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Transcriptional Analysis of Stearoyl-Acyl Carrier Protein Desaturase Genes from Olive (*Olea europaea*) in Relation to the Oleic Acid Content of the Virgin Olive Oil

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ABSTRACT

The specific contribution of different stearoyl-ACP desaturase (*SAD)* genes to the oleic acid content in olive (*Olea europaea*) fruit has been studied. Towards that end, we isolated three distinct cDNA clones encoding three SAD isoforms from olive (cv. Picual), as revealed by sequence analysis. The expression levels of olive *SAD* genes were determined in different tissues from Picual and Arbequina cultivars, including developing mesocarp and seed, together with the unsaturated fatty acid content. Lipid and gene expression analysis indicate that *OeSAD2* seems to be the main gene contributing to the oleic acid content of the olive fruit and, therefore, of the virgin olive oil. This conclusion was confirmed when the study was extended to Hojiblanca, Picudo and Manzanilla cultivars. Furthermore, our data indicate that the olive microsomal oleate desaturase gene *OeFAD2-2*, but not *OeSAD2*, is responsible for the linoleic acid content in the virgin olive oil.

KEYWORDS: *Olea europaea, olive, desaturase, SAD, gene expression, oleic acid, olive oil*

INTRODUCTION

Olive (*Olea europaea* L.) is one of the first plants to be cultivated for oil production and the second most important oil fruit crop cultivated worldwide.[1] The olive fruit is a drupe consisting of a fleshy pericarp and a woody endocarp that encloses a single seed. The pericarp, in turn, consists of an outer epicarp and an inner mesocarp. Triacylglycerols (TAG) are formed and stored in both, the mesocarp and the seed. Olive mesocarp possesses the remarkable characteristic of having high TAG content together with active chloroplasts,[2] which make it an interesting system to study fatty acid metabolism.

Virgin olive oil (VOO) is a natural fruit juice having excellent organoleptic and nutritional properties. These properties are determined by the metabolites initially present in the olive fruit and by the transformation of some of them occurring during the fruit processing to obtain the oil. Among those metabolites, TAG and their fatty acids which are the major components, do not suffer any transformation during processing. Fatty acids are the main responsible for the nutritional and technological properties of VOO,[3] which is highly enriched (55-83%) in oleic acid (18:1 Δ9). It also contains variable amounts (3-21%) of linoleic acid (18:2 Δ9,12), but less than 1% of α-linolenic acid (18:3 Δ9,12,15).[4] The relative contents of oleic, linoleic, and α-linolenic acids depend mainly on the cultivar but also on pedoclimatic and culture conditions,[5] affecting the nutritional and technological properties of the oil. Remarkably, oleic acid decreases the risk of cardiovascular diseases,[6] and suppresses tumorigenesis of inflammatory diseases.[7] In addition, oleic acid has 10-fold higher auto-oxidative stability than linoleic acid and, therefore, oils with higher oleic to linoleic acid ratio, such as olive oil, have longer shelf life.[8]

In higher plants, fatty acid biosynthesis occurs in the plastid by successive addition of two carbon atoms from acetyl-CoA, yielding primarily palmitoyl-acyl carrier protein (16:0-ACP) and stearoyl-ACP (18:0-ACP).[9] The first desaturation takes place in the plastid by the action of the soluble stearoyl-ACP desaturase (SAD), which produces oleoyl-ACP that is the main product of the plastidial fatty acid synthesis. SAD enzyme uses ferredoxin as electron donor. In addition, in some cases, it can desaturate other precursors, such as palmitoyl-ACP, to produce palmitoleoyl-ACP (16:1 Δ9-ACP).[10] The acyl-ACPs are then cleaved by specific thioesterases to free fatty acids, which are incorporated into glycerolipids, where can be further desaturate to linoleic and α-linolenic acids. Two sets of Δ12 and Δ15 membrane-bound fatty acid desaturases has been described, which differ in their cellular localization, lipid substrates, and electron donor system.[11] The microsomal Δ12 and Δ15 desaturases (FAD2 and FAD3, respectively) are located in the endoplasmic reticulum and use phospholipids as acyl substrates, as well as NADH, NADH-cytochrome b_5 reductase, and cytochrome b_5 as electron donors. On the other hand, the plastidial Δ12 and Δ15 desaturases (FAD6 and FAD7/8, respectively) are located in the chloroplast and use primarily glycolipids as acyl carriers, as well as NAD(P)H, ferredoxin-NAD(P) reductase, and ferredoxin as electron donors. In this respect, the fact that most plants lack other desaturases that utilize stearoyl-ACP as substrate, makes the activity of SAD of particular interest. This activity plays an essential role determining the overall levels of fatty acid desaturation,[12] which has a significant effect on the fluidity and rigidity of membrane system and the relationship of this to the adaption of plants to various environmental conditions.

Given the functional importance of SAD in plants, *SAD* genes from many plant species have been cloned and characterized, and a high correlation between SAD activity and levels of stearic and oleic acids has been observed. For instance, antisense

expression of *Brassica rapa SAD* gene in *Brassica napus* leads to dramatically increased stearate levels in the transgenic seeds[13] and, in the reverse, when the *SAD* gene from yellow lupine was overexpressed in tobacco, transgenic plants exhibited very high level of oleic acid in comparison with control plants.[14] On the other hand, the Arabidopsis genome carries seven *SAD* genes, and all of them except *DES6* are predicted to be localized in the plastid.[15] Furthermore, SSI2/FAB2 seems to be the major contributor of SAD activity responsible for oleic acid synthesis in Arabidopsis seeds, and is also regulated at post-translational level.[15]

The search for new olive cultivars with improved nutritional, organoleptic and technological quality is considered a priority in olive breeding programs. In particular, high oleic acid content is currently being used as quality trait to enhance the health promoting properties and oxidative stability of the corresponding VOOs. In olive, a single *SAD* gene encoding stearoyl-ACP desaturase has been characterized up to date[16], and a second SAD sequence has been deposited in the GenBank database. However, a comprehensive characterization of the olive *SAD* gene family, together with the determination of the oleic acid content in the different lipid classes during the development and ripening of the olive fruit mesocarp have not been reported so far.

In this sense, the aim of this work was the transcriptional analysis of olive fruit genes encoding stearoyl-ACP desaturase, in order to identify the main *SAD* gene or genes responsible for the oleic acid content in the VOO.

MATERIALS AND METHODS

Chemicals

Culture media components and agarose were purchased from Pronadisa (Torrejón de Ardoz, Spain), and liquid chromatography grade solvents were supplied by Merck (Darmstadt, Germany). IPTG, X-Gal, restriction enzymes, RNAse A, molecular markers for DNA, and dNTPs were purchased from Fermentas (Vilnius, Lithuania). Fatty acids, antibiotics, and buffer components were supplied by Sigma–Aldrich (St Louis, MO, USA).

Plant material

Olive (*Olea europaea* L. cv. Picual and Arbequina) trees were grown in the experimental orchard of Instituto de la Grasa, Seville (Spain), with drip irrigation and fertirrigation from the time of full bloom to fruit maturation. Young drupes, developing seeds, and mesocarp tissue were harvested at different times after blooming corresponding to different developmental stages of the olive fruit, frozen in liquid nitrogen, and stored at -80ºC. Young leaves were collected similarly. The dry weight (DW) was determined by incubating 25 g of olive fruit in the case of mesocarp tissue or 0.5 g of seeds at 110 ºC until constant weight.

Isolation of stearoyl-ACP desaturase full-length cDNA clones

Candidate olive *SAD* sequences were found in the olive EST database[17] using the tblastn algorithm. Based on these sequences, specific pairs of primers for each gene were designed and utilized for PCR amplification with ACCUZYME™ DNA polymerase (Bioline, Spain), which has proofreading activity. An aliquot of an olive Uni-ZAP XR cDNA library constructed with mRNA isolated from 13 weeks after

flowering (WAF) olive fruit of cultivar Picual,[16] was used as DNA template. One fragment with the expected size was generated in each reaction, subcloned into the vector pSpark® I (Canvax, Spain) and sequenced in both directions.

