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Molecular cloning and characterization of genes encoding two microsomal oleate desaturases (*FAD2*) from olive

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Abstract

Two different cDNA sequences, designated *OepFAD2-1* and *OepFAD2-2*, encoding two microsomal oleate desaturases (FAD2) have been isolated from olive (*Olea europaea* cv. Picual) using a PCR approach. Both deduced amino acid sequences showed the three histidine boxes characteristic of all membrane-bound desaturases, and possess a C-terminal endoplasmic reticulum retention signal. Phylogenetic analysis shows that OepFAD2-1 and OepFAD2-2 are grouped with other plant FAD2 sequences. Functional expression of the corresponding *FAD2* cDNAs in yeast confirmed that they encode microsomal oleate desaturases. Genomic Southern blot analysis is consistent with the presence of at least two copies of each *OepFAD2* gene in the olive genome. *OepFAD2-1* transcript was strongly detected in very young seeds and in leaves, showing low levels in mesocarps, while the transcript of the *OepFAD2-2* gene was moderately expressed in developing seeds, ripening mesocarp and leaves. These expression data suggest differential functions for the two olive microsomal oleate desaturase genes, with *FAD2-1* possibly responsible for the desaturation of reserve lipids in the young seed, while *FAD2-2* may be mainly involved in storage lipid desaturation in the mature seeds and the mesocarp.

Keywords: *Olea europaea*; Oleaceae; Olive; Gene expression; Microsomal oleate desaturase; *FAD2*; Oleic acid; Linoleic acid

1. Introduction

Plant lipids contain polyunsaturated fatty acids, mainly linoleic and α-linolenic acids, which play crucial roles in plant metabolism as storage compounds mainly in the form of triacylglycerols (TAG), as structural components of membrane lipids, and as precursors of signalling molecules involved in plant development and stress response (Ohlrogge and Browse, 1995; Weber, 2002). Linoleic acid, together with oleic acid, is a major fatty acid in vegetable oils and its content greatly affects technological properties such as their oxidative stability (Márquez-Ruíz et al., 1999) and nutritional characteristics (Cunnane, 2003).

In higher plants, the fatty acid biosynthesis is catalyzed in the plastid by a type II (dissociable) fatty acid synthase, leading primarily to the synthesis of palmitoyl-ACP and stearoyl-ACP by successive additions of two carbon atoms from acetyl-CoA (Harwood, 1996). Still in the plastid, most of the stearoyl-ACP is desaturated to oleoyl-ACP by the soluble stearoyl-ACP desaturase. This oleic acid, which is the main product of the plastidial fatty acid synthesis, is largely activated to oleoyl-CoA and exported to the cytosol, where is incorporated into glycerolipids and can be further desaturated to linoleic acid by the microsomal oleate desaturase (FAD2). This enzyme is located in the endoplasmic reticulum (ER), use phospholipids as acyl

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substrates, and NADH, NADH-cytochrome b_5 reductase and cytochrome b_5 as electron donors (Shanklin and Cahoon, 1998).

Olive (*Olea europaea*) was one of the first plants to be cultivated for oil production. 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. TAG are formed and stored in both, the mesocarp and the seed, with different purposes. In the seed, TAG are synthesized as storage compounds to be used to nourish the embryo during the first steps of germination, whereas TAG accumulated in the mesocarp attract animals, aiding seed dissemination (Sánchez, 1994). The exceptional nutritional properties of olive oil are due to its well balanced fatty acid composition, as well as the presence of minor components such as natural antioxidants and vitamins. According to the European Commission Regulation (2003), oleic acid is the major fatty acid in the olive oil (55–83%), while linoleic acid accounts for 3.5–21%, and linolenic acid for less than 1%. The relative contents of oleic and linoleic acids depend on the variety and growth conditions, but only slightly on growth temperature.

Unlike oil seeds, little information is available on the regulation of fatty acid biosynthesis in oil fruits. Particularly, although *FAD2* genes have been isolated and studied from numerous plants including *Arabidopsis thaliana* (Okuley et al., 1994), and the oil seeds soybean (Heppard et al., 1996) and sunflower (Martínez-Rivas et al., 2001), no cloning and characterization have been reported for this gene from an oil fruit so far. In addition, olive fruit represents an interesting system to investigate the regulation of FAD2 because it contains two oil accumulating tissues, the seed and the mesocarp. Furthermore, the mesocarp can be considered an alternative model to oil seeds to study the regulation of this gene and its contribution to the final linoleic acid content, since this tissue possesses the remarkable characteristic of having a high proportion of active chloroplasts together with a high amount of TAG (Sánchez, 1994).

In this paper we report the isolation of two *FAD2* genes in olive. Expression studies show that they are tissue and developmentally regulated. Possible differential physiological roles related to their contribution to the linoleic acid content in different tissues are discussed.

2. Results and discussion

2.1. cDNA isolation and sequence analysis of microsomal oleate desaturases from olive

On the basis of highly conserved regions outside of the three histidine boxes characteristic for desaturases, to avoid cloning of desaturases with other regioselectivities, two degenarate primers were designed from the comparison of known plant FAD2 amino acid sequences (Fig. 1). These primers together with aliquots of an olive cDNA library were used for PCR amplification reactions. The library was made using mRNA isolated from 13 weeks after flowering (WAF) olive fruits, which correspond to the beginning of oil accumulation in the mesocarp and the seed of the olive fruit, and immediately after the lignification of the endocarp (Sánchez, 1994). Two different fragments were obtained using the FAD2 deduced primers, both with the expected size of about 900 bp. Sequencing of these clones revealed open reading frames (ORF) of 294 amino acids, and the alignment of the two deduced amino acid sequences showed a high degree of identity to the central coding regions of known plant FAD2 sequences, therefore, they were designated *OepFAD2-1* and *OepFAD2-2*.

