 PCR cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 15 s, while monitoring the resulting fluorescence. These were used to estimate the transcript content of the calibrator gen. The Livak method (Livak and Schmittgen 2001) was applied to calculate comparative expression levels between samples. The *C. sativa* actin gen (GenBank accession number: KJ670375) was used as internal reference to normalize the

relative amount of cDNAs for all samples with the specific pair of primers CsActin_qPCR_F and CsActin_qPCR_R (Table 1).

Results.

Isolation and sequence analysis of acyl-ACP desaturase from Camelina sativa, Macadamia tetraphylla and Dolichandra unguis-cati.

Two different PCR products with a similar length (218 bp) were amplified using degenerated primer pairs (Table 1) and cDNA from developing *C. sativa* seeds and young *M. tetraphylla* leaves. They corresponded to internal fragments of putative *CsSAD* and *MtSAD* genes. Their sequences were used to obtain the full-length *CsSAD* and *MtSAD* cDNA clones of 1206 bp and 1191 bp, respectively, by the technique of RACE as described in the experimental section. The alignment of the deduced amino acid sequences showed a high degree of identity to internal coding regions of known acyl-ACP desaturase sequences. Using these sequences, specific primer pairs were designed to amplify all possible alleles of each gene in each species: CsSAD_SphI_F3 and CsSAD_XmaI_R3 for *CsSAD* genes and MtSAD_SphI_F3 and MtSAD_PstI_R3 for *MtSAD* genes (Table 1). Using this strategy we found three different alleles in *C. sativa*: *CsSAD1*, 1206 bp (GenBank accession number AFQ60943); *CsSAD2*, 1206 bp (GenBank accession number AFQ60944) and *CsSAD3*, 1206 bp (GenBank accession number AFQ60945), whereas only one allele was identified n the case of *M. tetraphylla*: *MtSAD1*, 1110 bp (GenBank accession number ADE06393). For the acyl-ACP desaturases from *D. unguis-cati* it was not necessary to amplify an internal fragment and apply RACE. Using an specific primer pairs for *DuSAD* gene, DuSAD_XmaI_F2 and DuSAD_KpnI_R1 (Table 1) and cDNA from young leaves, two different alleles were easily amplified: *DuSAD1*, 1191 bp (GenBank accession number KJ126791) and *DuSAD2*, 1191 bp (GenBank accession number KJ1268792).

The identified *CsSAD1*, *CsSAD2* and *CsSAD3* open reading frames (ORFs) encode predicted proteins of 401 amino acid residues (Fig. 1), which correspond to calculated molecular masses of 45.51, 45.55, 45.64 kDa, and similar pIs of 6.62, 6.62, 6.44, respectively. The ORF of *MtSAD*, encodes a protein of 396 amino acid residues (45.23 kDa and pI 6.28) and the ORFs of *DuSAD1* and *DuSAD2*, encode for predicted proteins of 396 amino acid residues (Fig. 1), with identical estimated molecular masses of 45.09 kDa, and different pIs 6.45 and 6.31, respectively.

The cleavage site of the chloroplast transit peptide of these enzymes was estimated using a prediction program for protein subcellular localization, WoLF PSORT (Horton et al. 2007), and the results reported about acyl-ACP desaturase of *Ricinus communis* (Shanklin et al. 1991), *Asclepias syriaca* (Cahoon et al. 1997), *Sesamum indicum* (Yukawa et al. 1996), *D. unguis-cati* (Cahoon et al. 1998), *Nerium oleander* (Gummeson et al. 1999) and *Macadamia integrifolia* (Gummeson et al. 1999). The Ala-36 was found to be, the best candidate to be the N-terminal amino acid of the mature protein for *CsSAD* alleles, corresponding with a signal peptide formed by 35 amino acid residues. In the case of *MtSAD* and *DuSAD* alleles we consider Ala-34 the first amino acid of mature protein; decoupling a signal peptide of 33 amino acids (Fig. 1). Taking into account the presence of signal peptides in these desaturases, predicted mature protein will have 366, 363 and 363 amino acids for CsSAD, *MtSAD* and *DuSAD*, with molecular masses of 41.65, 41.53 and 41.55 kDa and pI values of 5.65, 5.57 and 5.75, respectively.

A phylogenetic tree was generated for the novel acyl-ACP desaturase genes from *C. sativa*, *M. tetraphylla* and *D. unguis-cati* based on their deduced amino acid sequences and those from other known plant acyl-ACP desaturase proteins (Fig. 2). The acyl-ACP desaturase from *C. sativa* grouped very closely with those from *Arabidopsis thaliana*, *Brassica juncea*, *B. napus* and *B. rapa*; all these species belonging to the Brassicaceae family and so having a common phylogenetic origin. Acyl-ACP desaturases from *M. tetraphylla* and *D. unguis-cati* were phylogenetically very close and are pretty far from *C. sativa* desaturases. Other closely related sequences with *Mt*SAD and *Du*SAD were those from species *Camellia chekiangoleosa* belonging to the family Theaceae and *Sesamum indicum* corresponding to the family Pedaliaceae

Genomic organization of Camelina sativa acyl-ACP desaturase genes

To analyze the genomic organization of *CsSAD* gene alleles, a genomic DNA fragment of the locus was amplified using the specific primer pair, CsSAD_INTR_F1 and CsSAD_INTR_R1. Three different clones we obtained corresponding to *CsSAD1* (2371 nucleotides), *CsSAD2* (2427 nucleotides) and *CsSAD3* (2395 nucleotides), which were sequenced. The organization of introns and exons of the three alleles of *CsSAD* gene were revealed by aligning the cDNA sequences and these cloned fragments from genomic DNA. All alleles of *CsSAD* consisted of three exons separated by two introns; the lengths of exons are similar in the three copies, however some differences in the length of introns were observed (Fig. 3). Intron I of alleles of *CsSAD* gene is located in the putative transit peptide and accumulates high genetic variability, mostly consistent of insertion-deletion and single

nucleotide polymorphisms (SNPs). Furthermore, the differences of length (iICsSAD1: 1088 bp; iICsSAD2: 1145 bp; iICsSAD3: 1111 bp) become larger compared with the intron 2 (Fig. 3). The intron II is located in the fifth α-helix of *Cs*SAD, there were not significant differences in their size and the observed variability was only due to SNPs (Fig. 3).

Expression profile of acyl-ACP desaturase from Camelina sativa

The expression levels of acyl-ACP desaturase gen from *C. sativa* was studied by QRT-PCR in developing seeds and vegetative tissues. Results in Figure 4 show that the transcript accumulation profile was temporally regulated during embryo development. The highest levels of expression of *CsSAD* genes in developing seeds were found in 6 and 12 DAF which corresponds to the first part of phase of oil accumulation. With regard to vegetative tissues a level of expression fairly high was found in all of them out of root tissues, in which the lowest expression level was observed (Fig. 4).

