The olive *DGAT2* gene is developmentally regulated and shares overlapping but distinct expression patterns with *DGAT1*

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Received 6 January 2010; Revised 23 July 2010; Accepted 6 August 2010

Abstract

Diacylglycerol acyltransferases (DGATs) catalyse the final step of the triacylglycerol (TAG) biosynthesis of the Kennedy pathway. Two major gene families have been shown to encode DGATs, *DGAT1* (type-1) and *DGAT2* (type-2). Both genes encode membrane-bound proteins, with no sequence homology to each other. In this study, the molecular cloning and characterization of a type-2 *DGAT* cDNA from olive is presented. Southern blot analysis showed that *OeDGAT2* is represented by a single copy in the olive genome. Comparative transcriptional analysis revealed that *DGAT1* and *DGAT2* are developmentally regulated and share an overall overlapping but distinct transcription pattern in various tissues during vegetative growth. *DGAT2* is highly expressed in mature or senescing olive tissues. In flowers, the expression of *DGAT1* was almost undetectable, while *DGAT2* transcripts accumulated at the later stages of both anther and ovary development. Differential gene regulation was also detected in the seed and mesocarp, two drupe compartments that largely differ in their functional roles and mode of lipid accumulation. *DGAT1* appears to contribute for most of the TAG deposition in seeds, whereas, in the mesocarp, both *DGAT1* and *DGAT2* share an overlapping expression pattern. During the last stages of mesocarp growth, when TAGs are still accumulating, strong up-regulation of *DGAT2* but a marked decline of *DGAT1* transcript levels were detected. The present results show overlapping gene expression for olive *DGATs* during mesocarp growth, with a more prominent implication of *DGAT2* in floral bud development and fruit ripening.

Key words: Diacylglycerol acyltransferase, gene expression, *in situ* hybridization, olive development, triacylglycerols.

Introduction

A number of plants accumulate large amounts of triacylglycerols (TAGs) in their seeds as storage reserves for germination and seedling development. Key points in the accumulation of TAGs are the early events of fatty acid biosynthesis and the last and critical events of TAG synthesis (Bao and Ohlrogge, 1999; Jako*et al.*, 2001; Weselake, 2005; Lung and Weselake, 2006). There are few fruit crops that deposit most of the oil in the mesocarp tissues to attract animals for seed dispersal. Among them, olive is of predominant economic importance because its oil is ideal for direct consumption. It is therefore of great importance to elucidate the key-points in the olive oil biosynthesis pathway and storage. Such knowledge could speed up the breeding programmes aimed at selecting clones with superior fatty acid composition and is also essential for selecting high oil-yielding genotypes more efficiently and rapidly, thus improving decision-making processes. Nevertheless, the molecular basis of gene regulation underlying
olive oil production is far from complete. There is a significant amount of information concerning the regulation of several genes involved in fatty acid synthesis and modification (Hatzopoulos et al., 2002; Doveri and Baldoni, 2007; Banilas and Hatzopoulos, 2009), but much less is known about the cellular mechanisms governing the transfer of fatty acids into storage TAGs, not only in olive but generally in plants (Shockey et al., 2006).

TAG biosynthesis is principally accomplished by membrane-bound enzymes that operate in the endoplasmic reticulum through the glycerol-3-phosphate or the so-called Kennedy pathway (Kennedy, 1961; Browse and Somerville, 1991). The first step in the process involves the acylation of glycerol-3-phosphate (GP) at the sn-1 position to produce lysophosphatidic acid (LPA) by GP acyltransferase (GPAT). LPA is further acylated at the sn-2 position by LPA acyltransferase (LPAT) resulting in the formation of phosphatidic acid (PA). PA is dephosphorylated to produce diacylglycerol (DAG), which is further acylated to produce TAG by diacylglycerol acyltransferase (DGAT), the only enzyme in the pathway that is thought to be exclusively committed to TAG synthesis. Inasmuch as DGAT catalyses the final and most critical step for TAG synthesis, it has been suggested that it may constitute a rate-limiting factor in TAG bioassembly in developing seeds (Ichihara et al., 1988; Jako et al., 2001; Weselake, 2005; Lung and Weselake, 2006). However, TAGs could also be produced via the transfer of acyl groups from phospholipids to diacylglycerols, an acyl-CoA-independent reaction catalysed by the enzyme phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000; Stahl et al., 2004; Zhang et al., 2009).