To obtain the missing 5′-end of the partial cDNA clones, a PCR approach was performed. Two different reverse gene-specific primers deduced from the 5′-region of each clone, and the same forward SK primer that binds to the 5′-region of the polylinker of the Uni-ZAP XR vector, were used together with an aliquot of the olive cDNA library for PCR amplification. Two different fragments corresponding to each clone were amplified, isolated and sequenced. Both sequences showed a 5′-untranslated region (UTR) and a start codon (ATG) followed by an ORF matching the 5′-region of the previously known cDNA sequences.

Finally, specific forward primers deduced from the 5′-UTR sequences of each clone and the same reverse T7 primer that binds to the 3′-region of the polylinker of the Uni-ZAP XR vector, were used to amplify the corresponding full-length cDNA clones, with aliquots of the olive cDNA library as template and a DNA polymerase with proofreading activity to avoid sequencing mistakes due to the amplification. The two amplified fragments were isolated, sequenced in both directions and were found to be full-length cDNAs.

The *OepFAD2-1* and *OepFAD2-2* full-length cDNA clones, with sizes of 1430 and 1439 bp, revealed ORFs encoding predicted proteins of 381 and 383 amino acid residues, respectively, which correspond to a calculated molecular mass of 44.2 and 43.9 kDa, and p*I* of 8.5 in both cases. These ORFs were flanked by unique UTRs of 84 and 62 bp for the 5′-UTR, and 182 and 178 bp for the 3′-UTR, respectively, with a poly(A) tail at the 3′-end.

The sequences flanking the methionine start codon in the two cDNA clones were highly conserved, with only one base difference for *OepFAD2-2* (AACAATGGG), and two bases for *OepFAD2-1* (AAAAATGGG), from the proposed consensus sequence (AACAATGGC) for translation initiation in plants (Lutcke et al., 1987).

Alignment of the two deduced amino acid sequences (Fig. 1) showed that *OepFAD2-1* and *OepFAD2-2*

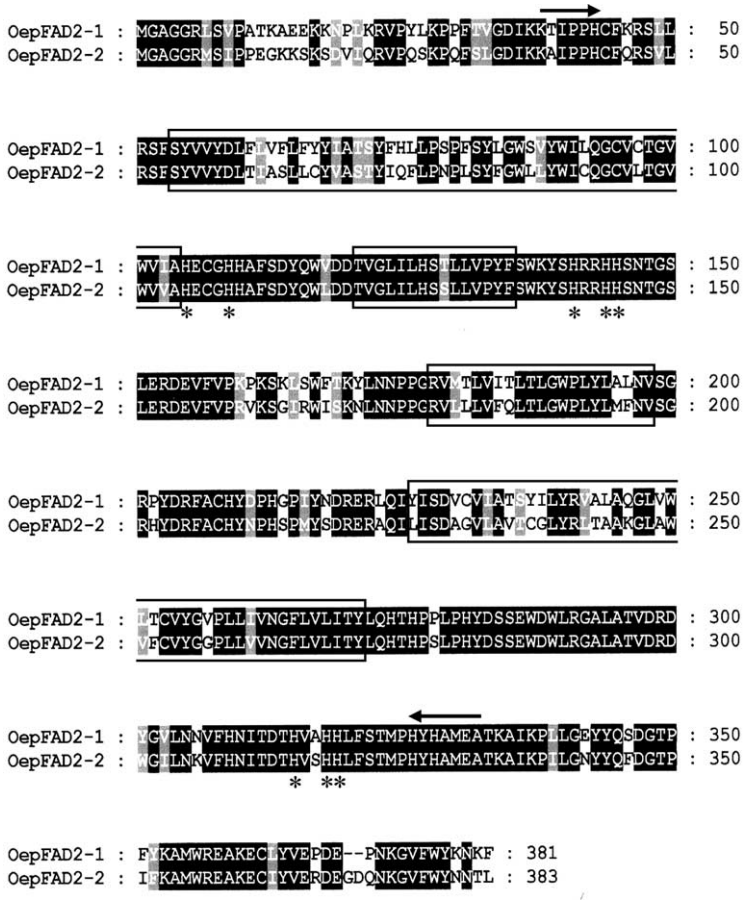

Fig. 1. Comparison of the deduced amino acid sequences of olive *FAD2* genes. Identical and similar residues are shown on a background of black and grey, respectively. The eight invariant histidines grouped in three different boxes characteristic for desaturases are indicated by asterisks, and the four hydrophobic regions are framed. Regions used for deducing degenerated oligonucleotides are indicated by arrows. The cDNA sequences corresponding to *OepFAD2-1* and *OepFAD2-2* have been deposited in the GenBank/EMBL/DDBJ database with the Accession Nos. AY733076 and AY733077, respectively.

shared 73% identity. *OepFAD2-1* and *OepFAD2-2* amino acid sequences displayed significant homology to the known plant FAD2 sequences (84–65% identity), suggesting that they encode microsomal oleate desaturases. A previously isolated *FAD2* cDNA clone from the olive cultivar Koroneiki (Accession No. AY083163) shows 98% identity to the *OepFAD2-2* cDNA clone described in this paper.