DNA sequence determination and analysis

DNA sequence determination was performed by SECUGEN (Madrid, Spain). The DNA sequence data were compiled and analyzed with the LASERGENE software package (DNAStar, Madison, WI). The hydropathy plots of the three SAD amino acid sequences were generated by the method of Kyte and Doolittle.[18] Multiple sequence alignments of olive SAD amino acid sequences were calculated using the ClustalX program and displayed with GeneDoc. Phylogenetic tree analysis was performed using the Neighbor-Joining method implemented in the Phylip package using Kimura's correction for multiple substitutions and a 1,000 bootstrap data set. TreeView was used to display the tree. Subcellular localization was predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/).

Total RNA extraction and cDNA synthesis

Total RNA isolation was performed as described by Hernández et al.[19] using 1-2 g of frozen olive tissue collected from at least three different olive trees and different olive fruit. RNA quality verification, removal of contaminating DNA and cDNA synthesis were carried out according to Hernández et al.[20]

Quantitative real-time PCR (qRT-PCR)

Gene expression analysis was performed by qRT-PCR as previously described.[20] Primers for gene-specific amplification (Table S1) were designed using the Primer3

146 program (http://bioinfo.ut.ee/primer3/). The housekeeping olive ubiquitin2 gene
147 (*OeUBQ2*, AF429430) was used as an endogenous reference for normalization.[20] Real-
148 time PCR data were calibrated relative to the corresponding gene expression level in 12
149 WAF mesocarp tissue from Picual. The relative expression level of each gene was
150 calculated following the $2^{-\Delta\Delta Ct}$ method for relative quantification.[21] Data are presented
151 as means ± SD of three reactions performed in different 96-well plates, each having two
152 replicates in each plate.
153
154 **Lipid analysis**
155 Olive fruit mesocarp tissue was heated at 70 ºC for 30 min with isopropanol to
156 inactivate endogenous lipase activity. Lipids were extracted as described by Hara and
157 Radin,[22] and lipid separation was carried out by thin layer chromatography according to
158 Hernández et al.[23] Fatty acid methyl esters of the different olive tissues and lipid
159 preparations were produced by acid-catalyzed transmethylation,[24] and analyzed by gas
160 chromatography.[25] Heptadecanoic acid was used as internal standard to calculate the
161 lipid and fatty acid content in the samples. Results are expressed either in mol percent
162 of the different fatty acids or in mg of the sum of unsaturated fatty acids per g of DW,
163 and are presented as means ± SD of three independent determinations.
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RESULTS AND DISCUSSION

cDNA isolation and sequence analysis of olive stearoyl-ACP desaturase genes

From the olive EST database,[17] three contigs were identified with a high degree of similarity to the olive stearoyl-ACP desaturase gene (*OeSAD*) previously characterized from cultivar Koroneiki.[16] One of them (*OeSAD1*) exhibited near 100% identity to the above mentioned gene, whereas the second one (*OeSAD2*) showed near 100% identity to another olive SAD sequence deposited in the GenBank database from cultivars Leccino (Accession No. U58141), Mari (Accession No. KP165083) and Shengeh (Accession No. KP165084). Finally, the third contig (*OeSAD3*) showed a lower degree of identity to all of them and turned out to correspond to a distinct SAD isoform described in olive. To obtain the three full-length cDNAs from cultivar Picual (*OepSAD1*, *OepSAD2* and *OepSAD3*), a pair of specific primers was designed based on each contig sequence and used for PCR amplification, together with an aliquot of an olive fruit (13 WAF) cDNA library. The three amplified fragments were isolated and sequenced in both directions.

The *OepSAD1*, *OepSAD2* and *OepSAD3* full-length cDNA clones, with sizes of 1477, 1494 and 1427 bp, revealed ORFs encoding predicted proteins of 390, 390 and 396 amino acid residues, respectively, which correspond to a calculated molecular mass of 44.6 kDa for OepSAD1 and OepSAD2, and 45.5 kDa for OepSAD3, and a p*I* of 6.36, 6.21 and 6.34, respectively. These ORFs were flanked by unique 5'- and 3'-UTRs of 92 and 212 bp respectively, for *OepSAD1*, 122 and 199 bp for *OepSAD2* and 82 and 154 bp for *OepSAD3*, with a poly(A) tail at the 3'-end.

Analysis of OepSAD deduced amino acid sequences with TargetP Server, predicted chloroplast localization for all of them, and an N-terminal transit peptide with

196 the characteristic features of a chloroplast targeting peptide (Figure 1). The putative
197 transit peptide for the translocation of the protein into the stroma of plastids showed a
198 similar predicted cleavage site for OepSAD1 and OepSAD2 after Arg at residue 49,
199 while OepSAD3 showed a shorter transit peptide with a predicted cleavage site after
200 Met at residue 33, as previously reported for SAD enzymes from safflower,[26] sesame,[27]
201 oil palm,[28] Jatropha,[29] and *Cinnamomum longepaniculatum*.[30] On the other hand,
202 hydrophobicity plot analysis of deduced amino acid sequences of OepSAD showed that
203 these enzymes are likely to be water-soluble proteins.

204 Alignment of the three deduced amino acid sequences (Figure 1) showed that
205 while OepSAD1 and OepSAD2 shared 97% identity, OepSAD3 displayed only 86%
206 identity with the other two olive SAD proteins. Among the conserved amino acids in
207 the three olive SAD sequences were two regions, namely, DETGASP/L and DYADILE
208 which are highly conserved in plant SAD sequences, of sequence identity to the two
209 peptides identified by direct N-terminal sequencing of tryptic fragments derived from
210 the purified avocado stearoyl-ACP desaturase.[12] In addition, two E/DEXXH motifs were
211 also found in olive SAD sequences, which are essential for catalytic activity, and their
212 amino acid sequence is characteristic of a soluble group of acyl-ACP desaturases.[31] In
213 fact, these two last amino acid sequences represent a biological motif used for the
214 creation of reactive catalytic intermediates. Thus, the process of fatty acid desaturation
215 may proceed via enzymatic generation of a high-valent iron-oxo species derived from
216 the diiron cluster.[31] In addition, the three olive SAD amino acid sequences displayed
217 significant identity to other plant SAD sequences (62-86%). All these data indicate that
218 the three olive *SAD* genes code for stearoyl-ACP desaturase enzymes, and they belong
219 to the acyl-ACP desaturase family and to the ferritin-like family.[32] Moreover, the high
220 amino acid sequence identity of all *SAD* genes suggests that SAD proteins have been

221 highly conserved during evolution and further demonstrates their critical enzymatic
222 roles in fatty acid biosynthesis in plants.

223 To elucidate the phylogenetic relationship of the olive *SAD* genes, their deduced
224 amino acid sequences were included in a dendrogram representing all known and
225 characterized plant SAD sequences, for comparison (Figure 2). The results revealed that
226 the phylogenetic tree grouped the SAD sequences into three different clusters. While
227 OepSAD1 and OepSAD2 were positioned in the same group with a close relationship,
228 OepSAD3 was located in a different group, showing the closest evolutionary
229 relationship with *Fraxinus americana* SAD (FaSAD).