TAGs are not only produced in seeds or mesocarps. Both TAG accumulation and DGAT activity have been reported in several other organs, such as flowers, developing siliques, stems, flowers, roots, and leaves of tobacco (Zhang et al., 2005). Based on those observations, it has been suggested that TAG may also be implicated in physiological roles other than as a carbon or energy source (Lu and Hills, 2002; Lu et al., 2003).

Two major unrelated gene families have been shown to encode DGATs, namely DGAT1 (type-1) and DGAT2 (type-2) both of which are ER-localized. DGAT1 genes have been cloned from several plant species, including olive (Giannoula et al., 2000). DGAT2 genes have been cloned from diverse eukaryotes, including the oleaginous fungus Mortierella ramanjiana (Lardizabal et al., 2001), human (Cases et al., 2001), and the plant species Arabidopsis (Lardizabal et al., 2001), castor bean (Kroon et al., 2006), and tung tree (Shockey et al., 2006). A third member of the DGAT family (type-3), highly unrelated to the previously reported, was identified in peanut that possesses a cytosolic localization (Saha et al., 2006).

Accumulating data suggest that DGAT activity may have a substantial effect on carbon flow into seed oil of Brassica napus (Perry et al., 1999; Weselake et al., 2008), Arabidopsis thaliana (Katavic et al., 1995; Zou et al., 1999; Jako et al., 2001), and maize (Zheng et al., 2008). In an attempt to gain further insight into the role(s) of DGATs in plant lipid biosynthesis, it is shown here that DGAT2 is highly expressed in mature or senescent olive tissues. The expression patterns of DGAT1 and DGAT2 during drupe development and in several other organs/tissues of the olive tree indicated that genes are differentially regulated to fulfil the needs for TAG accumulation at certain points of growth and development.

Materials and methods

Plant material

Leaves, buds, flowers, and drupes at different developmental stages were harvested from ‘Koroneiki’, an oil olive (Olea europaea L.) cultivar, grown in a natural environment at the Agricultural University of Athens (37°58’ N, 23°46’ E). Ovaries and anthers were dissected from flowers, while primary roots, hypocotyls, cotyledons, and shoot tips were dissected from seedlings grown in a growth chamber at 23 °C under a 16 h photoperiod. Samples were immediately frozen in liquid nitrogen and stored at –80 °C for RNA and DNA extractions.

RNA extraction and RT reactions

Total RNA was isolated from different olive tissues by a phenol:chloroform extraction procedure as described previously (Haralampidis et al., 1998). The concentration of total RNA was determined spectrophotometrically and verified following agarose gel electrophoresis by ethidium bromide staining. Total RNA was treated with RNase-free DNase I (Promega) and 2 μg were used as a template in first-strand cDNA synthesis using Superscript™ II RNase H Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. Unless otherwise stated, the first-strand cDNA was primed off by the poly-A tail with the reverse transcription primer T17XHO (5’-GTCGACCTCCAGGATTTTTTTTTTTTTTTTTTTTT3’).

cDNA cloning

To amplify a central fragment of olive diacylglycerol acyltransferase type-2 cDNA (OeDGAT2), the degenerate primers FOR1 (5’-CC C/T TA C/T GT A/C/T TT C/T GG A/G/T TATGA A/G/ CC-3’) and REV2 (5’-CC A/G/C/T ACCACCAC A/G TG A/C/G/ AT A/T GG-3’) were designed for conserved regions of orthologous genes from GenBank. A cDNA amount corresponding to 150 ng of total RNA from mesocarp tissue at 25 weeks after flowering (WAF) was used as a template in PCR together with 200 μM of each dNTP, 20 pmol of each primer, 2 U of DNA polymerase (Expand™ High Fidelity, Boehringer, Mannheim) and 1 × PCR buffer (provided by the manufacturer of the enzyme). Amplification was achieved in a thermal cycler (Model PTC-200, MJ Research, Waltham, MA) during 35 cycles of the following program: 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s. PCR products were gel extracted (QIAquick® Gel Extraction Kit), cloned into pGEM-T Easy (Promega) and sequenced by Macrogen Inc. (Seoul, Korea). That sequence was used to design primers for amplification of the 3’ and 5’ ends by standard RACE PCR methods (Frohman et al., 1990). For 3’-RACE, the first-strand cDNA was primed off with the T17XHO primer and the amplification was achieved by using in reverse the specific forward primer 3aDG2 (5’-TAACACCAAGCAGGAAAGG-3’) followed by the nested primer 3bDG2 (5’-ATGGGCAACCCTCGTGTTCC-3’). For 5’-RACE, the first-strand cDNA was synthesized by the reverse specific primer 5zDG2 (5’-TTTCCACATAGGCCTTCCAC-3’), and after dA-tailing the amplification was
achieved using the nested primers 5bDG2 (5'-AATTGCTCTCTT-GACTCCCCCT-3') and 5eDG2 (5'-TAGAACACAGCAGTAC-TAGCG-3') in combination with T17XHO. The full-length sequence was amplified using the primers 5UTRDGAT2 (5'-TCCCATCTACAAATTCACCTC-3') and 3UTRDGAT2 (5'-GCCTTTGTATCTGCAGCTATT-3'), designed from the 5'- and 3'-UTRs.