Among the conserved amino acids in the two olive microsomal oleate desaturase sequences were eight histidines that have been shown essential for desaturase activity (Shanklin et al., 1994). These invariant residues are arranged in three histidine boxes (HXXXH, HXXHH, and HXXHH) with conserved spaces between them (Fig. 1). This feature is characteristic of all membrane-bound desaturases. These histidine boxes are thought to comprise the catalytic centre of the enzyme, since they form ligands to a diiron cluster in the catalytic site (Shanklin and Cahoon, 1998).

In addition, hydropathy plots of the two FAD2 amino acid sequences were generated by the method of Kyte and Doolittle (1982). Four different hydrophobic regions were found (Fig. 1). The two flanking hydrophobic domains are long enough to span the membrane twice and correspond to the predicted membrane-spanning domains in desaturase integral membrane protein models. In contrast, the other two are too short, thus they may be single-pass monolayer segments (Shanklin et al., 1994). The three conserved histidine boxes were located in hydrophilic regions, and according to this topological model, all of them are exposed to the cytoplasmic side (Los and Murata, 1998).

On the other hand, similar to other plant *FAD2* genes, *OepFAD2-1* and *OepFAD2-2* lack an N-terminal transit peptide and also the carboxy-terminal -KDEL or -KXKXX motifs that have been suggested to represent the retention signal for integral membrane proteins in the ER (Jackson et al., 1990). On the contrary, an aromatic amino acid enriched signal was found at the C-terminus of both proteins (-YKNKF and -YNNTL for OepFAD2-1 and OepFAD2-2, respectively), which have been reported recently to be both necessary and sufficient for maintaining localization of the enzymes in the ER (McCartney et al., 2004).

To elucidate the phylogenetic relationships of the olive microsomal oleate desaturase genes, their deduced amino acid sequences were included in a dendrogram representing all known plant oleate desaturase sequences, either microsomal (FAD2) or plastidial (FAD6), for comparison (Fig. 2). Both OepFAD2 proteins are positioned in the group corresponding to the FAD2 desaturases. The large separation between FAD2 and FAD6 branches indicates that they should not diverge recently. Interestingly, OepFAD2-1 was located in a subgroup of FAD2 enzymes specifically or highly expressed in developing seeds, while OepFAD2-2 was positioned in a subgroup with FAD2 enzymes that exhibit a "housekeeping" pattern of expression. The seed-type *FAD2* genes may have evolved independently after separation by duplication from those *FAD2* genes that show a constitutive expression (Martínez-Rivas et al., 2001).

2.2. Functional expression of olive FAD2 genes in S. cerevisiae

To confirm the functional identity of the two olive *FAD2* cDNAs described above, we have cloned the corresponding ORFs into the expression vector pVT102-U behind the constitutive promoter *ADH1*, and transformed into *S. cerevisiae* cells. Yeast cells have been used successfully for functional expression of several plant microsomal desaturases, as they act as a very convenient host due to its simple fatty acid profile, the presence of only one major fatty acyl desaturase, and the appropriate redox chain in a suitable membrane (Reed et al., 2000). The fatty acid analysis of the transformant yeast cells showed the presence of a new fatty acid, which was not present either in the wild-type yeast or in the control cells transformed with the empty vector (Table 1). GC–MS analysis of the fatty acid DMOX derivative demonstrated that the novel peak was methyl linoleate. The spectrum of the DMOX derivative had a molecular ion at *m/z* 333, suggesting an octadecadienoic acid. The peaks at *m/z* 196, 208; and 236, 248 were consistent with double bonds at the Δ9 and Δ12 positions of the fatty acid chain. Thus, the olive *FAD2* genes have been functionally identified since they encode for isoforms that catalyze the desaturation of the endogenous oleate to linoleate. The FAD2 proteins are associated to the microsomal fraction of the transformant yeast cells, being able to interact with the *S. cerevisiae* cytochrome b_5 (Brown et al., 1998). Unlike previous reports for other plant FAD2 expressed in yeast (Sánchez-García et al., 2004), no palmitolinoleic acid was detected in the olive FAD2-expressing yeast cells, possibly due to the preferential use of oleic acid as substrate as compared to palmitoleic acid and the small amount of linoleic acid synthesized (Brown et al., 1998). The maximal accumulation of linoleic acid occurred during late exponential and early stationary phase, staying constant afterwards. This period of time coincides with that of maximal expression of the *ADH1* promoter (Sánchez-García et al., 2004).

A temperature-dependent content of linoleic acid in *S. cerevisiae* cells expressing other plant *FAD2* genes has been previously reported (Kirsch et al., 1997). Low temperatures increase the percentage of linoleic acid and this has been attributed to the low thermal stability of a particular FAD2 isoform (Sánchez-García et al., 2004). However, little temperature effect was observed when the olive FAD2-expressing yeast cells were grown at 15 °C (Table 1), in comparison to the standard growth temperature (30 °C). It may well be that both olive FAD2 isoforms possess similar activity levels at both temperatures, indicating comparable thermal stabilities.

In accordance with previous reports (Sánchez-García et al., 2004), attempts to measure in vitro FAD2 activity in microsomal preparations isolated from transformed *S. cerevisiae* cells were unsuccessful.

2.3. Genomic organization of olive microsomal oleate desaturase genes

The coding sequences of the two olive *FAD2* genes were homologous, but the 5′- and 3′-UTRs of the two sequences were unique. This fact allowed us to obtain gene-specific probes corresponding to each olive *FAD2* gene by PCR and suggested that *OepFAD2-1* and *OepFAD2-2* were two distinct members of the olive microsomal oleate desaturase gene family. The occurrence of *FAD2* gene families has been frequently reported in plants (Heppard et al., 1996; Martínez-Rivas et al., 2001).