*Fatty acid analysis of E. coli expressing Cs*SAD, *Mt*SAD *and Du*SAD *enzymes.*

The mature acyl-ACP desaturases were expressed in *E. coli* using the pQE-80L protein expression vector. Only one allele was expressed in the case of *CsSAD* and *DuSAD*, assuming that the other alleles displayed similar characteristics due to the minor observed differences. Thus, the three alleles of *CsSAD* present two changes in their amino acids sequence, one conservative change (Asp-167-Arg) and one semi-conservative change (Gly-350-Asp). The differences between the two alleles of *DuSAD* were minimal, showing only one conservative change (Gln-74-Lys). Moreover, the amino acid residues forming the hydrophobic pocket and coordinating the diiron center of *CsSAD* and *DuSAD* are identical for all their desaturase alleles (Fig. 1).

The fatty acid composition of *E. coli* expressing acyl-ACP desaturase genes from *C. sativa, M. tetraphylla* and *D. unguis-cati* acyl-ACP were analyzed and compared to that in control cells bearing the empty pQE-80L vector (Table 2). The results suggest that expressed *Cs*SAD, *Mt*SAD and *Du*SAD are functional and that they interfere in the normal fatty acid biosynthetic pathway. The expression of *Cs*SAD and *Mt*SAD enzymes caused an increase in the total content of unsaturated fatty acid in *E. coli*, around 6.0 % and 2.2 % respectively. The fatty acid profile of *E. coli* with pQE80L::*Cs*SAD shows a significant decrease of palmitic acid (from 45.3 % to 38.6 %) and a relevant increase of cis-vaccenic acid (from 15.5 % to 24.7 %) if compared with the control (Table 2). A similar effect was observed for pQE80L::MtSAD, although the changes in the fatty acid profile were not as pronounced. Therefore, the ratio

unsaturated/saturated fatty acids UFA/SFA is higher in *E. coli* cells expressing pQE80L::*Cs*SAD and pQE80L::*Mt*SAD than in control bacteria (Table 2). However, the fatty acid profiles of *E. coli* bearing pQE80L and pQE80L::*Du*SAD were similar (Table 2).

On the other hand, the contents of cyclic fatty acids, 17:0Δ (cis-9,10-Methylen-hexadecanoic acid, cyclopropane derivative from $16:1^{\Delta 9}$) and 19:0Δ (cis-11,12-Methylen-octadecanoic acid, cyclopropane derivative from $18:1^{\Delta 9}$) did not importantly change with the expression of desaturases.

Substrate specificity and kinetic parameters of acyl-ACP desaturase from Camelina sativa, Macadamia tetraphylla and Dolichandra unguis-cati.

The kinetic parameters of *Cs*SAD, *Mt*SAD and *Du*SAD were investigated in protein extracts from transgenic *E. coli* cultures to evaluate the contribution of these enzymes to oil biosynthesis and the possible uses of them in plant biotechnology. This method allowed to obtain the recombinant acyl-ACP desaturase at high levels in the soluble fraction of *E.coli* , accounting for about 25 % of total protein for *Cs*SAD; 23 % for *Mt*SAD and 19 % *Du*SAD (Fig. 5A), which was confirmed by western blot with monoclonal anti-polyhistidine antibodies(Fig. 5B). Several attempts to purify the proteins from the crude bacterial extracts were carried out using techniques of NTA-Ni affinity, ion exchange and exclusion chromatograpies but in all cases most of activity was lost after purification. Therefore, the enzymes were characterized using the crude *E. coli* extracts.

Substrate specificity of *Cs*SAD, *Mt*SAD and *Du*SAD was studied by assaying the activity these enzymes on different acyl-ACP at constant substrate concentration (Fig. 6). *Cs*SAD showed a conventional profile of Δ^9-stearoyl-ACP desaturase; with activities towards 18:0-ACP 300-fold higher than for 16:0-ACP (Fig. 6). The enzyme *Mt*SAD showed a similar profile, with high activity on 18:0-ACP, which was desaturated at 179.7 pkat mg $prot^{-1}$, whereas 16:0-ACP was at 1.5 pkat mg $prot^{-1}$ (Fig. 6).

Furthermore, the enzyme *Du*SAD showed an unusual desaturase profile, being a Δ^9-palmitoyl-ACP desaturase for which activity towards 16:0-ACP is twice that found for 18:0-ACP (Fig. 6.). The plant *D. unguis-cati* accumulates approximately 72 % ω-7 fatty acid in their seeds (comprising 55 % $16:1^{\Delta 9}$ and 17 % $18:1^{\Delta 11}$; Chisholm and Hopkins, 1965).

To gain insight into the mechanism underlying in substrate specificity, we performed a comparative kinetic analysis on *Cs*SAD, *Mt*SAD and *Du*SAD (Table 3). The differences between the specificity profile of these enzymes apparently not affected affinity for substrates, because they display similar K_m values for both acyl-ACPs, which were in the micromolar order, except for *Du*SAD where it was even lower (0.14 µM for 16:0-ACP and 0.52 µM for

18:0-ACP) (Table 3). Moreover, the amino acids involved in the hydrophobic pocket binding acyl-ACP and those implied in the coordination of di-iron center are identical in these enzymes (Fig. 1). The values of V_{max} show the major differences. In the case of *Cs*SAD and *Mt*SAD they were 143.4 and 50.2 pkat mg prot^{-1} for 18:0-ACP and 1.7 and 0.34 pkat mg prot^{-1} for 16:0-ACP (Table 3). However, V_{max} of *Du*SAD for 16:0-ACP and 18:0-ACP is similar, around 12.4 and 10.3 pkat mg prot^{-1} (Table 3).

Together these changes in V_{max} values result in an increase in turnover number (K_{cat}) and in specificity factor (K_{cat}/K_m) for *CsSAD* and *MtSAD* towards 18:0-ACP and for *DuSAD* for 16:0-ACP. The Δ^9 stearoyl-ACP desaturase from *M. tetraphylla* shows the highest specificity factor when is assayed with 18:0-ACP, 0.69 s^{-1}, being similar to that reported by Whittle and Shanklin (2001) for Δ^9 acyl-ACP desaturase from *Ricinus communis* (0.71 s^{-1}). Moreover, the *Du*SAD presents a K_{cat} value for 16:0-ACP 0.054 s^{-1}, around 9 times higher than that in *Cs*SAD and approximately 27 times higher than *Mt*SAD $K_{cat.}$ The specificity factor obtained for Δ^9 palmitoyl-ACP desaturase from *D. unguis-cati* for 16:0-ACP is 0.34 s^{-1} µM^{-1}, similar order of magnitude reported for *RcSAD* with 18:0-ACP (Whittle and Shanklin 2001) and around 42 and 172 times higher than *Cs*SAD and *Mt*SAD, respectively.