**Sequence analysis**

Nucleotide and deduced amino acid sequences were identified by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Prediction of open reading frame (ORF) and molecular weight estimation of the deduced polypeptide (GenBank accession number: GU357635) were made by the EditSeq program (version 3.88). Sequence comparisons were made by the MegAlign program (DNASTAR Inc., London UK). Transmembrane regions were predicted by the TMHMM Server ver. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and subcellular localization of the deduced polypeptide by PSORT (http://psort.imst.ac.jp/form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) algorithms. Amino acid multiple alignments were made with the ClustalW program (http://www.ebi.ac.uk/clustalw) under default parameters. A phylogenetic tree was constructed using the Neighbor-Joining algorithm included in the ClustalW program and visualized by TreeView, version 1.6.6.

DNA gel blot analysis was extracted from olive leaves by the CTAB method (Murray and Thomson, 1980) and 5 μg were digested with the restriction endonucleases EcoRI, HindIII or EcoRI/HindIII. DNA was then fractionated on an 0.8% agarose gel, transferred to nylon membrane, probed with a 32P-labelled OeDGAT2 cDNA fragment of 482 bp, corresponding to the 3'-RACE, and washed under high stringency conditions at 65 °C (Church and Gilbert, 1984).

RNA gel blot and semi-quantitative RT-PCR

For RNA gel blot analysis (Sambrook et al., 1989), total RNA (10 μg per lane, standardized by spectrophotometric analysis and gel electrophoresis of equivalent amounts of rRNA), was separated on denaturing 1.4% formaldehyde-agarose gel, transferred onto nitrocellulose membranes without treatment, probed and washed. The full-length cDNA was cloned by conducting 3''- and 5''-RACE PCRs. Sequence comparisons of the 3'- and 5'-ends with the central part of the gene showed that the overlapping regions match perfectly. Based on the above sequence data, primers were designed from the 5'- and 3'-UTRs and the full-length cDNA was cloned, amplified, and sequenced, revealing 100% identity to the expected sequence.

The full-length OeDGAT2 cDNA consisted of a 100 nt 5'-UTR, a 277 nt 3'-UTR, and a putative 1008 nt ORF (Fig. 1), encoding a predicted polypeptide of 335 amino acid residues with a calculated molecular mass of 37.9 kDa and an isoelectric point of 9.6. The encoded polypeptide contains two putative transmembrane domains close to the N-terminus, at amino acid residues 34–56 and 61–83, as predicted by the TMHMM server (Fig. 1). Hydrophobicity plot analysis of other type-2 DGAT proteins from both plants and mammals is consistent with the presence of at least one membrane spanning domain close to the N-terminus (Shockey et al., 2006; Kroon et al., 2006). As opposed, hydropathy plots of various type-1 DGAT proteins, including OeDGAT1 (Giannoulia et al., 2000), consist of 9–10 putative transmembrane domains and a relatively hydrophilic N-terminus (Lung and Weselake, 2006). By using two different algorithms (PSORT and TargetP), no plastoideal or other signal transit peptide was predicted at the N-terminal region. At the C-terminus a sequence motif was detected (Fig. 1) that resembles the recently described endoplasmic reticulum (ER) retrieval motif -Φ-X-X-K/R/D/E-Φ-COOH, where -Φ- are large hydrophobic amino acid residues (McCarty et al., 2004). A similar motif seems to be highly conserved in both DGAT1 and DGAT2 polypeptides of different plant species (Kroon et al., 2006) including tung tree (Shockey et al., 2006).