Genomic Southern blot analysis using *OepFAD2-1* and *OepFAD2-2* gene-specific probes confirmed that these genes were non allelic (Fig. 3). Two bands were observed with olive genomic DNA digested with different restriction enzymes. Since there is not a restriction site for either enzyme within the probes used, these data suggest that at least two copies of each *FAD2* gene should be present in the olive genome. The possibility that the three restriction enzymes used cut in an intron present in the genomic sequence encoding the probe sequences is unlikely, but it cannot be completely discounted.

2.4. Tissue and developmental expression of olive microsomal oleate desaturase genes

In order to investigate the physiological role of the two olive microsomal oleate desaturase genes, we have determined the linoleic content and measured their steady-state transcript levels in olive tissues characterized by a highly active lipid biosynthesis. Particularly, we have studied young leaves, where the formation of membrane lipids for the photosynthetic machinery is

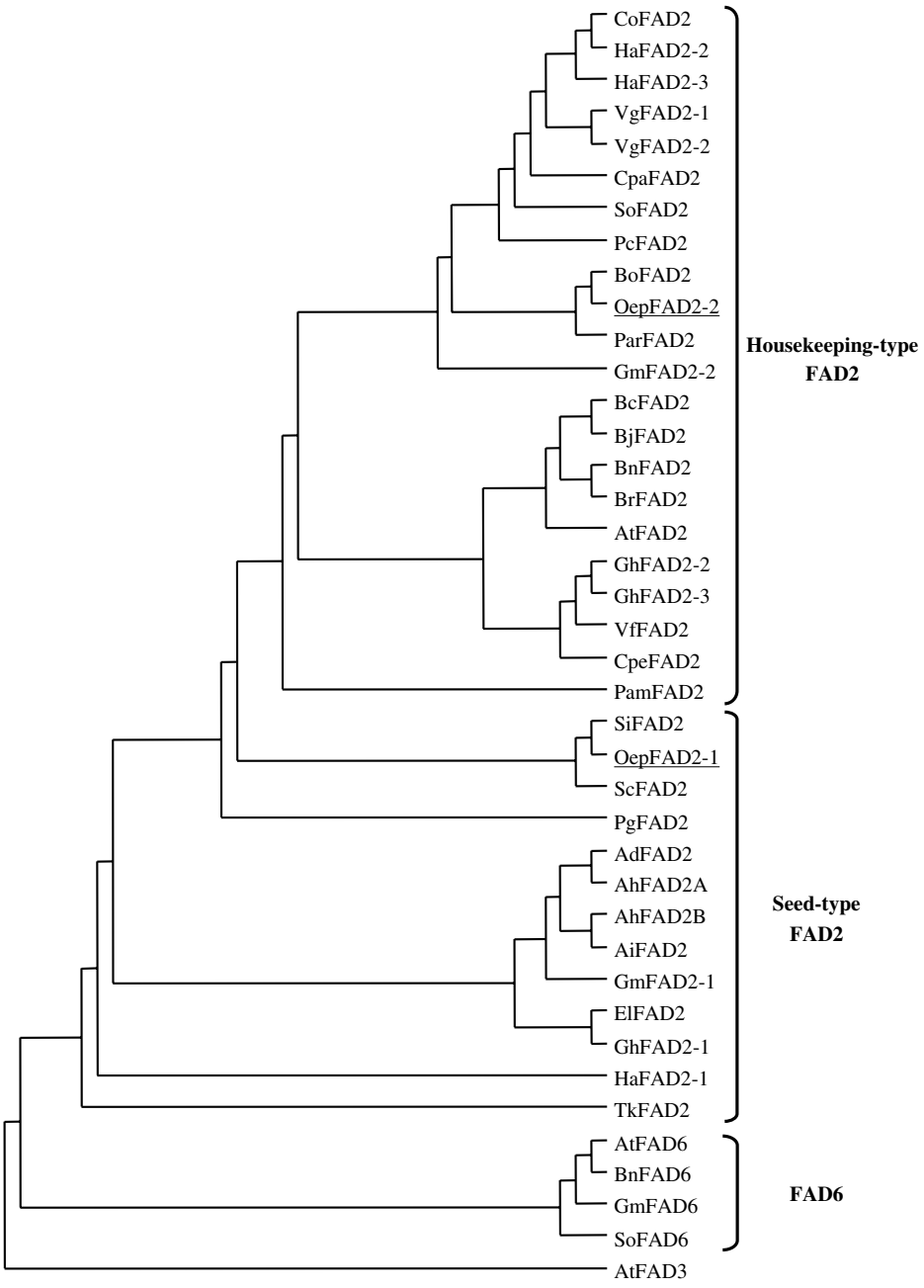

Fig. 2. Phylogenetic analysis of plant FAD2 and FAD6 enzymes. The dendrogram was arbitrarily rooted with the *Arabidopsis thaliana* FAD3 sequence. Distances along the horizontal axes are proportional to sequence differences. Positions of the olive microsomal oleate desaturase genes are underlined. Accession numbers of the different desaturases included in the analysis: *Arabidopsis thaliana* (AtFAD2, L26296; AtFAD3, D17579; AtFAD6, U09503), *Arachis duranensis* (AdFAD2, AF272951), *Arachis hypogaea* (AhFAD2A, AF030319; AhFAD2B, AF272950), *Arachis ipaensis* (AiFAD2, AF272952), *Borago officinalis* (BoFAD2, AF074324), *Brassica carinata* (BcFAD2, AF124360), *Brassica juncea* (BjFAD2, X91139), *Brassica napus* (BnFAD2, AF243045; BnFAD6, L29214), *Brassica rapa* (BrFAD2, AJ459107), *Calendula officinalis* (CoFAD2, AF343065), *Crepis palestina* (CpaFAD2, Y16284), *Cucurbita pepo* (CpeFAD2, AY525163), *Euphorbia lagascae* (ElFAD2, AY486148), *Glycine max* (GmFAD2-1, L43920; GmFAD2-2, L43921; GmFAD6, L29215), *Gossypium hirsutum* (GhFAD2-1, X97016; GhFAD2-2, Y10112; GhFAD2-3, AF331163), *Helianthus annuus* (HaFAD2-1, AF251842; HaFAD2-2, AF251843; HaFAD2-3, AF251844), *Persea americana* (PamFAD2, AY057406), *Petroselinum crispum* (PcFAD2, U86072), *Punica granatum* (PgFAD2, AJ437139), *Sesamum indicum* (SiFAD2, AF192486), *Solanum commersonii* (SoFAD2, X92847), *Spinacia oleracea* (SoFAD2, AB094415; SoFAD6, X78311), *Trichosanthes kirilowii* (TkFAD2; AY188445), *Vernicia fordii* (VfFAD2, AF525535), *Vernonia galamensis* (VgFAD2-1, AF188263; VgFAD2-2, AF188264).