Discussion

Nowadays there is increasing interest in enhancing plant oil for human nutrition and using plant oils as renewable sources of industrial chemical feedstocks (Damude and Kinney, 2008) (Salas et al., 2004). The ability to manipulate the fatty acid profile of oil and the content of monounsaturated fatty acids offers a way altering the physical properties and commercial uses of conventional vegetable oils. In this regards, acyl-ACP desaturase are a primary targets for the production of transgenic oilseed crops with an unusual fatty acid profile rich in 16:1$^{\Delta 9}$ and 18:1$^{\Delta 9}$. For this reason, in the present work we studied fatty acid acyl-ACP desaturase present in *C. sativa, M. tetraphylla* and *D. unguis-cati.*

In this work, we were identified three different alleles of *Cs*SAD (GenBank accession numbers: AFQ60943; AFQ60944; AFQ60945) with minimal differences in their genomic sequence, made evidence that *C. sativa* is a triploid species. The work published by Gehringer et al. (2006) showed that the triplication of *C. sativa* genome might result from two allopolyploidy events; firstly a combination of two diploid (2n) plus other diploid (2n) species, resulting a tetraploid (4n) one, and then a combination of this hypothetical tetraploid species with a other diploid one, generating a hexaploid (6n) organismplant. Other possibility reported by Hutcheon et al. (2010) was that *C. sativa* could be derived from the combination of autotetraploid (4n) and a diploid (2n) species, in an autopolyploidized (6n) genome. Ours

analysis of introns sequences supported the last hypothesis, because the introns sequence of *CsSAD1* and *CsSAD2* presented high level of identity between then and both significant difference with *CsSAD3* introns sequence. Thus, *C. sativa* genome resulted from combination of autotetraploid and diploid species and the diploid parental species contributing to *C. sativa* genome of 20 chromosomes could probably be of the from 7+7+6 or 6+6+8 (Hutcheon et al. 2010). The alignment of our sequences with the published sequences for "*Camelina sativa* Genome Project" included in the "The Prairie Gold Project" allows to locate the different alleles of desaturase genes in the *C. sativa* genome: *CsSAD1*, *CsSAD2* and *CsSAD3* in chromosome 5, 4 and 6, respectively.

The analysis of amino acid sequence from *Cs*SAD, *Mt*SAD and *Du*SAD showed that the residues involved in active site are highly conserved between these acyl-ACP desatuares. The studies about the structure of the castor stearoyl-ACP desaturase (Lindqvist et al. 1996) showed that six amino acids residues are involved in coordination of the two iron atoms. In the case of stearoyl-ACP desaturase from castor (*RcSAD*), Fe1 is coordinated by Glu-138 in a bidentate manner and by a single interaction with His-176. Fe2 is coordinated in the same condition with Glu-229 and His-265 respectively. Both Glu143 and Glu229 bridge the two iron ions, with each residue forming one interaction with Fe1 and one with Fe2 (Fig. 1). All these residues are very preserved between *Cs*SAD, *Mt*SAD and *Du*SAD enzymes. Similarly, residues participating in the hydrophobic pocket of the active center where the carbon chain of the substrate interact with enzyme are also highly conserved. In castor these amino acid are Thr-137, Tyr-144, Met-147, Leu-148, Trp-165, Tyr-224, Thr-225, Gln-228, Thr-232, Met-298, Pro-297, Phe-312, Ser-316, and Tyr-325 (Fig. 1). However, the residues involved in the interaction between the two subunits of acyl-ACP desaturase, marked in Figure 1 with starts, present two semi-conservative changes (Cys-98-Lys and Lys-200-Thr) and one conservative change (Arg-196-Lys) between *RcSAD* and the desaturase enzymes investigated in this work (Fig. 1).

The heterologous expression of *Cs*SAD and *Mt*SAD produced changes in fatty acid profile of *E.* coli cultures, which showed a higher ratio UFA/SFA than control strain. The excess of $18{:}1\Delta^{11}$ of cells that bearing pQE80L::*Cs*SAD and pQE80L::*Mt*SAD (Table 2) indicated that these recombinant desaturasa are functional. The high level of *cis*-vaccenic acid was due that *Cs*SAD and *Mt*SAD desaturases are able to desaturate the 16:0 to $16{:}1\Delta^{9}$ and the latter is elongated to $18{:}1\Delta^{11}$ by ketoacyl-ACP syntahase I/II from *E. coli* (fabF/B). On the other hand, the fatty acid profiles of *E. coli* with pQE80L and pQE80L::*Du*SAD are similar (Table 2). A careful analysis of the codons that encode for the acyl-ACP desaturase from *D. unguis-cati* showed that there is six leucine residues encoded by CTA, leucine codon used less than 20 % in *E. coli* (Fig.1). These differences in codon usage preference among *D. unguis-cati* and *E. coli*

can be cause a variety of problems concerning heterologous gene expression, for example, a low translation and low *DuSAD* desaturase activity *in vivo* in *E. coli.*

The enzymatic characterization showed that acyl-ACP desaturases found in *C. sativa* and *M. tetraphylla* were Δ^9-stearoyl-ACP desaturases (Fig. 6). This results fitted well with the fatty acid profile of this *C. sativa* crop. The *C. sativa* oil accumulated around 31.8 % ω-9 fatty acid (mainly oleic and eicosanoic acid) and only 1.7 % ω-7 fatty acids, moreover *C. sativa* oil has a high content of linoleic and linolenic acids, 21.9% and 29.3 %, respectively. All of them are derivated of oleic acid ($18:1^{\Delta 9}$), the main product of stearoyl-ACP desaturase from *C. sativa* (Rodríguez-Rodríguez et al. 2013). Despite *M. tetraphylla* seeds are a natural source of ω-7 fatty acid (palmitoleic and asclepic acid represented 16.9 % and 4.8 %, respectively, in "Cate" variety), they still accumulates a high levels of ω-9 fatty acid, mainly oleic acid (around 56 %; Moreno-Pérez et al., 2011). Therefore, the presence of a palmitoyl-ACP desaturase in this crop is probable, although it has not been yet found. The *Mt*SAD cDNA studied in this work was isolated from leaf tissues, which contain mostly ω-9 fatty acids so its specificity profile fits well with that acyl lipid composition.