**Results**

Cloning and sequence analysis of OeDGAT2 cDNA

Based on conserved amino acid sequences of different type-2 DGATs, degenerate oligonucleotide primers were designed to amplify a central fragment of the homologous gene in olive. PCR employing cDNA from mesocarp tissue generated a fragment of 496 bp. BLAST searches of both nucleotide and deduced amino acid sequences predicted this fragment to be a central part of a type-2 DGAT gene. The full-length cDNA was cloned by conducting 3'’- and 5'’-RACE PCRs. Sequence comparisons of the 3'’- and 5'’-ends with the central part of the gene showed that the overlapping regions match perfectly. Based on the above sequence data, primers were designed from the 5'- and 3'-UTRs and the full-length cDNA was cloned, amplified, and sequenced, revealing 100% identity to the expected sequence.

BLAST search showed high similarities of the predicted OeDGAT2 amino acid sequence to type-2 DGATs from other plants, like grapevine (70% identity and 84% similarity) and Arabidopsis (61% and 77%, respectively).
No homology (8% identity, 19% similarity) was shared between OeDGAT2 and its type-1 isoform from olive (Giannoulia et al., 2000). To elucidate phylogenetic relationships of OeDGAT2, the deduced amino acid sequence was aligned with other plant type-2 and type-1 DGATs and a phylogenetic tree was constructed. The Neighbor–Joining tree was composed of two distinct branches representing the two types of DGAT families, with OeDGAT1 and OeDGAT2 being clustered within their expected groups (Fig. 2).

To determine the copy number of OeDGAT2 within the olive genome, genomic DNA was digested with EcoRI, HindIII or EcoRI/HindIII and separated on an agarose gel. Within the target sequence there is a unique EcoRI restriction site, but there are no restriction sites for HindIII (data not shown). Single digest with EcoRI revealed two hybridizing bands, suggesting the presence of a single gene within the olive genome (Fig. 3). This was also confirmed with the HindIII digestion of gDNA. A single hybridizing
band was detected (Fig. 3). Double digest also confirmed that the olive genome contains a single copy of a type-2 DGAT gene.

**DGAT1 and DGAT2 are differentially regulated during olive drupe development**

To determine whether the pattern of mRNA synthesis of the type-2 *OeDGAT* gene shares any similarities to that of type-1 *OeDGAT*, the expression profile of the two genes was analysed in different tissues. The temporal expression and developmental accumulation of olive type-1 and -2 DGAT transcripts were investigated during olive fruit development. Total RNA was isolated from embryos, endosperms, and mesocarps at different times and analysed by RNA gel blotting. At the very early stages of drupe development (5–11 WAF) RNA was isolated from intact drupes, since zygotic embryos at early globular and heart stages could not be excised without injury. Embryos at the early torpedo, early mid-torpedo, mid-torpedo, and late torpedo stages (13, 16, 19, and 22 WAF, respectively) were dissected out from the surrounding endosperm and collected. As it is shown in Fig. 4, both *OeDGAT*s are developmentally regulated. At the early stages of drupe development (5–11 WAF), low levels of mRNA accumulation were observed for *DGAT1*. The *DGAT2* mRNA was not detected in drupes from 5–9 WAF, while transcripts were just detectable at 11 WAF. *In situ* mRNA hybridization on 7 WAF drupes revealed that *DGAT1* is expressed in both seed and mesocarp tissues of the drupe, confirming the above results. Consistent with RNA blotting results, no hybridization signal was observed for the *DGAT2* anti-sense probe (Fig. 5). The expression of *DGAT1* occurred throughout the cell types of the drupe. A prominent staining was observed in the mesocarp, the developing seed coat tissues and the perisperm. However, globular embryos showed high levels of expression (Fig. 5). The higher expression level of the *DGAT1* gene observed in globular embryos than in the mesocarp (Fig. 5) was also apparent during the later stages of drupe growth (Fig. 4).