very important; developing seeds, characterized by an active storage lipid biosynthesis; and also mesocarps, which have both characteristics, active chloroplasts for thylakoid membrane lipid biosynthesis and a very important accumulation of TAG, which are the major components of the olive oil.

Table 1
Fatty acid composition of *S. cerevisiae* cells overexpressing olive *FAD2* genes[a]

Plasmid	Temperature (°C)	Fatty acid composition (mol%)				
		16:0	16:1	18:0	18:1	18:2
PVT102-U	30	19.7	35.3	8.5	36.4	–
	15	14.6	38.6	9.3	37.5	–
PVTOepFAD2-1	30	19.2	31.7	9.6	36.8	2.6
	15	14.5	37.1	9.1	38.3	0.9
PVTOepFAD2-2	30	18.5	33.4	9.9	36.7	1.5
	15	15.1	34.7	10.1	38.9	1.1

[a] Yeast cultures were grown at the indicated temperatures until stationary phase. Then, yeast cells were harvested and the fatty acid composition determined in whole cells as described in Section 4. Results are mean of three independent experiments, with duplicate determinations of fatty acid composition. In all cases, the SD was <3% of mean value. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid.

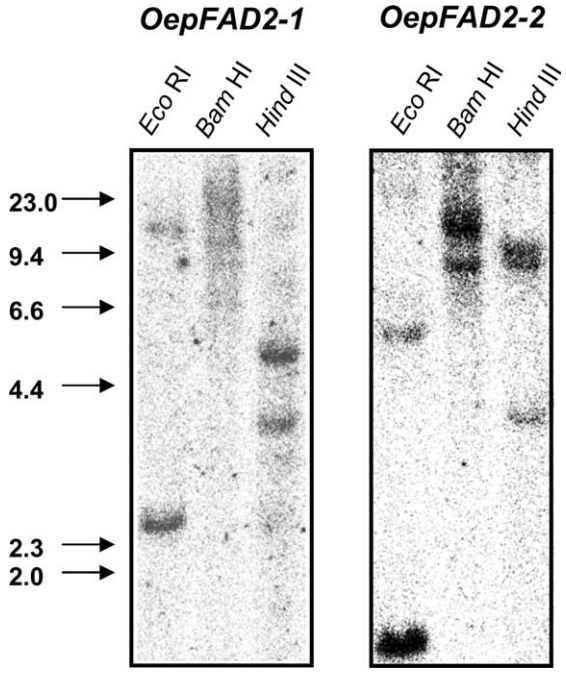

Fig. 3. Southern blot analysis of olive genomic DNA digested with the indicated restriction enzymes, and probed with *OepFAD2-1* and *OepFAD2-2* gene-specific probes. Molecular size (kb) of the marker is indicated on the left.

In olive fruits, proper and differentiated seed and mesocarp tissues do not appear until the lignification of the endocarp occurs (10–12 WAF). Since then, the TAG biosynthesis and accumulation takes place in both tissues, with no oil deposition observed during the first 10 WAF, either in the seed or the mesocarp (Sánchez, 1994). Very young seeds (13 WAF) show a relatively high linoleate content that slightly decrease later (20 WAF), remaining constant afterwards (Fig. 4). In contrast, the percentage of linoleic acid is very low in the very young mesocarp, increasing a little during fruit development, and slightly more during fruit ripening (after 30 WAF). Therefore, the fatty acid composition and the degree of unsaturation of olive developing seeds and mesocarps are regulated by the developmental stage as it has been reported in oil seeds (Heppard et al., 1996). In addition, young leaves are characterized by a moderate percentage of linoleic acid (10%) and a high content of its desaturation product α-linolenic acid (35%).