230

231 **Tissue specificity of olive stearoyl-ACP desaturase genes**

232 To investigate the physiological role of the three olive *SAD* genes (*OeSAD1*,
233 *OeSAD2*, and *OeSAD3*) and their specific contribution to the biosynthesis of oleic acid
234 and its polyunsaturated derivatives, we have measured their steady-state transcripts
235 levels and determined the unsaturated fatty acids content in tissues from Picual and
236 Arbequina cultivars characterized by a highly active lipid biosynthesis (Figure 3).
237 Particularly, we have studied young leaves, where the biosynthetic machinery is
238 directed toward the synthesis of membrane lipids, largely represented by galactolipids;
239 developing seeds, with a high rate of accumulation of storage lipids; mesocarp tissue,
240 which possesses both active chloroplasts, where the lipid biosynthesis of the thylakoid
241 membranes takes place and a high accumulation of TAG which are the major
242 components of the olive oil; and finally young drupes, which can be considered a tissue
243 with intermediate characteristics between leaf and mesocarp. The young drupe (9 WAF)
244 showed oleic, linoleic and α-linolenic acids percentages around 20% in both cultivars
245 (Figure 3A). On the other hand, oleic acid was the main fatty acid in mesocarp and seed

246 tissues, either in Picual or Arbequina cultivar. The percentage of linoleic acid was
247 higher in Arbequina mesocarp than in Picual, and in the seed than in the mesocarp in
248 both cultivars. The total unsaturated fatty acids content in the mesocarp increased from
249 12 to 31 WAF, while in the seed the levels were similar in both stages of development.
250 On the other hand, in the leaves, the unsaturated fatty acids content represents about
251 60% of total fatty acids, being α-linolenic acid the major fatty acid in both cultivars.

252 When the expression levels of the olive *SAD* genes were analyzed in the above
253 mentioned tissues from Picual and Arbequina cultivars (Figure 3B), it was observed that
254 olive *SAD* genes were expressed in all studied tissues but at different levels, indicating a
255 spatial regulation of *SAD* genes in olive. Similar results have been described for the
256 *SAD* genes from oil palm,[28] Jatropha,[29] *C. longepaniculatum*,[30] and *Pongamia*
257 *pinnata*.[33] In the case of *OeSAD1*, expression levels remained low in all studied tissues.
258 With respect to *OeSAD2* gene, transcript levels increased considerably in the mesocarp
259 of Picual and Arbequina cultivars at 31 WAF compared to the same tissue at 12 WAF.
260 On the contrary, the expression levels of *OeSAD2* in the seed from both cultivars at the
261 beginning of development (16 WAF) were high, and decreased significantly at 31
262 WAF. On the other hand, *SAD3* gene showed higher expression levels in young drupe
263 and mesocarp than in the seed, especially at the beginning of development in Arbequina
264 cultivar. In the leaves, despite the higher transcript levels of *OeSAD3* compared to
265 *OeSAD1* and *OeSAD2,* the expression levels of the three olive *SAD* genes were lower
266 than in the other studied tissues. These data indicate that olive *SAD* genes are expressed
267 mainly in oil bearing tissues such as mesocarp and seeds or rapidly growing tissues such
268 as the drupe. These results are in agreement with those previously reported for other
269 *SAD* genes, as in the case of castor,[12] rape,[34] sesame,[27] and *P. pinnata*.[33]
270

Developmental expression of stearoyl-ACP desaturase genes in olive fruit in relation to the oleic acid content

In order to identify the olive *SAD* gene or genes mainly involved in the oleic acid biosynthesis in olive fruit and, consequently, responsible for its content in the VOO, we have measured their expression levels and the unsaturated fatty acids content in the mesocarp and seed tissues from Picual and Arbequina cultivars at different times during olive fruit development and ripening (Figures 4 and 5). To look for correlations between olive *SAD* gene transcript levels and unsaturated fatty acids content, we have calculated the sum of oleic acid and palmitoleic acid, which can be produced in the plastid by means of SAD activity, and also linoleic and α-linolenic acids that are synthesized by further desaturation of oleic acid. During the mesocarp development and ripening, the unsaturated fatty acids content increased considerably for both cultivars from approximately 20.0 μg/mg DW, reaching 379.73 and 343.61 μg/mg DW at the end of maturity for Picual and Arbequina, respectively (Figure 4A).

To further investigate a possible correlation between olive *SAD* genes expression levels and the unsaturated fatty acids content in Picual and Arbequina cultivars, we have analyzed the percentage of unsaturated fatty acids in the different lipid classes from mesocarp tissue of both cultivars at different stages of development and ripening (Table 1). We did not observe any significance difference in the unsaturated fatty acids content between Picual and Arbequina cultivars during mesocarp development and ripening. In particular, the highest unsaturated fatty acids percentage in the mesocarp tissue from both cultivars was shown in diacylglycerol and TAG, with a slightly increase during the mesocarp development, and reaching about 80% at the end of the ripening period. In addition, phosphatidylcholine and phosphatidate showed a slightly increase in the

295 unsaturated fatty acids percentage during the mesocarp development and ripening, while
296 the levels in galactolipids remained unchanged.

297 The expression analysis of olive *SAD* genes (Figure 4B) revealed that *OeSAD1*
298 transcript levels remained practically constant during mesocarp development and
299 ripening, while *OeSAD2* gene expression increased, with a maximum at the beginning
300 of fruit ripening (28 WAF), and then slightly declined. This expression pattern was
301 similar in both cultivars, although the *OeSAD2* transcripts in Arbequina reached higher
302 levels than in Picual. With respect to *OeSAD3* gene, the expression levels decreased
303 during the mesocarp development and ripening, being the effect more pronounced in
304 Arbequina than in Picual cultivar. Therefore, unlike *OeSAD1* and *OeSAD3* genes which
305 are characterized by a steady or decreased transcript levels in the mesocarp tissue during
306 the development and ripening of the olive fruit, the expression levels of *OeSAD2* gene
307 increased similarly to the unsaturated fatty acids content in the mesocarp tissue of both
308 Picual and Arbequina cultivars (Figure 4). However, the higher expression levels of
309 *OeSAD2* gene detected in Arbequina mesocarp compared to Picual are not accompanied
310 by higher unsaturated fatty acids content neither in TAG, nor in other microsomal or
311 chloroplastic lipids (Table 1), suggesting the possible existence of translational or post-
312 translational regulatory mechanisms.

313 A similar study was conducted in developing seeds (Figure 5). In this tissue, the
314 unsaturated fatty acids content showed a rapid and significant increase in both cultivars
315 at the beginning of fruit development, immediately after the lignification of the stone
316 (Figure 5A). Then, these levels remained constant during the fruit development, without
317 reaching the levels observed in the mesocarp. Gene expression analysis revealed that
318 *OeSAD1* and *OeSAD3* transcript levels remain constant in the seed during olive fruit
319 development and ripening (Figure 5B). Regarding *OeSAD2* gene, the expression levels

detected in the seed at 16 WAF were higher than in the mesocarp. Then, these levels decreased considerably during seed development in both cultivars, being almost undetectable at the beginning of fruit ripening (28 WAF). At the end of the ripening period, *OeSAD2* expression levels recovered, reaching similar levels than initial values in Picual cultivar. In this tissue, the high *OeSAD2* expression levels at the beginning of seed development are consistent with the increase observed in the unsaturated fatty acids content in Picual and Arbequina cultivars (Figure 5).

All these data reveal a temporal regulation of olive *SAD* genes and suggest that *OeSAD2* is the gene that contributes mainly to the oleic acid biosynthesis in both the mesocarp and seed tissues and, therefore, to its content in the VOO. Interestingly, the present olive *SAD* genes expression data in the mesocarp and seed tissues during the development and ripening of the olive fruit are in discrepancy with those reported previously for the olive *SAD* genes. However, the occurrence of non-specific hybridization in the Northern blots due to the use of a non-specific probe in one case,[16] or non-specific amplification in the qRT-PCR because of the use of non-specific primers in the second case,[35] cannot be discarded, since the nucleotide sequences of *OeSAD1* and *OeSAD2* exhibit a very high degree of identity and, in neither of these two cases, it was known the existence of additional *SAD* genes in olive.