RNA gel blotting (Fig. 4) showed relatively high *DGAT1* transcript accumulation in embryos, starting as early as the early torpedo stage (13 WAF). The signal increased gradually at the later stages, peaked at 19 WAF (mid-torpedo stage), and finally declined substantially at 22 WAF. A similar bell-shaped accumulation pattern was observed in endosperms, with a peak at 19 WAF, but the signal was less intense throughout the time-course. The respective expression of *DGAT2* had a different pattern of mRNA accumulation, nevertheless the signal was weak throughout the time-course. Higher expression could be detected during the early- to mid-torpedo embryos. In endosperms, a transient slight increase at 19 WAF was also detected (Fig. 4). At the early stages of mesocarp development (13 and 16 WAF), the expressions of both *DGAT1* and *DGAT2* were very low, almost undetectable...
At 19 WAF, however, both transcript levels increased rapidly, particularly for DGAT1 that reached almost maximum levels. DGAT1 transcripts peaked at 22 WAF and thereafter decreased gradually until the end of the time-course. At 28 WAF the OeDGAT1 transcript levels were very low. As opposed, transcription of DGAT2 remained at rather low levels, similar to that detected at 19 WAF through 25 WAF. Notably, a sharp up-regulation was observed at 28 WAF, the late maturity stage. During that time (28 WAF), the olive drupes are starting to turn in colour from green to purplish-black. The results show that the two types of DGATs have distinct developmental regulation of gene expression in olive drupes.

Tissue-specific expression patterns in various reproductive or vegetative organs

To uncover whether this differential regulation of the two DGATs was also apparent in other parts of the plant, their expression patterns were compared in other than drupe tissues. Semi-quantitative RT-PCR analysis was carried out with total RNA extracted from various seedling parts (roots, hypocotyls, cotyledons, and shoot tips), leaves at different developmental stages (expanding, young, mature), floral buds (1.5 and 2.0 mm in length), anthers and ovaries of open flowers (2.5–3.0 mm in length). For comparison reasons, two samples from RNA gel blotting, i.e. embryos at 13 WAF and mesocarps at 22 WAF, were also included in the analysis. The results obtained from RT-PCR analysis (Fig. 6) were similar to the ones observed in the Northern blot analysis (Fig. 4). DGAT1 expression level in embryos was almost half of the mRNA accumulation detected in mesocarp, while DGAT2 mRNA transcripts in the mesocarp were at much higher levels than those of embryos (Figs 4, 6).

Both DGATs were transcribed in all the vegetative tissues examined (Fig. 6). DGAT1 transcript levels were very low in all seedling parts and relatively constant, as compared to embryo and mesocarp tissues. Root, hypocotyl, and cotyledons had similar levels of DGAT1 mRNAs. By contrast, relatively high DGAT2 expression was recorded in both cotyledons and hypocotyls. Different expression patterns
between the two genes were also observed during leaf development. The expression of \textit{DGAT1} was almost undetectable in expanding leaves, peaked at the young stage, and was strongly down-regulated in mature leaves (Fig. 6). The \textit{DGAT1} mRNA accumulation in young leaves was the highest among the vegetative tissues. The expression of \textit{DGAT2} in expanding leaves was low, as per \textit{DGAT1}, increased during the young stage and remained at similar high levels at maturity. \textit{In situ} mRNA hybridization analysis on expanding leaves revealed that \textit{DGAT1} was expressed in almost every cell type including palisade and spongy parenchyma cells. However, the respective signal for \textit{DGAT2} was most prominent in phloem cells of the vascular tissue of veins (Fig. 7). The expression level of \textit{DGAT2} in mature leaves was similar to that of the cotyledons, but nevertheless lower than that of the 28 WAF mesocarp (Fig. 6).

Distinct pattern of expression between \textit{DGAT1} and \textit{DGAT2} genes was also observed in flower tissues. Based on RT-PCR analysis, no transcripts were detected for \textit{DGAT1} and \textit{DGAT2} genes in young floral buds (1.5 or 2.0 mm). At later stages of bud development (2.5–3.0 mm) relatively weak signals corresponding to \textit{DGAT2} were observed both in anthers and ovules, while no transcripts were detected for \textit{DGAT1} (Fig. 6). The levels of DGAT2 mRNAs in both

| seedlings | leaves | drupes | flowers |
|-----------|--------|--------|---------|
| R | H | C | ST | EL | YL | ML | E | M | B1 | B2 | A | O |

\textit{DGAT1} and \textit{DGAT2} genes. First-strand cDNAs were synthesized from total RNA extracted from roots (R), hypocotyls (H), cotyledons (C), and shoot tips (ST) of olive seedlings; expanding (EL), young (YL), and mature (ML) leaves; embryos (E) at 13 weeks after flowering (WAF) and mesocarps (M) at 22 WAF; flower buds 1.0 mm (B1) and 2.0 mm (B2) in length; anthers (A) and ovaries (O) of flowers 2.5–3.0 mm in length. To ensure equal amounts of template, olive \(\beta\)-tubulin was used as a reference gene.