The *Du*SAD activity on 16:0-ACP (121.5 pkat mg $prot^{-1}$) in crude *E. coli* extracts was higher than reported previously for other Δ^9-desaturase from cat´s claw seed (7.8 pkat mg $prot^{-1}$) and so could be a major contributor to this fatty acid composition. However, this activity was much lower than that shown by L118W mutant Δ^9-desaturase from castor (*Rc*SADL118W) with high activity on palmitic acid bond to ACP (19716.7 pkat mg $prot^{-1}$) (Cahoon et al. 1998). However, *DuSAD* characterized in this work has the lysine in the position 118 like Δ^9-stearoyl-ACP desaturase from *C. sativa*, *M. tetraphylla* and *R. communis*, so that this change is not sufficient to provide the ability to desaturated 16:0-ACP with high efficiency at *Rc*SADL118W and DuSAD*. Probably the dissimilarity between *Du*SAD and others forms previously published relies on five conservative changes and twenty no-conservative changes, making the hydrophobic pocket more accessible to the substrate and increasing the catalytic efficiency for 16:0-ACP (Fig. 1).

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Tables

Table 1
Degenerated and non-degenerated primers used in this work

Primer name	Sequence 5´- 3´
Degenerated primers	
Deg_SAD_F1	GGNGAYATGATHACNGARGA
Deg_SAD_R1	ARRTATTGDATNGTYTTYTC
***Cs*SAD**	
CsSAD_F1	GACCTATCAAACTATGTTGAACACTTTGG
CsSAD_F2	GGACTGCTGAAGAAAATCGACATGG
CsSAD_R1	TTCAACACGACCAGACAAGTAAAGG
CsSAD_R2	AATAGCCCATGAAGTAAGGACTAGCACC
CsSAD_SphI_F3[a]	TAGCAGCATGCATGGCTCTAAAGCTTAACC
CsSAD_SphI_F4	TAGCAGCATGCGCTTCATCTTCTCCG
CsSAD_XmaI_R3	CAGAGAAGTGCAGCTCTAACCCGGGCT
CsSAD_INTR_F1	TCATCTTCTCCGGCTCTCAG
CsSAD_INTR_R1	CGAAGTATAGATGAAGCCAAGG
CsSAD_qPCR_F	TTTTTGGTTGGTAGGTGGAA
CsSAD_qPCR_R	TCCAGCTGAAAGGAATCTTG
***Cs*Actin**	
CsActin_qPCR_F	TTGGAAGGATCTGTACGGTAAC
CsActin_qPCR_R	TGTGAACGATTCCTGGACC
***Mt*SAD**	
MtSAD_F1	CCTTCCAACTTACCAAACAATGC
MtSAD_F2	GGCTGTGTGGACAAGGGCA
MtSAD_R1	TTGCTTCATGTCTACTCGTCCAG
MtSAD_R2	CCACACAGCCCAGGAAGTGAGG
MtSAD_SphI_F3	ATCTCGCATGCATGGCTCTCAAGCTTAATC
MtSAD_SphI_F4	TCTTAGCATGCGCATCCACCCTTCGTTC
MtSAD_PstI_R3	AGCTGCAGTCAAAGCATCACTTCTTTATC
***Du*SAD**	
DuSAD_XmaI_F1	ATCCCGGGATGGCCTTGAAGCTG
DuSAD_KpnI_R1	TGGTACCTCAGAGTTGCACCTCTC
DuSAD_SphI_F2	TAGATGCATGCGCTGCCACTCTTCGG
DuSAD_HindIII_R2	ATCATAAGCTTTCAGAGTTGCACCTCTC

[a] Restriction sites introduce 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).

Table 2
Fatty acids composition of *E. coli* cells containing recombinant plasmid. Dates are average of three independent samples.

	Fatty Acids (mol %)											
	14:0	**14:1$^{\Delta 7}$**	**16:0**	**16:1$^{\Delta 9}$**	**17:0Δ[a]**	**18:0**	**18:1$^{\Delta 9}$**	**18:1$^{\Delta 11}$**	**19:0Δ[b]**	**% UFA[c]**	**% SFA[d]**	**UFA/SFA**
pQE80	1.1 ± 0.1	4.61 ± 0.1	45.3 ± 0.8	19.9 ± 1.44	8.1 ± 0.2	3.3 ± 0.8	1.2 ± 0.4	15.5 ± 0.3	0.5 ± 0.1	50.1 ± 1.2	49.7 ± 1.2	1.0 ± 0.0
pQE80::*Cs*SAD	1.0 ± 0.0	4.06 ± 0.1	38.6 ± 1.4	16.0 ± 0.46	8.9 ± 0.2	4.5 ± 0.9	1.2 ± 0.1	24.7 ± 0.1	0.8 ± 0.1	56.0 ± 0.6	43.9 ± 0.6	1.3 ± 0.0
pQE80::*Du*SAD	1.0 ± 0.2	4.77 ± 0.1	46.6 ± 0.6	16.2 ± 1.1	9.7 ± 0.6	4.8 ± 0.9	1.9 ± 0.7	13.7 ± 0.2	0.7 ± 0.0	47.6 ± 0.4	52.4 ± 0.4	0.9 ± 0.0
pQE80::*Mt*SAD	1.0 ± 0.1	3.99 ± 0.5	42.0 ± 0.7	16.8 ± 1.18	8.9 ± 0.3	4.8 ± 0.8	2.5 ± 1.2	18.2 ± 0.9	0.9 ± 0.0	52.2 ± 0.6	47.9 ± 0.6	1.1 ± 0.0

a cis-9,10-Methylen-hexadecanoic acid, cyclopropane derivate from 16:1Δ9
b cis-11,12-Methylen-octadecanoic acid, cyclopropane derivate from 18:1$^{\Delta 9}$
c Unsaturated fatty acids and derivatives; 14:1$^{\Delta 7}$ + 16:1$^{\Delta 9}$ + 17:0Δ + 18:1$^{\Delta 9}$ + 18:1$^{\Delta 11}$ + 19:0Δ
d Saturated fatty acids; 14:0 + 16:0 + 18:0

Table3
Kinetic parameters of the recombinant *Cs*SAD, *Mt*SAD and *Du*SAD proteins acting on different acyl-ACP substrate. Dates are average of three independent samples.