The present study was extended to other cultivars of economic importance (Picudo, Hojiblanca and Manzanilla), using mesocarp tissue corresponding to three different stages of fruit ripening which cover the harvest period of the olive fruit for the olive oil production. The percentage of unsaturated fatty acids remained practically constant in all cultivars studied, with values around 80% (Figure 6A). With respect to the oleic and linoleic acids content, Picual, Hojiblanca and Manzanilla showed relatively low percentage of linoleic acid (3.3, 4.6 and 4.0%, respectively), whereas

345 Arbequina and Picudo cultivars are characterized by a higher linoleic acid content
346 (approximately 13% in both cultivars). The linolenic acid percentage was very low in
347 all studied cultivars. *OeSAD1* and *OeSAD3* gene expression levels in the five cultivars
348 remained almost constant during the mesocarp ripening, showing no significant changes
349 (Figure 6B). In contrast, the *OeSAD2* gene expression levels showed an important
350 increase during fruit ripening in all cultivars, and then slightly decreased at the end of
351 the ripening period in the case of Hojiblanca and Manzanilla cultivars. The highest
352 *OeSAD2* expression levels were observed in Arbequina and Manzanilla cultivars.
353 Therefore, all cultivars studied showed an increase in *OeSAD2* gene transcripts during
354 this period in contrast to *OeSAD1* and *OeSAD3* transcripts, which remained at low
355 levels, further confirming that the *OeSAD2* gene is mainly involved in the oleic acid
356 biosynthesis in the olive fruit and, therefore, in its content in the olive oil.

357 The presence of several SAD isoforms has been described in many plants species.
358 Two different *SAD* cDNA clones have been reported in sunflower, which were strongly
359 expressed in developing seeds.[36] Shah et al.[28] also detected two different SAD isoforms
360 in oil palm and, as reported in sunflower, both genes exhibited similar expression
361 pattern, although in this case the highest expression levels were detected in mature
362 mesocarp. In contrast, in Arabidopsis,[15] peanut,[37] linseed,[38] and cacao,[39] where more
363 than one SAD isoforms have also been described, only one of them seems to be the
364 main responsible for oleic acid biosynthesis in oily tissues, as we have observed in
365 olive.

366 On the other hand, a full correlation between expression levels of a *SAD* gene and
367 oil accumulation have been reported in peanut developing seeds.[37] Similar results were
368 obtained in *Brassica napus* seeds,[40,41] where the accumulation of *SAD* transcripts during
369 seed development occurred at the same time, but different to oleosin gene expression.

370 Liu et al.[42] also reported a correlation between *SAD* expression and oil concentration in
371 high-oil maize, and in sesame seeds the *SAD* gene was identified as an SSR marker
372 associated to oil content.[43] In olive, we have also observed a parallel behavior between
373 *OeSAD2* expression levels and oil accumulation in the mesocarp (Figures 4B and 7). In
374 addition, the rapid rate of oil biosynthesis at the beginning of seed development
375 correlates well with the high *OeSAD2* expression levels detected in the olive seed at 16
376 WAF (Figures 5B and 7). However, although the expression pattern of *OeSAD2* was
377 similar in Picual and Arbequina olive fruit, the expression levels during mesocarp
378 ripening and at the beginning of seed development were higher in Arbequina cultivar,
379 whereas the oil content was higher in Picual, in both tissues (Figure 7). Therefore, our
380 results suggest that, during mesocarp development and ripening, expression of *OeSAD2*
381 gene, which seems to be the main responsible for the oleic acid biosynthesis, was not
382 directly associated with TAG accumulation, in contrast to what Kilaru et al.[44] reported
383 in avocado, another oil fruit such as olive fruit.

384 In a previous study using different cultivars with distinct oleic and linoleic acid
385 contents,[20] it was shown that the expression levels of the microsomal oleate desaturase
386 gene *OeFAD2-2* in the mesocarp correlated adequately with the linoleic acid content in
387 this tissue. As a consequence, *OeFAD2-2* was identified as the gene mainly responsible
388 for the linoleic acid content in olive fruit mesocarp and, therefore, in the VOO. In
389 addition, because *OeFAD2-2* expression levels were always inversely correlated with
390 the oleic acid content, *OeFAD2-2* was also involved in the regulation of the quantity of
391 this monounsaturated fatty acid. Recently, it has been proposed that the higher oleic and
392 lower linoleic acids contents in Mari cultivar compared to Shengeh are due to higher
393 expression levels of *OeSAD2* and lower expression levels of *OeFAD2-2*.[35] However, in
394 the present work, we could not observe a correlation between *OeSAD2* transcript levels

and the linoleic acid content. In fact, among the cultivars studied, the highest *OeSAD2* expression levels were observed in Arbequina and Manzanilla cultivars, which are characterized by contrasting linoleic acid contents, high and low, respectively (Figure 6). These results support the hypothesis that *OeFAD2-2*, but not *OeSAD2* gene, is involved in the control of the linoleic acid content in olive fruit. In a similar way, it has been reported that the differential linolenic acid accumulation in high and low linolenic acid groups of linseed is mainly due to the contribution of *FAD2-2* and *FAD3* genes, with insignificant contribution of *SAD* genes.[38]

In conclusion, we have isolated and characterized three members of the olive *SAD* gene family. Sequence analysis of these genes (*OepSAD1, OepSAD2* and *OepSAD3*) indicates that they code for three SAD enzymes. Gene expression data and lipid analysis in olive fruit during development and ripening identify *OeSAD2* as the main gene responsible for the biosynthesis of oleic acid in mesocarp and seed tissues, and therefore, for its content in the olive oil. In addition, our results suggest that *OeFAD2-2*, but not *OeSAD2* gene, is responsible for the linoleic acid content in the olive oil. Finally, the present study will allow the development of molecular markers for *OeSAD2* gene to be used in the marker-assisted selection of new olive cultivars with higher unsaturated fatty acid content in their corresponding oils. Furthermore, knowledge of the factors involved in the regulation of this gene will help to establish optimum conditions for olive tree cultivation and olive fruit harvesting.

ABBREVIATIONS

ACP, acyl carrier protein; DW, dry weight; SAD, stearoyl-ACP desaturase; TAG, triacylglycerol; VOO, virgin olive oil; WAF, weeks after flowering.

ASSOCIATED CONTENT

Supporting Information

Table S1 shows gene accession numbers and sequences of primers pairs used for qRT-PCR; Table S2 shows accession numbers of SAD sequences included in the phylogenetic tree.

AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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580 **FIGURE CAPTIONS**

581

582 **Figure 1.** Comparison of the deduced amino acid sequences of olive *SAD* genes. The
583 sequences were aligned using the ClustalX program and displayed with GeneDoc.
584 Identical and similar residues are shown on a background of black or gray, respectively.
585 The conserved motifs of SAD and soluble plant desaturases are underlined with
586 continuous or dashed lines, respectively. The putative cleavage site of the chloroplast
587 transit peptide is indicated by a filled (OepSAD1 and OpSAD2) or open (OpSAD3)
588 arrow. The cDNA sequences corresponding to *OepSAD1, OepSAD2* and *OepSAD3* have
589 been deposited in the GenBank/EMBL/DDBJ database with accession numbers
590 KX196198, KX196199 and KX196200, respectively.

591

592 **Figure 2**. Phylogenetic tree analysis of plant stearoyl-ACP desaturases. Alignments
593 were calculated with ClustalX and the analysis was performed using the Neighbor-
594 Joining method implemented in the Phylip package using Kimura's correction for
595 multiple substitutions, and a 1,000 bootstrap dataset. TreeView was used to display the
596 tree. Positions of the olive *SAD* genes isolated in this work are in bold and underlined.

597

598 **Figure 3.** Unsaturated fatty acid composition (A) and relative expression levels of
599 *OeSAD1*, *OeSAD2* and *OeSAD3* genes (B) in different tissues of Picual and Arbequina
600 cultivars. Fatty acid composition and relative expression levels were determined in the
601 indicated tissues as described under Materials and Methods.

602

603 **Figure 4.** Evolution during olive fruit development and ripening of the unsaturated fatty
604 acids content (A) and relative expression levels of *OeSAD1*, *OeSAD2* and *OeSAD3*
605 genes (B) in the mesocarp tissue of Picual and Arbequina cultivars. The beginning of
606 fruit ripening, corresponding to the appearance of pink-purple colour, is marked by an
607 arrow. Fatty acid composition and relative expression levels were determined in the
608 indicated tissues as described under Materials and Methods.