To study the expression levels of the *OepFAD2-1* and *OepFAD2-2*, northern blot analysis were performed with total RNAs isolated from different tissues at different stage of development, and using gene-specific probes (Fig. 5). *OepFAD2-1* was strongly expressed in very young seeds (13 WAF) and in leaves, showing low levels in the mesocarp. In contrast, the *OepFAD2-2* gene was moderately expressed in developing seeds during the late stages of development, in ripening mesocarp (31 WAF), and in leaves. Interestingly, this pattern of expression for *FAD2* genes of an oil fruit is different from that usually described in oilseed plants, where one gene is predominantly expressed in developing seeds and a second one shows low expression in all plant tissues (Heppard et al., 1996; Martínez-Rivas et al., 2001).

The enhanced linoleic content observed at early stages of development of the olive seeds (13 WAF) (Fig. 4) correlates well with the high transcript level detected for *OepFAD2-1* in this tissue (Fig. 5), suggesting that this gene could be responsible for this fact. This hypothesis is supported by the high expression of the stearoyl-ACP desaturase gene, responsible for the biosynthesis of oleic acid, and the cytochrome b_5 gene, that encodes the electron donor of the FAD2 enzyme, reported in olive very young seeds (Haralampidis et al., 1998; Martsinkovskaya et al., 1999). Furthermore, this early oleate desaturation coincides with the rapid oil accumulation produced in the olive seed (12–22 WAF) compared with the more slow oil deposition found in the mesocarp (12–40 WAF) (Sánchez, 1994). On the other hand, the increase in linoleic acid content (Fig. 4) found at the last stage of seed development (31 WAF) could be also associated with the enhanced expression of *OepFAD2-2* observed in this tissue (Fig. 5).

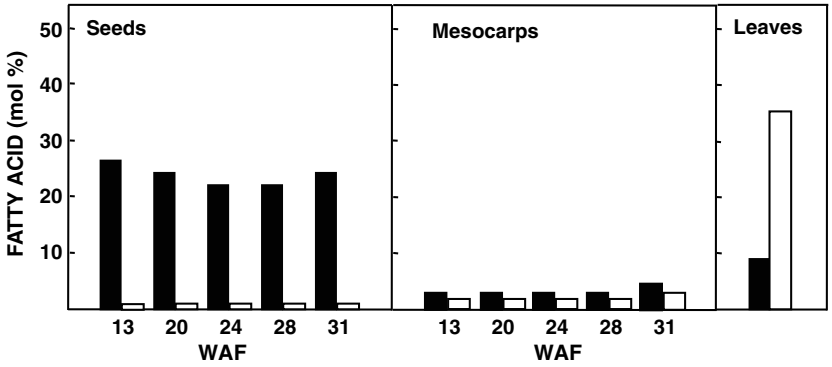

Fig. 4. Linoleic and α-linolenic acid content from different olive tissues. Fatty acid composition was determined as indicated in Section 4 in seeds and mesocarps of olive fruits at different stages of development and ripening, and in young leaves (solid bar, linoleic acid; open bar, α-linolenic acid). Results are mean of five independent tissue samples, with duplicate determinations of fatty acid composition. In all cases, the SD was <3% of mean value.

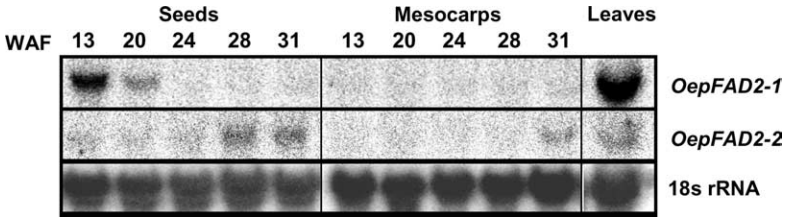

Fig. 5. Northern blot analysis of total RNA isolated from different olive tissues. Total RNA (30 μg) samples were probed with labeled DNA fragments specific for *OepFAD2-1* and *OepFAD2-2* genes. Even loading was tested by re-probing the filters with maize 17S DNA which detects olive 18S rRNA.

Regarding the mesocarp, the increase in the percentage of linoleic acid detected at late ripening stage (31 WAF) (Fig. 4) correlates well with the increased expression found in this tissue for *OepFAD2-2* (Fig. 5). However, the start of the enhanced expression of the stearoyl-ACP desaturase (Haralampidis et al., 1998) and the cytochrome b_5 genes (Martsinkovskaya et al., 1999) in the mesocarp occurs before (19 WAF). These data explain why the mesocarp has high oleate content before it starts the maturation process, thus yielding olive oils richer in oleic acid when the olive fruit is collected before ripening and, on the contrary, with increased linoleic acid when it is obtained from late-ripening fruits (Gutiérrez et al., 1999). Moreover, the low level of expression found for both *FAD2* genes in the mesocarp is not surprising since the linoleate content is low, particularly in the cv. Picual, and the times for development and ripening of the olive fruit are extremely long.