	Substrate	K_m (μM)	V_{max} (pkat/mg prot s)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} μM^{-1})
***Cs*SAD**	**16:0-ACP**	0.75	1.7	0.006	0.008
	18:0-ACP	1.39	143.4	0.48	0.34
***Mt*SAD**	**16:0-ACP**	1.01	0.34	0.002	0.002
	18:0-ACP	1.08	50.2	0.69	0.64
***Du*SAD**	**16:0-ACP**	0.14	12.4	0.054	0.34
	18:0-ACP	0.52	10.3	0.045	0.086

Figure Legends

Figure 1. Alignment of deduced amino acid sequences of acyl-ACP desaturase enzymes of *Camelina sativa* (*CsSAD1*, AFQ60943; *CsSAD2*, AFQ60944; *CsSAD3*, AFQ60945), *Macadamia tetraphylla* (*MtSAD1*, ADE06393), *Dolichandra unguis-cati* (*DuSAD1*, KJ126791; *DuSAD2*, KJ1268792), *Dolichandra unguis-cati* characterized in 1998 by Dr. Cahoon *(DuSAD(1998),* AF051134*) Arabidopsis thaliana* (*AtSAD*, AAK82496), *Ricinus communis* (*RcSAD*, XP_002531889) and *Oryza sativa* (*OsSAD*, NP_001045215). Identical amino acids are shaded in black, whereas conserved residues are shaded in grey. The amino acids considered to coordinate di-iron centre are indicated by black dots, the residues that form the hydrophobic pocket are designated by arrows, and the amino acid involved in the recognizing between monomer are indicated by stars. The conservative and semi-conservative change in the amino acid sequence between three *CsSAD* alleles (Asp-167-Arg and Gly-350-Asp) and two *DuSAD* alleles (Gln-74-Lys) are marked by white dots. The leucine residues of *Du*SAD enzyme (Leu-131, 183, 189, 276, 286 and 346) with putative translation problems in *E. coli* are designated by gray dots. The semi-conservative changes and no conservative changes between *Du*SADs reported in this work and that described by Dr. Cahoon et al. (1998) are marked by square gray and black, respectively.

Figure 2: Phylogenetic comparison of plant acyl-ACP desaturase enzymes. Plant species included in the phylogenetic tree are: Araceae (*Pinellia ternata*) Asteraceae (*Carthamus tinctorius*, *Helianthus annuus*), Brassicaceae (*Arabidopsis thaliana*, *Camelina sativa*, *Brassica juncea*, *Brassica napus*, *Brassica rapa*), Briophyta (*Physcomitrella patens*), Bignoniaceae (*Dolichandra unguis-cati*), Cucurbitacea (*Cucumis sativus*), Euphorbiaceae (*Jatropha curcas*, *Ricinus communis*, *Manihot esculenta*, *Vernicia montana*), Fabaceae (*Glycine max, Medicago truncatula*, *Millettia pinnata*, *Phaseolus vulgaris*), Lauraceae (*Persea Americana*), Linaceae (*Linum_usitatissimum*), Malvaceae (*Theobroma cacao*), Pedaliaceae (*Sesamum indicum*), Poaceae (*Oryza sativa*), Proteaceae (*Macadamia tetraphylla*), Salicaceae (*Populus trichocarpa*) and Theaceae (*Camellia chekiangoleosa*)

Figure 3. Acyl-ACP desaturase genes from *Camelina sativa.* Diagram showing the genomic structure of the three acyl-ACP desaturase alleles for *CsSAD* gene. Boxes represent exons while introns are represented by lines and numbered in Roman.

Figure 4. Expression of *CsSAD* gene in developing seeds and vegetative tissues of Camelina sativa. Values represent mean values ± SD of three independent samples

Figure 5. Comassie blue stained SDS-PAGE (panel A) and western blotting (panel B) showing recombinant acyl-ACP desaturase from *Camelina sativa, Macadamia tetraphylla and Dolichandra unguis-cati* (panel A). Lane 1, molecular weight standards; lane 2, soluble fraction of *E. coli* transformed with pQE80L empty; lanes 3, 4 and 5, soluble fraction of *E. coli* bearing pQE80L::*Cs*SAD, pQE80L::MtSAD and pQE80L::DuSAD, respectively.

Figure 6. Substrate specificity of acyl-ACP desaturase from *Camelina sativa*, *Macadamia tetraphylla* and *Dolichandra unguis-cati*. The activity was measured with soluble fraction of *E. coli* that content majority acyl-ACP desaturase. Data represent the mean (±SD) from three independent experiments.

Figure 1

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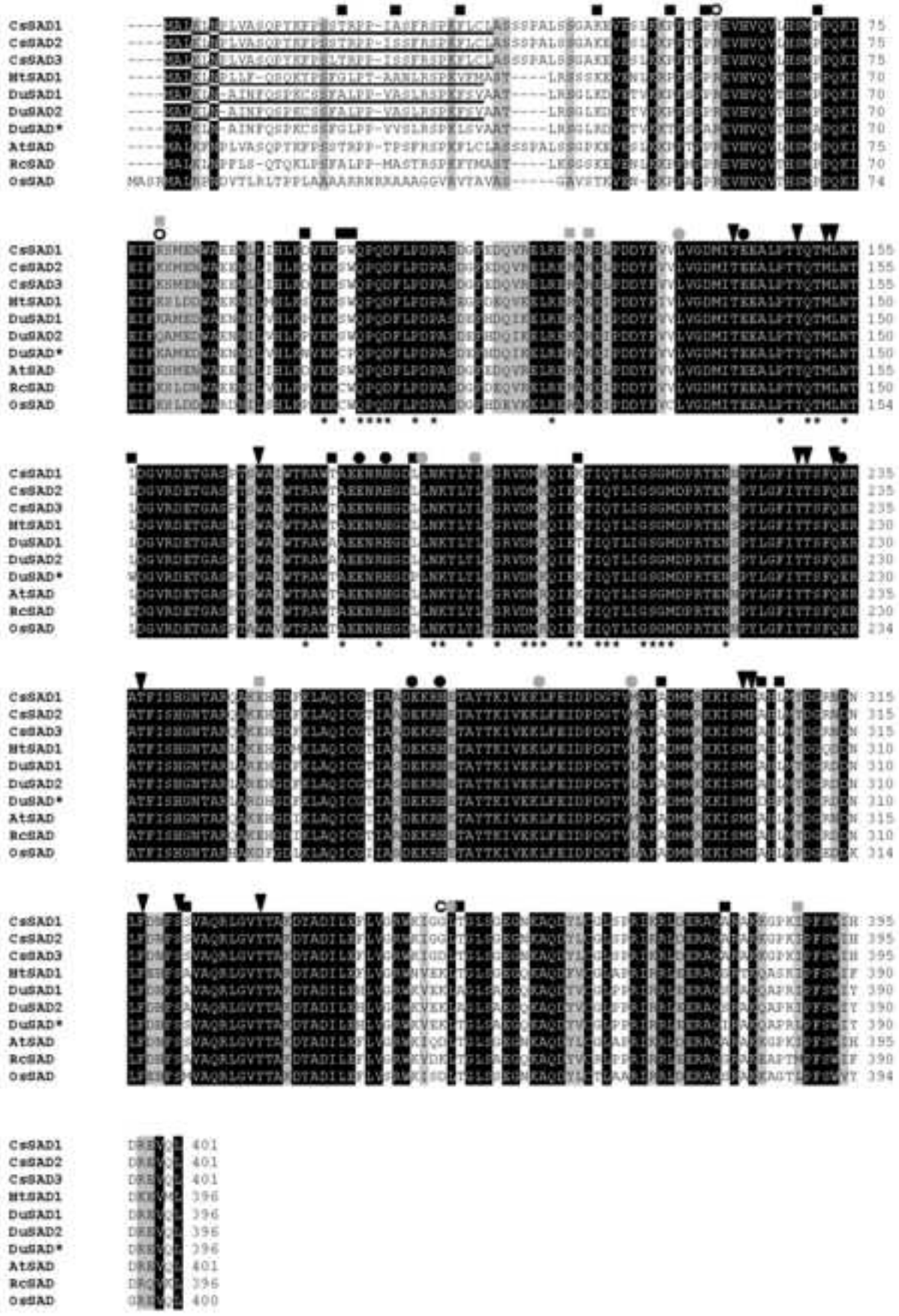