609

610 **Figure 5.** Evolution during olive fruit development and ripening of the unsaturated fatty
611 acids content (A) and relative expression levels of *OeSAD1*, *OeSAD2* and *OeSAD3*
612 genes (B) in seeds of Picual and Arbequina cultivars. The beginning of fruit ripening,
613 corresponding to the appearance of pink-purple colour, is marked by an arrow. Fatty
614 acid composition and relative expression levels were determined in the indicated tissues
615 as described under Materials and Methods.

616

617 **Figure 6.** Unsaturated fatty acids percentages (A) and relative expression levels of
618 *OeSAD1*, *OeSAD2* and *OeSAD3* genes (B) in the mesocarp tissue from distinct olive
619 fruit cultivars during development and ripening. Fatty acid composition and relative
620 expression levels were determined in the indicated tissues as described under Materials
621 and Methods. Oleic acid, black bars; linoleic acid, white bars; and linolenic acid, gray
622 bars.

623

624 **Figure 7.** Evolution during olive fruit development and ripening of the oil content in the
625 mesocarp tissue (A) and seeds (B) of Picual (Black squares) and Arbequina (White
626 squares) cultivars.

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TABLES

Table 1. Unsaturated fatty acids content in different lipid classes from Picual and Arbequina mesocarp tissue at different stages of development and ripening

Lipid class	Cultivar	Unsaturated fatty acids content (mol%)			
		16 WAF	23 WAF	31 WAF	35 WAF
DAG	Picual	72.30 ± 4.29	79.83 ± 1.62	79.91 ± 1.02	78.99 ± 0.53
	Arbequina	63.61 ± 3.06	71.96 ± 1.41	78.42 ± 0.45	80.39 ± 0.19
TAG	Picual	79.59 ± 0.67	80.90 ± 0.35	80.90 ± 0.21	81.93 ± 0.04
	Arbequina	76.63 ± 0.25	80.11 ± 1.02	79.77 ± 0.04	82.79 ± 0.81
PI	Picual	48.61 ± 3.52	46.96 ± 5.59	46.55 ± 0.14	55.97 ± 0.28
	Arbequina	17.51 ± 3.86	17.25 ± 2.11	17.49 ± 3.48	40.35 ± 2.61
PS	Picual	34.87 ± 5.30	41.70 ± 1.52	35.19 ± 2.22	49.43 ± 4.25
	Arbequina	21.02 ± 7.27	8.86 ± 8.62	11.07 ± 3.77	42.68 ± 4.65
PC	Picual	68.88 ± 1.96	72.43 ± 1.34	63.68 ± 2.90	76.09 ± 1.46
	Arbequina	62.52 ± 5.58	66.15 ± 7.93	62.89 ± 0.63	65.06 ± 3.78
PE	Picual	53.13 ± 3.45	42.76 ± 0.60	38.09 ± 1.79	47.71 ± 3.08
	Arbequina	38.28 ± 4.21	26.07 ± 13.24	29.42 ± 0.74	39.90 ± 4.06
PA	Picual	67.44 ± 2.88	68.15 ± 1.07	68.20 ± 3.92	77.98 ± 0.43
	Arbequina	50.32 ± 17.38	66.30 ± 2.74	70.06 ± 1.52	65.05 ± 5.24
DGDG	Picual	65.55 ± 1.69	64.61 ± 1.30	60.06 ± 2.21	66.77 ± 1.24
	Arbequina	60.99 ± 3.64	54.77 ± 4.08	56.01 ± 2.00	54.72 ± 4.42
MGDG	Picual	53.14 ± 0.55	59.02 ± 1.56	66.48 ± 0.16	71.50 ± 2.36
	Arbequina	52.95 ± 0.63	36.05 ± 0.13	53.75 ± 2.51	37.61 ± 16.09

Lipid classes are abbreviated as follows: DAG, diacylglycerol; TAG, triacylglycerol; PI, phosphatidylinosytol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidate; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol.