3. Conclusions

We have isolated and characterized two oleate desaturase genes from olive, both containing the three histidine boxes typical of all membrane-bound desaturases. Sequence analysis of the two genes (*OepFAD2-1* and *OepFAD2-2*) indicates that they code for two microsomal oleate desaturase enzymes. The identity of the *FAD2* genes was confirmed by functional expression of the corresponding cDNAs in yeast. Genomic Southern blot data are consistent with the presence of at least two copies of each *FAD2* gene in the olive genome. This is the first time that the cloning and expression analysis of *FAD2* genes from an oil fruit is reported. The expression data obtained suggest a differential physiological role for the two olive microsomal oleate desaturase genes, with *OepFAD2-1* possibly involved in the desaturation of storage lipids in the young seed, whereas *OepFAD2-2* may be mostly responsible for reserve lipid desaturation in the mature seed and the mesocarp. However, additional functions cannot be ruled out. The unexpected high level of expression found for the *OepFAD2-1* gene in leaves may indicate its possible participation in the linoleate biosynthesis required for thylakoid membrane formation. In fact, the rapid and transient induction of a parsley *FAD2* gene by a fungal elicitor has been described (Kirsch et al., 1997), indicating the contribution of not only *FAD6*, but also *FAD2* genes, in the biosynthesis of plastidial polyunsaturated fatty acids which act as precursors of signalling molecules involved in plant defence mechanisms. The precise localization of expression of the two genes in olive mesocarp and seed tissues may provide new information about the role of each gene product in controlling polyunsaturated fatty acid synthesis in membrane and storage lipids in olive fruit. Further characterization of the

mechanisms that regulate the olive microsomal oleate desaturases, either at gene expression or enzyme activity level, is currently under way in our group.

4. Experimental

4.1. Plant material

Olive (*O. europaea* cv. Picual) trees were grown in an orchard near Sevilla (Spain), which had been given drop irrigation and fertirrigation (irrigation with suitable fertilizers in the solution) from the time of full bloom to fruit maturation (April–December). Developing seeds and mesocarps were harvested at different times after flowering corresponding to different developmental stages of the olive fruit, chilled in liquid nitrogen, and stored at −80 °C. Young leaves were also collected in the same way.

4.2. Isolation of microsomal oleate desaturase partial cDNA clones

Two degenerate primers, LH1 (5′-AA[AG]GCGA-T[ACT]CCGCCGCA[CT]TG-3′) and LH3 (5′-GC[CT]TCCATGGC[AG]T[GT][AG]TA[AG]TG-3′), were designed from the comparison of known plant FAD2 amino acid sequences, which correspond to the highly conserved sequences KTIPPH for LH1, and HYHAMEA for LH3 (Fig. 1). This pair of primers, together with an aliquot of an olive Uni-ZAP XR cDNA library constructed with mRNA isolated from 13 WAF olive fruits cv. Picual (Haralampidis et al., 1998), were used for PCR amplification with the Thermo-Start DNA Polymerase (ABgene, Epsom, UK). Two different DNA fragments with the same expected size were generated, subcloned into the vector pGEM-T Easy (Promega, Madison, WI, USA) and sequenced.

4.3. PCR amplification of the 5′-end

Two different reverse primers deduced from the 5′-region specific for each of the above mentioned clones, and the same forward vector-specific primer SK (5′-CGCTCTAGAACTAGTGGATC-3′) that binds to the 5′-region of the multiple cloning site of the Uni-ZAP XR vector, were used together with an aliquot of the olive cDNA library previously described for PCR amplification with the Thermo-Start DNA Polymerase. One fragment was generated in each reaction, subcloned into the vector pGEM-T Easy and sequenced.

4.4. Isolation of microsomal oleate desaturase full-length cDNA clones

Two different forward primers deduced from the 5′-UTR specific for each clone and the same reverse vector-specific primer T7 (5′-GTAATACGACTCACTA-TAGGGC-3′) that binds to the 3′-region of the multiple cloning site of the Uni-ZAP XR vector, were used together with an aliquot of the olive cDNA library previously described for PCR amplification with Ecozyme DNA polymerase (Ecogen, Barcelona, Spain), which has proofreading activity. One fragment was generated in each reaction, subcloned into the vector pCR-Script Amp SK(+) (Stratagene, La Jolla, CA, USA) and sequenced in both directions.

4.5. DNA sequence determination and analysis

DNA sequencing was performed by GATC Biotech, Konstanz, Germany. The DNA sequence data were compiled and analyzed with the LASERGENE software package (DNAStar, Madison, WI, USA). For alignment and dendrogram creation, ClustalX, GeneDoc and TREEVIEW programs were used for create multiple sequence alignments of FAD2 and FAD6 amino acid sequences from various organisms.

4.6. DNA and RNA isolation

Olive genomic DNA was isolated from young leaves by the CTAB method (Murray and Thomson, 1980).

Total RNA isolation was performed using solutions previously treated with diethyl pyrocarbonate (DEPC) to inhibit RNases. Approximately 1–2 g of the frozen olive tissues was ground in a pre-cooled mortar with liquid nitrogen. After homogeneization, 5-ml of extraction buffer (100 mM Tris–HCl, pH 9.0; 100 mM NaCl; 10 mM Na_2EDTA, pH 8.0, and 2% SDS), 50 μl 2-mercaptoethanol and 2.5 ml phenol were added. After gentle shaking for 5 min, the mixture was supplemented with 2.5 ml chloroform, shaken gently for another 5 min, and centrifuged at 2500*g* for 10 min. The upper phase was extracted twice with phenol and chloroform in a similar way. Nucleic acids were precipitated adding 0.1 volumes of 3 M NaAc, pH 5.2, and 3 volumes of absolute ethanol for 30 min at −80 °C. After centrifugation at 2500*g* for 30 min at 4 °C, the pellet was resuspended in 2.5 ml DEPC–water, and 2.5 ml of 5 M LiCl were added to precipitate the RNA overnight at 4 °C. The preparation was centrifuged at 2500*g* for 30 min at 4 °C, and the pellet was resuspended in 1 ml DEPC–water. The RNA was precipitated as described above for the nucleic acids, and the pellet was washed twice with 70% ethanol and resuspended in 25 μl DEPC–water.