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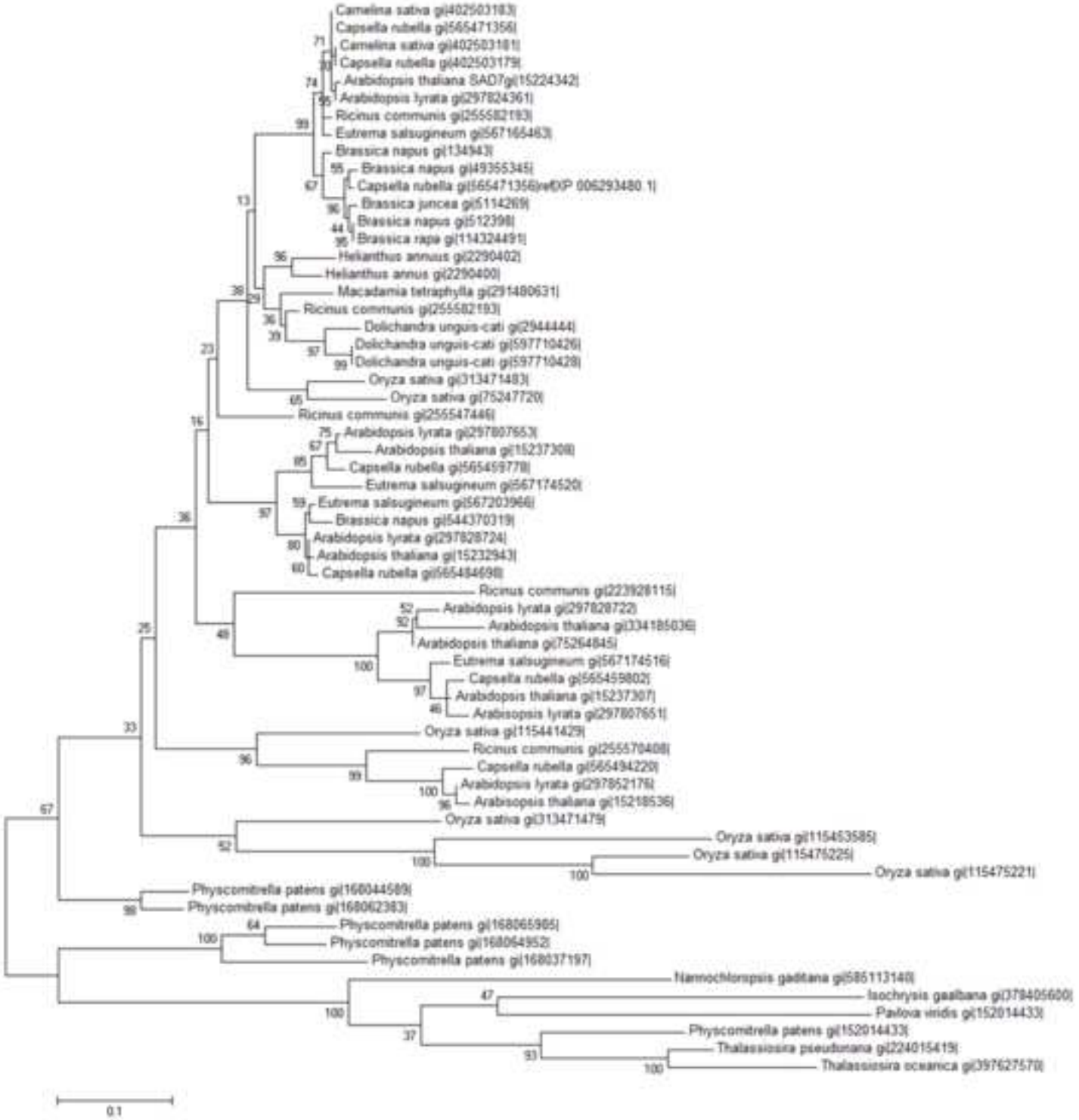

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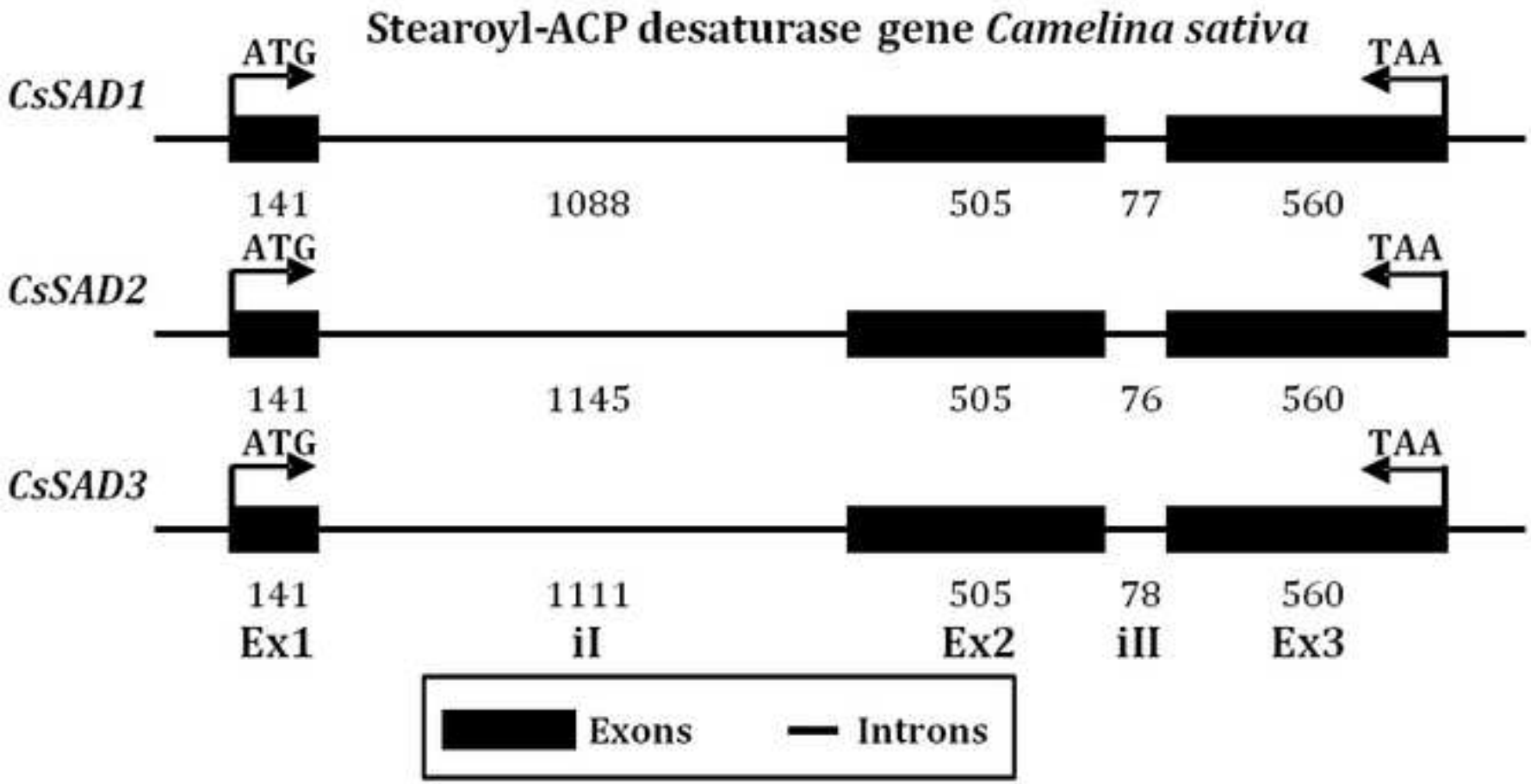