![Fig. 5. Localization of OeDGAT1 and OeDGAT2 transcripts in young drupes at 7 weeks after flowering (WAF). Longitudinal sections were processed for \textit{in situ} hybridization with DIG-labelled antisense RNA probes of OeDGAT1 (C, D) and OeDGAT2 (E, F). Negative controls were included by using two respective DIG-labelled riboprobes sense probes. Only representatives using OeDGAT1 sense-probe are presented here (A, B). DGAT1 expression was detected in mesocarp (me), the developing seed coat tissues (sc), the perisperm (pe), and the globular embryo (em), while the expression was relatively lower in endosperm (en) and the integuments (in). Sites of positive hybridization signals are shown as blue/violet regions. Scale bar represents 300 \(\mu\)m.](image1.png)

![Fig. 6. Semi-quantitative RT-PCR expression analysis of olive \textit{DGAT1} and OeDGAT2 genes. First-strand cDNAs were synthesized from total RNA extracted from roots (R), hypocotyls (H), cotyledons (C), and shoot tips (ST) of olive seedlings; expanding (EL), young (YL), and mature (ML) leaves; embryos (E) at 13 weeks after flowering (WAF) and mesocarps (M) at 22 WAF; flower buds 1.0 mm (B1) and 2.0 mm (B2) in length; anthers (A) and ovaries (O) of flowers 2.5–3.0 mm in length. To ensure equal amounts of template, olive \(\beta\)-tubulin was used as a reference gene.](image2.png)

![Fig. 7. \textit{In situ} localization of DGAT1 and DGAT2 transcript accumulation in transverse sections of expanding olive leaves. A representative negative control using the OeDGAT1 sense-probe is presented here (A). Signals of DGAT1 expression are prominent in almost every cell type including palisade parenchyma (pp), spongy parenchyma (sp), and phloem (ph) of the central vein, while DGAT2 mRNA is mostly localized to the phloem tissue of the vascular bundle. Sites of positive hybridization signals are shown as blue/violet regions. Scale bar represents 50 \(\mu\)m.](image3.png)
anthers and ovules were comparable with those observed in root tissues. In situ hybridization verified the above results and further showed that DGAT2 transcripts are largely localized in the tapetum cells and vascular bundles of anthers and mostly in the stigma and the vascular bundles of ovaries (Fig. 8). The above results show a prominent differential spatial and temporal gene expression of DGAT1 and DGAT2 in most organs/tissues examined.

**Discussion**

Although several type-1 DGATs have been isolated and characterized from various eukaryotes, to date, the physiological functions of type-2 DGATs have been much less determined (Shockey et al., 2006). In order to understand the mechanisms of TAG biosynthesis in olive better, a novel DGAT cDNA was cloned from olive drupe mesocarp. The potential contribution of type-1 and -2 DGATs in olive TAG biosynthesis were investigated through tissue-specific transcriptional analysis in various vegetative and reproductive organs, at different times during development. DGAT activity exerts strong control over flux in the Kennedy pathway of oleaginous seeds and in olive tissue cultures as well (Ramli et al., 2005), therefore particular emphasis was given to the temporal regulation of olive DGATs during drupe development. In olive fruit, TAGs are formed and stored in both the mesocarp and the seed, two drupe compartments that have different physiological functions and roles and also display differences in the mode of TAG accumulation. Storage TAGs in seeds are proposed to provide energy for germination. They are present in small (0.5–2 mm diameter) subcellular oil bodies completely covered by oleosins to prevent them from coalescence (Hsieh and Huang, 2004). By contrast, TAGs in the mesocarp have no such clear physiological role for the plant per se, but may attract animals for seed dissemination. The fleshy olive mesocarp possesses much larger (about 30 mm diameter) lipid particles of TAGs, which are devoid of surface oleosins (Murphy, 2001; Giannoulia et al., 2007). Accumulation of TAG in olive seeds is relatively fast, compared with the mesocarp, being completed within a relatively short period (Sanchez, 1994). Although massive TAG storage in seeds starts at about 11 WAF, coinciding with endocarp lignification, DGAT1 transcripts were present as early as 5 WAF, albeit at low levels. By contrast, DGAT2 transcripts were almost undetectable until 11 WAF, pointing to a principal role of DGAT1 in early TAG accumulation in olive drupes, especially in the seed.