FIGURE GRAPHICS

Figure 1

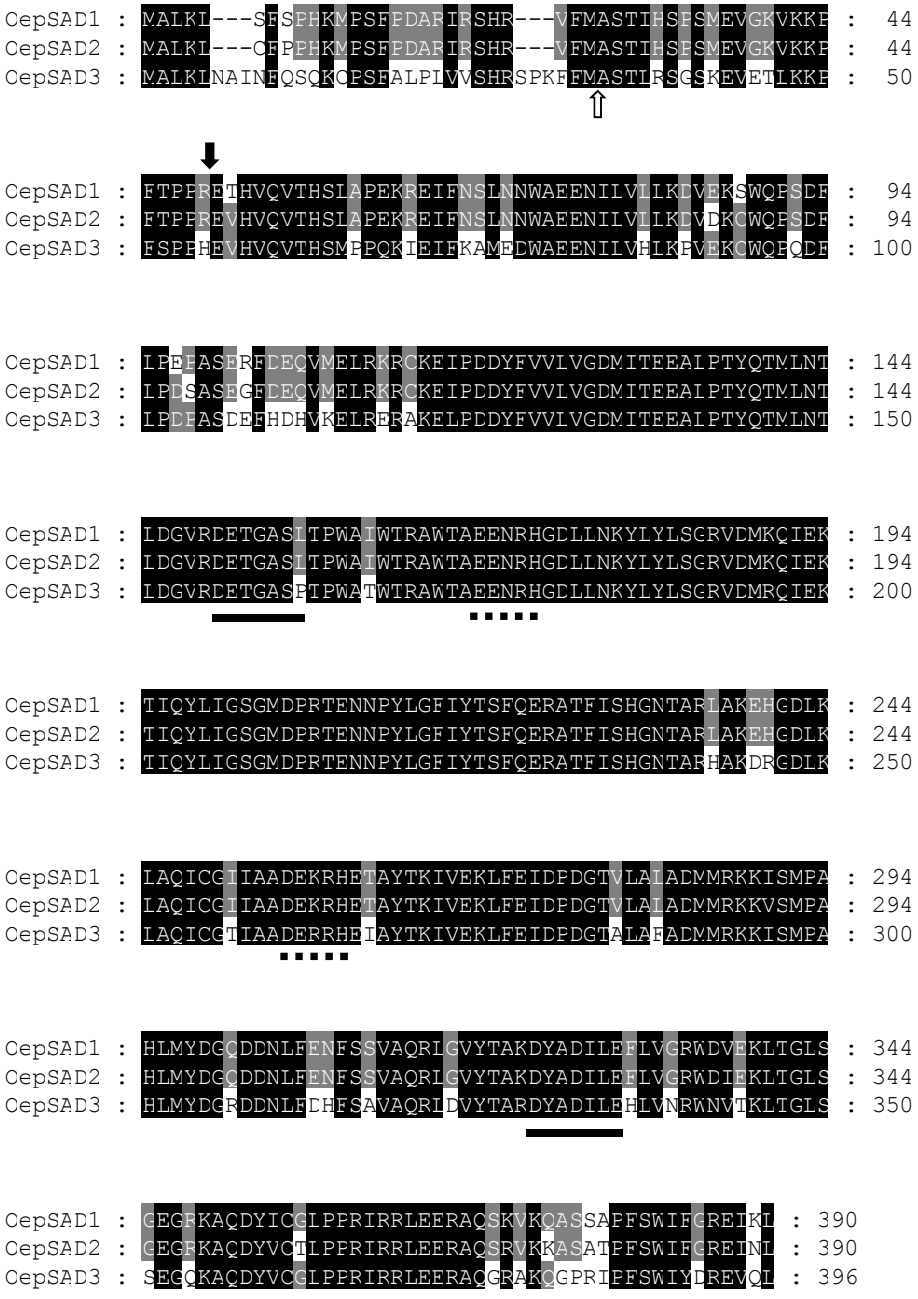


Figure 2

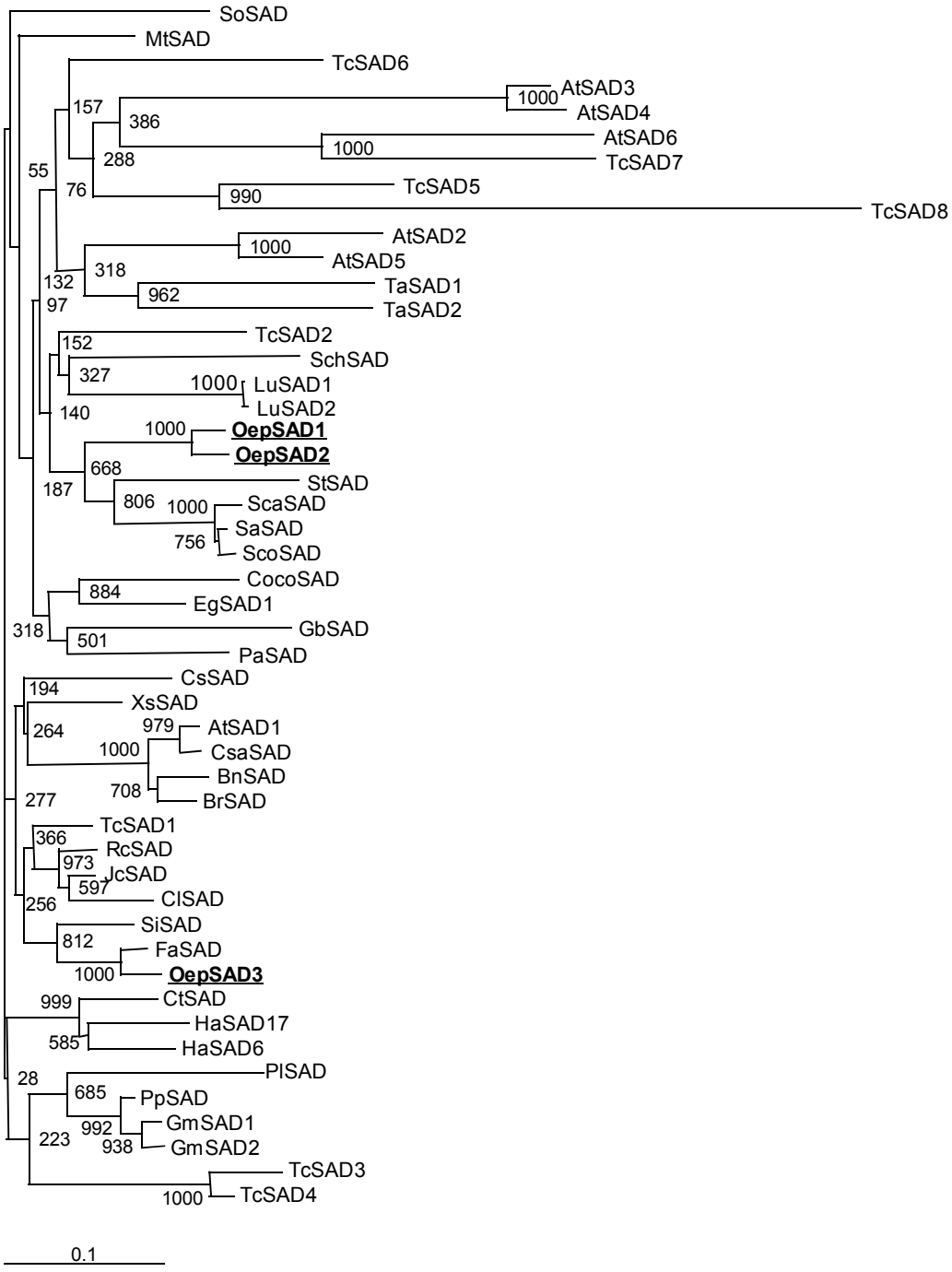

SoSAD
MtSAD
TcSAD6
1000
AtSAD3
AtSAD4
157
386
AtSAD6
1000
TcSAD7
288
55
76
TcSAD5
990
TcSAD8
1000
AtSAD2
AtSAD5
318
132
TaSAD1
962
97
TaSAD2
TcSAD2
152
SchSAD
327
1000
LuSAD1
LuSAD2
140
1000
OepSAD1
OepSAD2
668
StSAD
187
806
1000
ScaSAD
SaSAD
756
ScoSAD
CocoSAD
884
EgSAD1
318
GbSAD
501
PaSAD
CsSAD
194
XsSAD
264
979
AtSAD1
1000
CsaSAD
BnSAD
708
BrSAD
277
TcSAD1
366
RcSAD
973
JcSAD
597
ClSAD
256
SiSAD
812
FaSAD
1000
OepSAD3
999
CtSAD
HaSAD17
585
HaSAD6
PlSAD
28
685
PpSAD
992
GmSAD1
223
938
GmSAD2
TcSAD3
1000
TcSAD4
0.1