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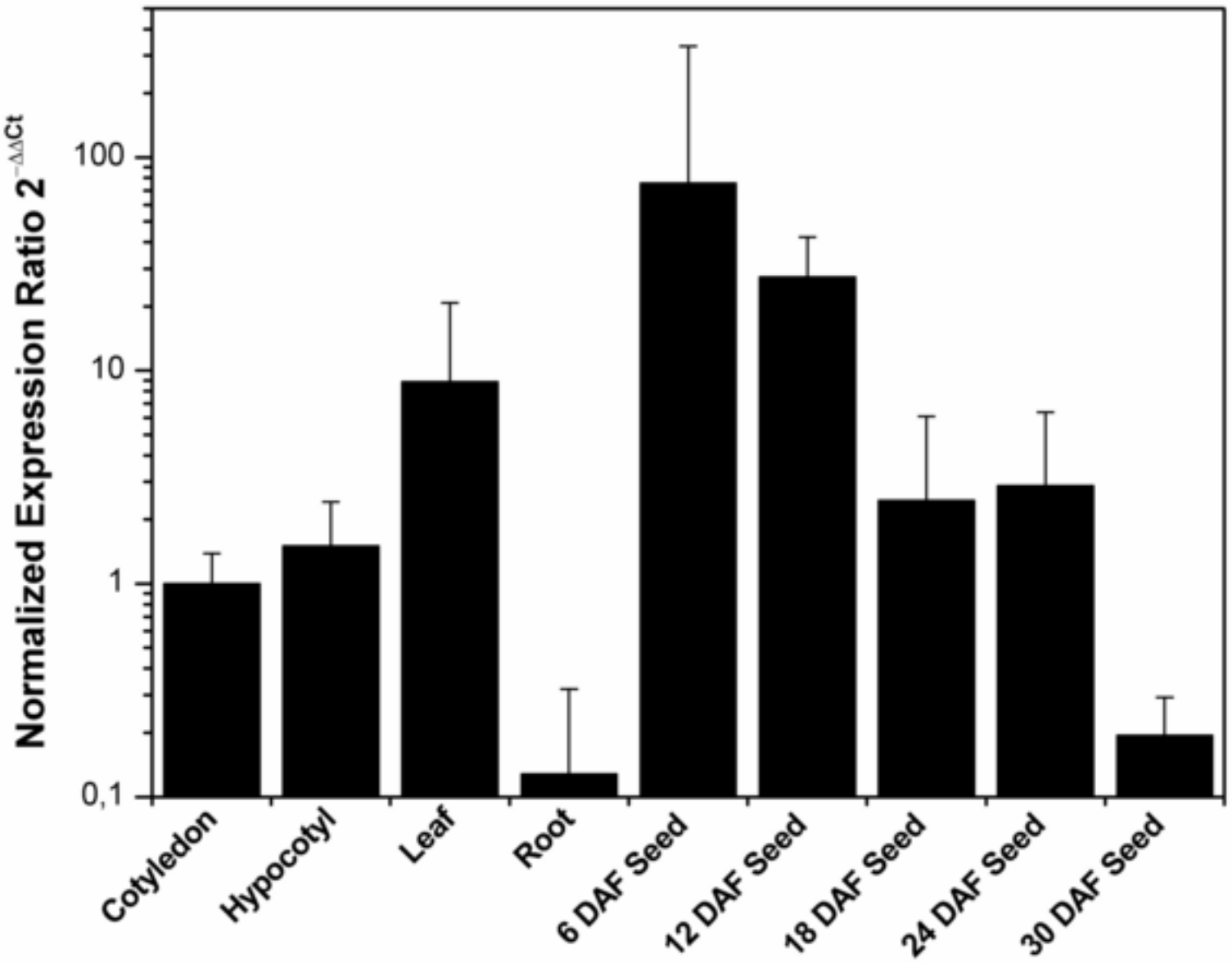