As the drupe grows further, the rate of oil synthesis in seed tissues accelerates reaching a plateau at about 22 WAF (Sanchez, 1994). The pattern of oil deposition in seeds correlates well with DGAT1 regulation both in embryo and endosperm, whereas the relative expression of DGAT2 was barely detectable. The bell-shaped expression pattern of DGAT1 coincides well with the relative expression of the olive oleosin gene in seed tissues (Giannoulia et al., 2007). Similarly, in oilseed species, a transient increase of DGAT activity occurs at the stage of active oil accumulation, but when the lipid content reaches plateau the activity decreases markedly (Tzen et al., 1993; Weselake et al., 1993). Taken together, the present results suggest a prominent role of DGAT1 in seed olive oil accumulation compared with DGAT2. This is in contrast to oleogenic seed crops that contain unusual fatty acids, where DGAT2 may play a more central role than DGAT1 in oil production (Yu et al., 2006; Shockey et al., 2006; Burgal et al., 2008; Li et al., 2010).

Olive oil does not contain unusual fatty acids and olive is one of the few exceptions of commercially important oil-producing crops in that most of the oil is produced in the mesocarp. Oil accumulation in the mesocarp follows a typical sigmoidal curve (Sanchez, 1994). The major proportion of oil generally starts to accumulate at 16–19 WAF and reaches a plateau at about 28 WAF. However, the pattern of accumulation may vary due to environmental conditions, different agricultural practices and/or the olive variety (Connor and Fereres, 2005). Both DGAT1 and DGAT2 share an overlapping expression pattern from 19–25 WAF, suggesting that they probably function together at those stages. However, following maximal mRNA levels at 22 WAF, DGAT1 transcription declined substantially. As opposed to DGAT1, a sharp up-regulation of DGAT2 was detected at 28 WAF reaching by far its maximum mRNA accumulation in the time-course. As this

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**Fig. 8.** In situ localization of OeDGAT1 and OeDGAT2 transcript accumulation in longitudinal sections of anthers (A–C) and pistils (D–F) from buds of about 2.5 mm in length. A representative negative control using the OeDGAT2 sense-probe is presented (A, D). DGAT1 exhibited no labelling or negligible background reaction in pollen grains (p) (B) or in ovaries (E). DGAT2 transcripts are largely localized in the tapetum cells (t) of anthers (C) and in the stigma (s) and the vascular bundles (vb) of ovaries (F). Sites of positive hybridization signals are shown as blue/violet regions. Scale bar represents 500 μm.
is also the case in planta, the present data suggest that DGAT2 predominates at the onset of ripening. Although most of TAG accumulation in olives takes place before the onset of ripening, it has been demonstrated that oil cultivars, like ‘Koroneiki’ in this study, have much longer oil-filling periods than those of table cultivars (Garcia-Martos and Mancha, 1992; Farinelli et al., 2002). Indeed, as shown previously, the transcripts of stearoyl-ACP desaturase, the responsible enzyme for oleate production, accumulates as the growth of the mesocarp proceeds, reaching maximum levels at 28 WAF (Haralampidis et al., 1998). It is still unclear why DGAT2 expression overwhelms DGAT1 at the late stages of mesocarp growth. DGAT2 implication in altering the fatty acid composition of TAGs is unlikely here, because there are not sufficient structural changes in olive oil composition during ripening (Gutierrez et al., 1999; Ayton et al., 2001; Cossignani et al., 2001). Some plant DGATs exhibit a broad acyl-CoA preference as revealed by specificity and selectivity studies (Lung and Weselake, 2006; Shockey et al., 2006). Therefore, it is plausible that the acyl composition of TAG in oilseeds is predominantly dictated by the availability of specific DAG and acyl-CoA pools (Kamisaka et al., 1997; Lung and Weselake, 2006; Shockey et al., 2006). The higher proportion of oleoyl-CoA throughout olive fruit growth dictates a higher incorporation of oleic acid, which is consistent with analytical data for the fatty acid composition of olive oils (Sanchez and Harwood, 2002).