Figure 3

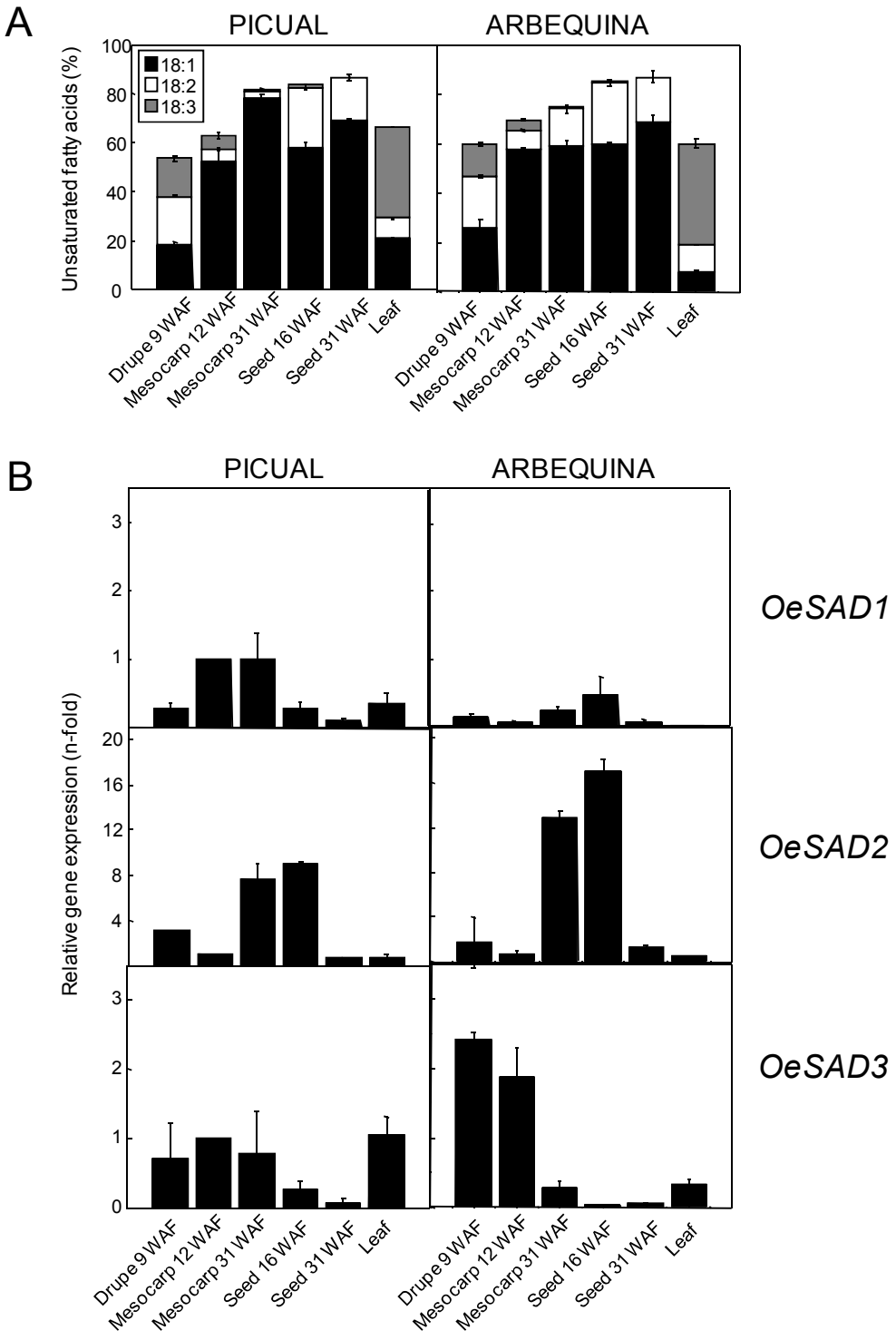

A
PICUAL
ARBEQUINA
Unsaturated fatty acids (%)
18:1
18:2
18:3
Drupe 9 WAF
Mesocarp 12 WAF
Mesocarp 31 WAF
Seed 16 WAF
Seed 31 WAF
Leaf
B
PICUAL
ARBEQUINA
Relative gene expression (n-fold)
OeSAD1
OeSAD2
OeSAD3

Figure 4

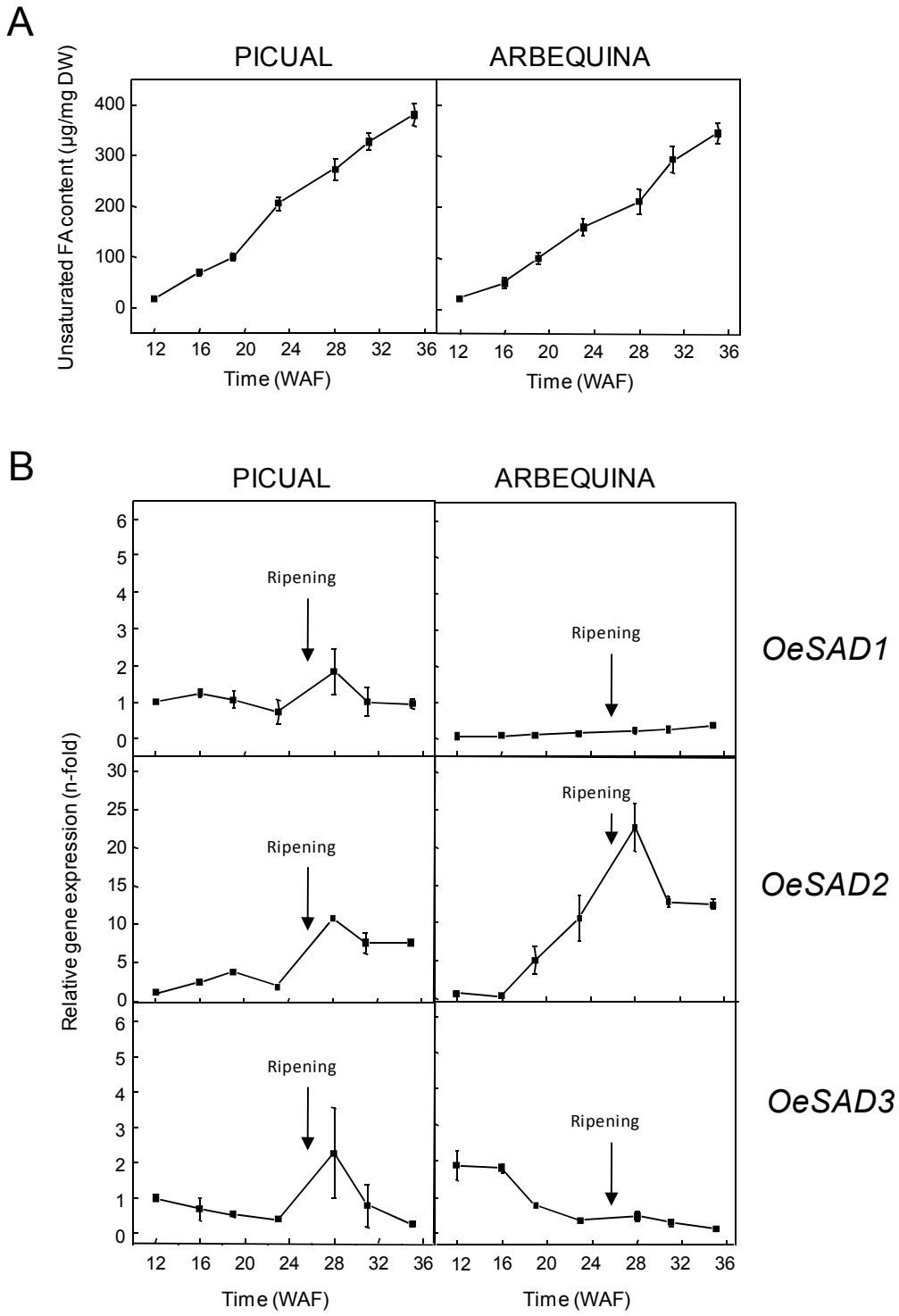

A
PICUAL
ARBEQUINA
Unsaturated FA content (µg/mg DW)
Time (WAF)
B
PICUAL
ARBEQUINA
Relative gene expression (n-fold)
Ripening
OeSAD1
OeSAD2
OeSAD3
Time (WAF)