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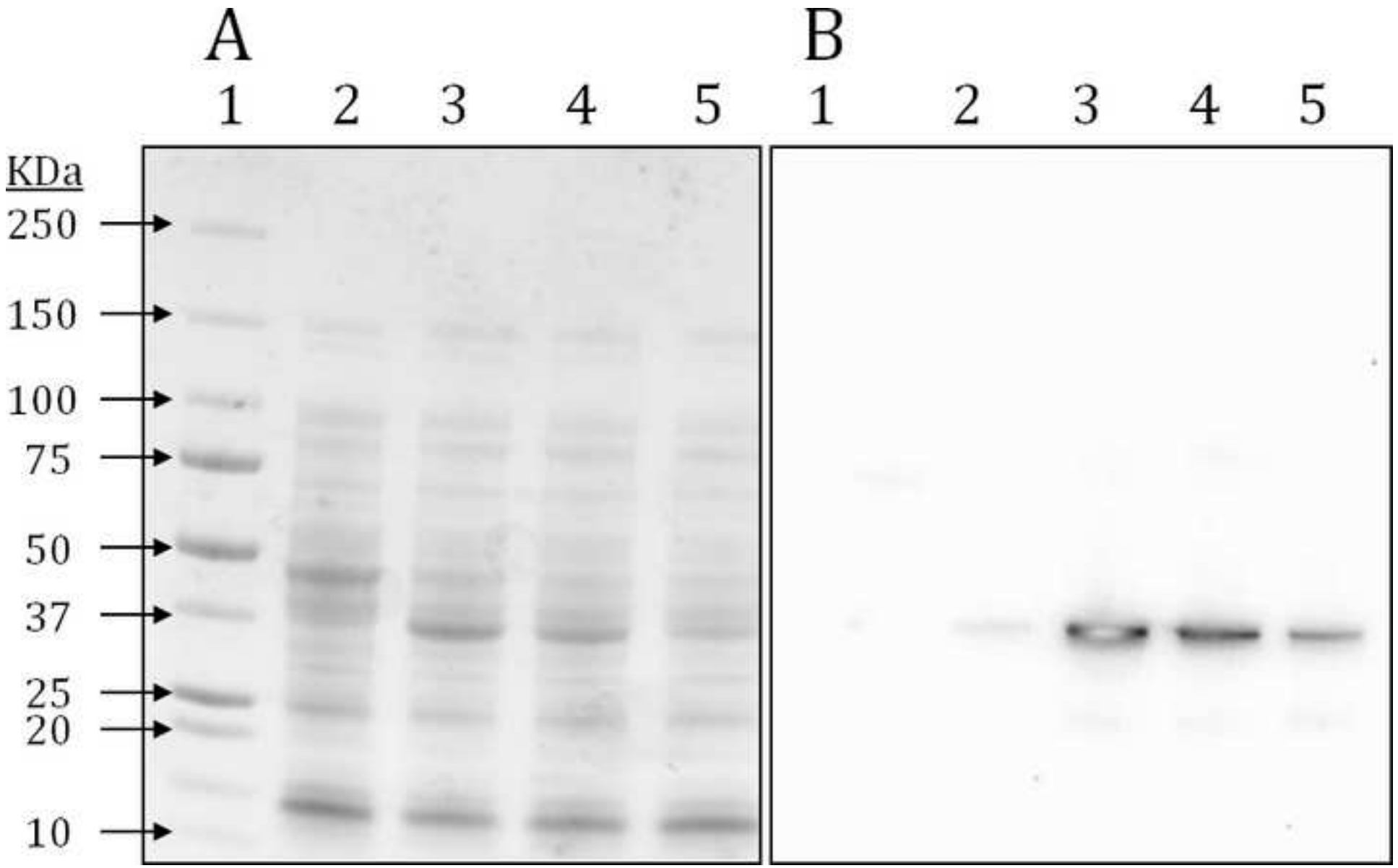

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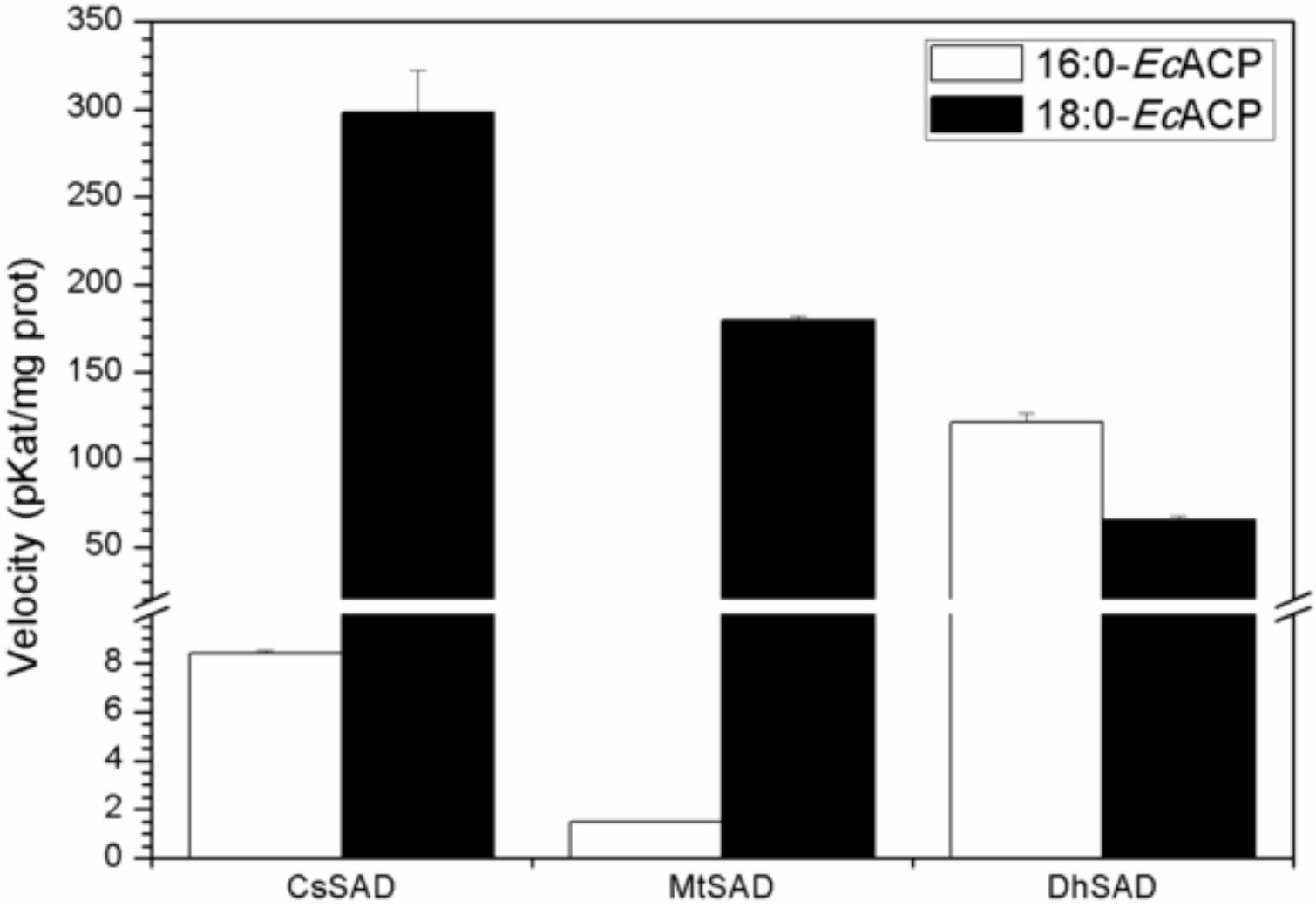

Graphical abstract

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