It is believed that the increase in lipid droplet size in olive mesocarpic cells is facilitated through the coalescence of smaller lipid bodies (Rangel et al., 1997). Concomitantly, during the olive fruit-ripening phase, oil biosynthesis continues together with an increase in dry matter, albeit at a slower rate than in previous phases (Conde et al., 2008). The mechanism of lipid-droplet formation and the growth in size in eukaryotes is largely unknown (Guo et al., 2009). Recent data revealed that DGAT2, but not DGAT1, may be dynamically associated with lipid droplets and mitochondrial compartments, promoting active synthesis and storage of TAGs in oleate-treated mammalian (COS-7) cells (Stone et al., 2009). It is unknown as yet whether the plant DGAT2 enzyme may have a similar function on lipid droplet enlargement in olive mesocarp cells. In any case, present data suggest that olive DGAT2 is a key player in the late production of mesocarp oil, the time of lipid droplet enlargement. By contrast, in both embryos and endosperm the oil bodies remain extremely small (Ross et al., 1993) and in those tissues the DGAT2 expression is almost negligible. This raises the possibility that OeDGAT2 may be useful for breeding programmes aimed at high oil-yielding olive genotypes through marker-assisted selection or by genetic engineering. In Arabidopsis, DGAT1 and PDAT1 have overlapping functions in TAG biosynthesis (Zhang et al., 2009). PDAT may also contribute to TAG biosynthesis in olive callus cultures (Hernandez et al., 2008). Nevertheless, the activity of this enzyme as measured in subcellular fractions of olive calli was rather low, indicating that DGAT exerts significant flux control over TAG formation in olives (Ramli et al., 2005; Hernandez et al., 2008).

Both DGAT genes were expressed in all seedling organs examined, with mRNA accumulation of DGAT2 being the highest in the cotyledons and hypocotyls. To our knowledge, this is the first time that the induction of DGAT2 has been reported in seedling tissues. Transcriptional analysis in Arabidopsis seedlings has revealed high mRNA levels of type-I DGAT during the post-embryonic stages in both cotyledons and hypocotyls (Lu et al., 2003) and DGAT1 activity has been previously correlated with de novo TAG synthesis in the cotyledons of castor seedlings (He et al., 2006). It is likely that excess sugar production is channelled into TAGs for internal use or for storage to prevent osmotic effects (He et al., 2006). In the latter case, glyoxysomes and other machineries may convert degraded lipids into sugar for export to non-senescing tissues (Pracharoenwattana and Smith, 2008).

Huang et al. (2009) showed that oil bodies, primarily composed of steryl esters and triacylglycerols, were abundant in the Physcomitrella photosynthetic vegetative gametophyte. In this study, relatively high levels of transcription of both OeDGATs were detected in olive leaves, where the regulation of expression was clearly developmentally regulated. Transcripts of both DGATs and, possibly, the accompanied neutral lipid accumulation, start as the leaf reaches its final size, but is still young. Cross-sections of young leaves followed by Sudan III staining and microscopic observation revealed the presence of lipid droplets throughout the mesophyll but mostly close to the epidermis and the vascular tissue (data not shown). Accumulating data, as stated above, suggest that DGAT genes also play roles other than its ‘classical’ role to synthesize TAGs in the storage organs (Zhang et al., 2009). The present results point to a differential contribution of each DGAT gene in various organs in a temporal-related manner. This notion was further justified in flowers. OeDGAT2, but not OeDGAT1, was highly expressed in the tapetum cells of anthers and in different ovule tissues. Neither DGAT expression was detected in floral buds while, in actively dividing cells like shoot tips and young seedling roots, both genes were down-regulated. DGAT2 expression in tapetum cells is in concert with the deposits of TAGs (Murphy, 2001). Taken together, the high levels of DGAT2 expression in leaves and in vascular and tapetum cells of flowers, point to its prominent role in the synthesis of TAGs in those organs possibly for later lipid mobilization and sugar transport.

In conclusion, both DGAT1 and DGAT2 share overlapping but distinct transcription patterns during vegetative and reproductive growth, suggesting that they are differentially regulated in a developmental and cellular manner. They probably have similar functions but they also serve different purposes. Distinct expression patterns of DGAT1 and DGAT2 were observed between the seed and mesocarp, with DGAT1 contributing most of the TAG deposition in seeds, reflecting the large differences in the mode of TAG accumulation between the two fruit compartments. Important differences between the expression profiles of the two genes were also apparent during drupe ripening. Our
findings in this study provide evidence that DGAT2 may be a key mediator of higher oil yields in ripening mesocarps, where oil droplets increase in size and TAGs are still accumulating. This important finding, however, remains to be further justified by measuring the respective enzymatic activities. Apart from the ripening mesocarp, DGAT2 may also serve for the accumulation of TAGs in senescing organs, like cotyledons and flower tissues, possibly for further lipid mobilization or other internal uses.

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