Figure 5

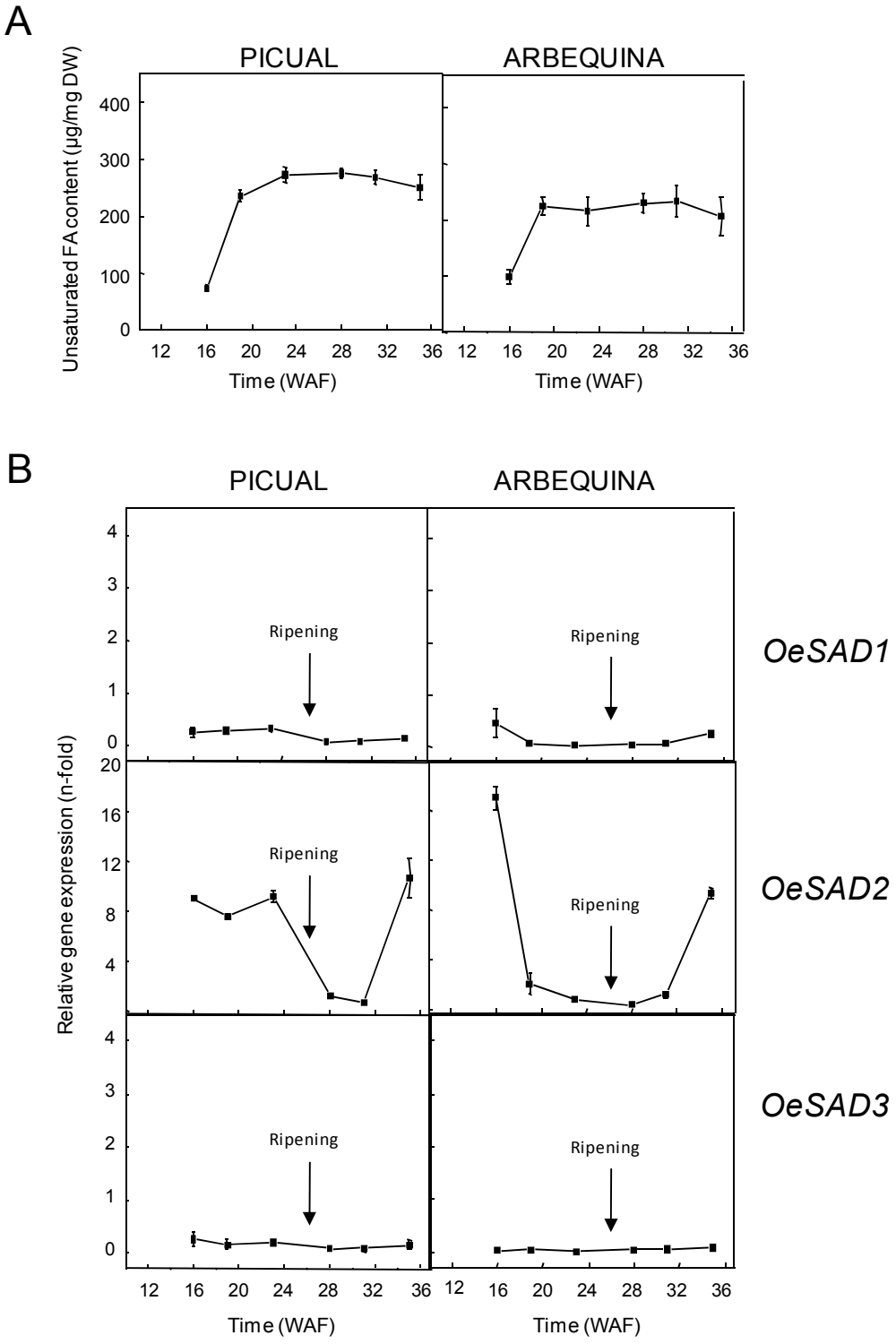

A
PICUAL
ARBEQUINA
Unsaturated FA content (µg/mg DW)
0
100
200
300
400
12 16 20 24 28 32 36
Time (WAF)
B
PICUAL
ARBEQUINA
Relative gene expression (n-fold)
Ripening
OeSAD1
OeSAD2
OeSAD3
Time (WAF)

Figure 6

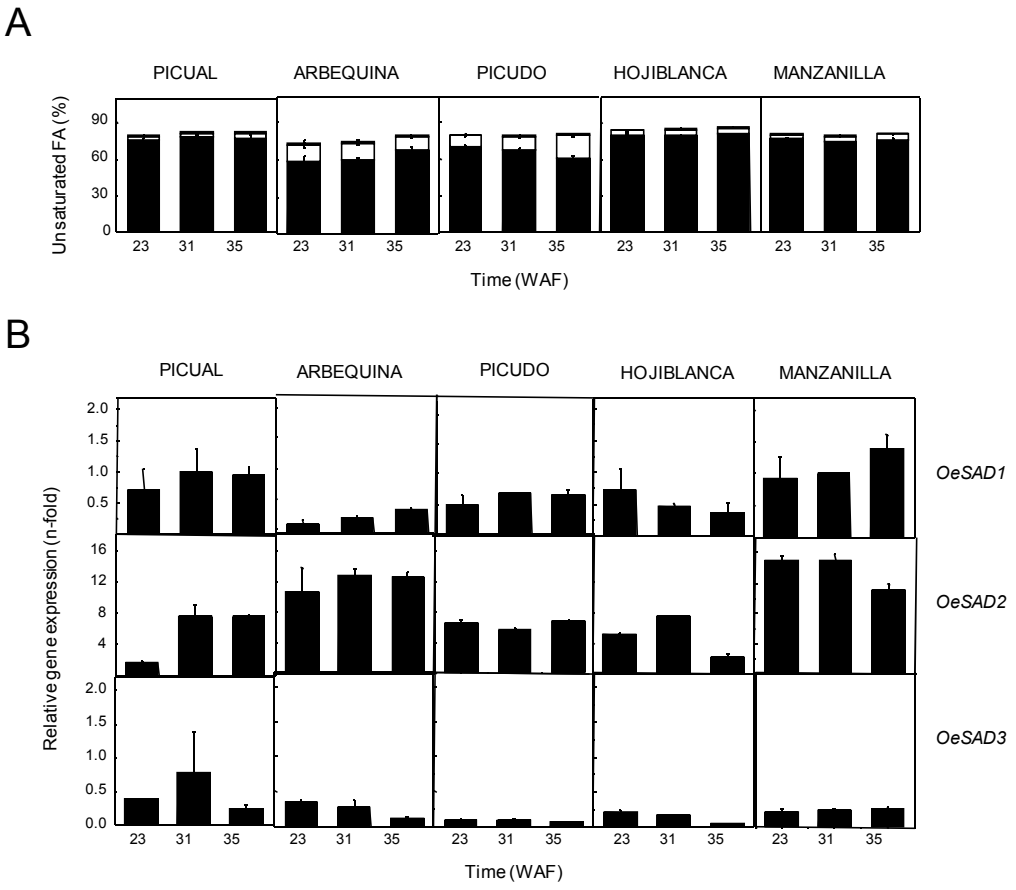
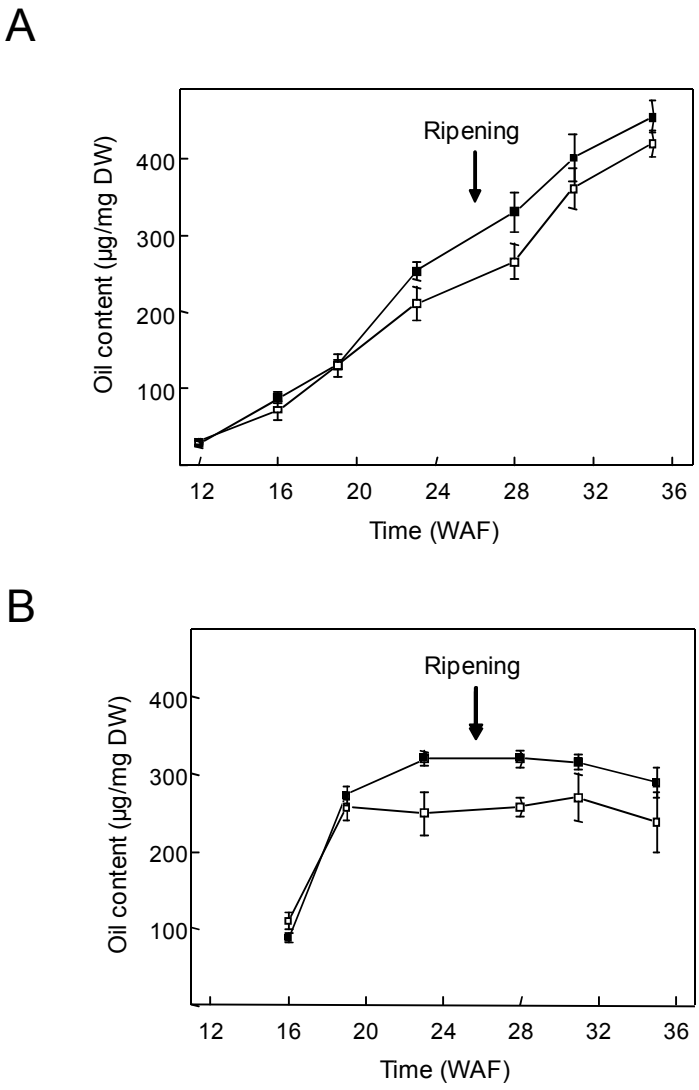
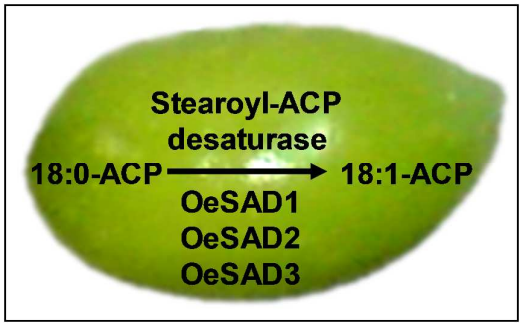


Figure 7


A
Ripening
Oil content (μg/mg DW)
100
200
300
400
12
16
20
24
28
32
36
Time (WAF)
B
Ripening
Oil content (μg/mg DW)
100
200
300
400
12
16
20
24
28
32
36
Time (WAF)

TOC graphic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OeSAD1
OeSAD2
OeSAD3