4.7. Genomic Southern blot analysis

Samples of olive genomic DNA (5 μg) were digested with restriction enzymes and electrophoresed through a 0.7% agarose gel. The gel was soaked in 0.25 M HCl

for 10 min, then in 0.5 M NaOH, 1 M NaCl for 30 min, then in 1.5 M Tris–HCl, pH 7.5, 3 M NaCl, and finally blotted onto a Zeta-Probe membrane (Bio-Rad, Hercules, CA, USA), and probed with [α-^{32}P]dCTP-labeled *OepFAD2-1* and *OepFAD2-2* gene-specific DNA fragments corresponding to the 3′-UTR unique to each gene. The olive *FAD2* gene-specific probes with a size of 150 and 146 bp, respectively, were obtained by PCR amplification with the following pairs of specific primers: LH47 (5′-ATTTTCGTTAGTGAAGTTGTG-3′) and LH27 (5′-TAACAGTCCCTATTTTATTTC-3′) for *OepFAD2-1*; and LH46 (5′-ATAGCGGC GCGAATTTCTGG-3′) and LH31 (5′-TTCAACAAC-CAGTTTCAATCC-3′) for *OepFAD2-2*. Hybridization was performed in 0.5 M Na_2HPO_4, pH 7.2, 1 mM Na_2EDTA, 7% SDS, overnight at 65 °C. The filters were washed twice in 40 mM Na_2HPO_4, pH 7.2, 1 mM Na_2EDTA, 1% SDS for 15 min at room temperature.

4.8. Northern blot analysis

About 30 μg per lane of total RNA were electrophoresed in 1% agarose gel containing formaldehyde, transferred to Zeta-Probe membrane and probed with [α-^{32}P]dCTP-labeled *OepFAD2-1* and *OepFAD2-2* gene-specific DNA fragments corresponding to the 3′-UTR unique to each gene, as mentioned before. Loading of RNA was controlled by probing the blots with maize 17S DNA for detection of the olive 18S rRNA. Hybridization and washing conditions were as described above for Southern blot hybridization.

4.9. Expression of olive FAD2 genes in S. cerevisiae

The corresponding open reading frames (ORF) of the two olive *FAD2* genes described above were amplified by PCR using Ecozyme DNA polymerase and the following pairs of specific primers: LH44 (5′-GAGCTCACATA**ATG**GGAGCAGGAGGACGATTGTC TGTTC-3′) and LH45 (5′-GAGCTCTCT**TCA**A AACTTGTTCTTATACCAGAAAACAC-3′) for *OepFAD2-1*; and LH40 (5′-CGGGATCCACATA**A TG**GGTGCTGGAGGCCGAATGTCCATTC-3′) and LH41 (5′-CGGGATCCAAG**CTA**AAGCGTGTTAT TGTACCAGAAGACG-3′) for *OepFAD2-2*. For ligation behind the constitutive *ADH1* gene promoter of the yeast expression vector pVT102-U (Vernet et al., 1987), the primers for *OepFAD2-1* or *OepFAD2-2* were extended by a *Sac*I or a *Bam*HI restriction site (underlined), respectively. The resulting 1.2-kb PCR product was subcloned into the vector pCR-Script Amp SK(+), digested with the corresponding restriction enzyme and ligated into *Sac*I- or *Bam*HI-digested pVT102-U. The sense orientation of the corresponding inserts relative to the *ADH1* promoter was confirmed by restriction mapping. The *Saccharomyces cerevisiae* strain UTL-7A was transformed with these plasmids by the lithium acetate method and selected on minimal agar plates lacking uracil (Ausubel et al., 1995). Complete minimal drop out-uracil medium containing 2% glucose as the exclusive carbon source, (CM glucose -ura medium) was inoculated with a single colony and grown at the corresponding temperatures until stationary phase. Yeast cells were harvested by centrifugation at 1500*g* for 5 min at 4 °C, and washed once with distilled water.

4.10. Fatty acid analysis

Fatty acid composition of olive tissues and whole yeast cells was determined using the one-step method of Garcés and Mancha (1993). Following the addition of 13.2 or 3.3 ml of methanol–toluene–dimethoxypropane–H_2SO_4 (39:20:5:2, vol/vol/vol/vol) and 6.8 or 1.7 ml heptane to 300 mg of olive tissue or to the pellet of yeast cells, respectively, the mixture was incubated for 1 h at 80 °C, forming a single phase. After cooling, the upper phase containing the fatty acid methyl esters was separated, washed with 5 ml 6.7% Na_2SO_4, and evaporated to dryness with nitrogen. The methyl esters were dissolved in the appropriate volume of heptane and analyzed by gas–liquid chromatography using a HP-5890 (Hewlett–Packard, Palo Alto, CA, USA) fitted with a capillary column (30 m length; 0.25 mm id; 0.20 μm film thickness) of fused silica (Supelco, Bellafonte, PA, USA) and a FID detector. Hydrogen was used as carrier gas with a linear rate of 28 cm s^{-1} and a split ratio of 1/50. The injector and detector temperature was 220 °C and the oven temperature 170 °C. The methyl ester corresponding to the novel fatty acid detected in the transgenic yeast cells was converted to fatty acid 4,4-dimethyloxazoline (DMOX) derivatives, and analyzed by GC–MS using a Fisons mass selective detector (model MSD-800) operating at an ionization voltage of 70 eV with a scan range of 30–500 amu. The mass spectrum of the new peak was compared with those of authentic standards.

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