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Uses Semi–quantitative and Relative Quantity Methods to Analysis Gene Expression of *DGAT1* Gene Responsible for the Olive Diacylglcerol Acyltransferases in 10 Cultivars of Olive (*Olea europaea*. L)

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Abstract

In this study gene expression for *DGAT1* gene was analyzed. Diacylglycerol acyltransferases (DGATs) catalyze 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). Gene expression were analyzed for 10 Olive cultivars (*Olea europaea* L.) (Khaderi, Qaysi, Manzenillo, Baashiqi, Arabqween, Nabali, Labeeb, Dahkan, Shami and Sorani). Different plant organs as plant materials (mature leaves, mesocarp and seeds for drups) used for analysis. Two methods for analysis gene expression were used, first method was called semi – quantitative and second method was called relative – quantitative, used in relative method (Real time PCR and Actine gene as Housekeeping gene). On the other hand chemical analysis was used on fruits like moisture % and oil % of dry and fresh weigh. The results revealed the following: *DGAT1* gene expression in leaves, mesocarp and seeds by two methods (semi- quantitative and relative quantity) were the convergent results and clear, also if this results compared with chemical analysis shows that the best cultivars were Arabqween, Khaderi, Qaysi and Labeeb. The cultivars Shami and Khaderi then were contain in fruits desirable qualities of olive oil, low moisture and high oil percentages ratios. While Nabali, Manzanello, and Sorani cultivars middle desirable quantity, and Baashiqi and Dahkan cultivars had undesirable because of low oil quantity and high moisture in contain fruits. Some cultivars have low intensity in semi- quantitative and little fold in relative quantity but it have high oil in contain fruits that may be indicate that these cultivars were complete gene expression and begin to accumulation and save oil in tissue. 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 difference in the mode of TAG accumulation. DGATI share an overlapping expression pattern after 28 WAF, suggesting that they probably function at those stages. However, following maximal mRNA levels at 22 WAF, DGAT1 transcription declined substantially.

Keywords: olive; *DGAT1;* diacylglycerol acyltransferases; gene expression.

Introduction

The olive tree *(Olea europaea* L.) is an evergreen species that ranks sixth in the world's production of vegetable oils [1]. Since ancient times it has been cultivated in the Mediterranean Basin, where ~2600 cultivars have been identified based on morphological traits [2]. In more recent years, interest in olive oil production has been extended to countries outside the Mediterranean region such as Argentina, the USA, Mexico, South Africa, western Africa, Australia, Azerbaijan, China and Japan [3, 4]. Olive trees lack dormancy, and are sensitive to low temperatures [5].

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 signaling molecules involved in plant development and stress response [6,7]. Linoleic acid, together with oleic acid, is a major fatty acid in vegetable oils and its content greatly affects the technological properties such as their oxidative stability [8] and nutritional characteristics [9].

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 [10, 11, 12, 13]. 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 programs 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 [2,14,15], 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 [16].

TAG biosynthesis is principally accomplished by membrane-bound enzymes that operate in the endoplasmic reticulum (ER) through the glycerol-3-phosphate or so called Kennedy pathway [17, 18]. 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. As much 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 [19, 20, 13]. However, TAGs could also be produced via the transfer of acyl groups from phospholipids to diacylglycerols, an acyl-CoA-independent reaction catalyzed by the enzyme phospholipid: diacylglycerol acyltransferase (PDAT) [21, 22, 23]. 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, germinating seeds, young seedlings, and senescing leaves of *Arabidopsis* [24, 25], and in stems, flowers, roots, and leaves of tobacco [26]. 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 [27, 28]. Two major unrelated gene families have been shown to encode DGATs, namely *DGATI* (type-I) and *DGAT2* (type-2) both of which are ER-localized. *DGATI* genes have been cloned from several plant species, including olive [29]. *DGAT2* genes have been cloned from diverse eukaryotes, including the oleaginous fungus *Mortierella ramanniana* [30], human [31], and the plant species *Arabidopsis* [30], castor bean [32], and tung tree [16]. A third member of the DGAT family (type-3), highly unrelated to the previously reported was identified in peanut that possesses a cytosolic localization [33]. Accumulating data suggest that DGAT activity may have a substantial effect on carbon flow into seed oil of *Brassica napus* [34, 35], *Arabidopsis thaliana* [24, 11], and maize [36]. In an attempt to gain the further insight into the role(s) of DGATs in plant lipid biosynthesis the expression patterns of *DGATl* in several other organs/tissues of the olive tree indicated that genes

are differentially regulated to fulfill the needs for TAG accumulation at certain points of growth and development.

Materials and methods

Plant Material

Used the mature leaves (ML), mesocarp fruit (ME) and seeds (S) included emberyo and endosperm of 10 olive cultivers (*Olea europaea*) were: (Khaderi, Qaysi, Manzenillo, Baashiqi, Arabqween, Nabali, Labeeb, Dahkan, Shami and Sorani) trees grown in Iraqi orchard near Mosul, and chilled in liquid nitrogen, and stored at 80℃.

Total RNA Extraction and cDNA Synthesis

Total RNA for samples were isolated by uses (SV Total RNA Isolation kit/Promega.USA). The quality of RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis. DNA was removed from RNA samples using the DNase I Mix/Promega USA (DNase I, MnCl2, yellow core buffer). First-strand cDNA was synthesized from (16 µl) of total RNA using the (power cDNA Syntheses kit/IntronBio.Inc. USA) with Oligo (dT) 15 primer, following the manufacturer's instructions and quantified using gel electrophoresis.

Semi- Quantitative Real Time PCR

Analysis were carried out by change PCR program respectively with Primer for gene-specific (*DGAT1*) amplification and cDNA Syntheses from 25th to 35th cycle. PCR products were also checked for purity by 1 % agarose gel electrophoresis and the following program shows the method.

Cycle	Time	Tm	Stage	
1	5 min	95	Pre - Denaturation	1
25	45 sec	95	Denaturation	2
	45 sec	55	Annealing	3
	45 sec	72	Extension	4
1	10 min	72	Final extension	5
Cycle	Time	Tm	Stage	
1	5 min	95	Pre - Denaturation	1
35	45 sec	95	Denaturation	2
	45 sec	55	Annealing	3
	45 sec	72	Extension	4
1	10 min	72	Final extension	5

*Tm (melting temperature)

Quantitative Real Time PCR (qRT -PCR)

Gene expression analysis was performed by qRT-PCR using an Mini Opticon System real-time PCR and GO Taq Master Mix SYBR Green kit Q-PCR/ IntronBio.Inc.USA. Primers for gene specific amplification were designed to generate a product of 100-200 bp and to have a Tm (melting temperature) of 60 ℃. PCR reactions were carried out in duplicate in plate. Reaction mix (22.5 µl per well) contained 12.5 µl, Master Mix SYBR Green, 2.5 µl forward and reverse primers,7.5 µl DEPC-D.W and 2.5 µl of cDNA. The thermal cycling conditions consisted of an initial denaturation step of 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 30 s, 60 ℃ for 1 min, and 72 ℃ for 30 s. The specificity of the PCR amplification was monitored by melting curve analysis following the final step of the PCR products were also checked for purity by agarose gel electrophoresis. The housekeeping olive Actin gene (*OeActin*) was used to normalize as endogenous reference. The real-time PCR data were analyzed by GeneX program. The gene of the olive tree, used for the design of primers are available in Gen Bank (www.ncbi.nih.nlm.gov/ Gen Bank/EMBL/DDBJ. the primers sequences (*DGAT1*) for QRT-PCR amplification were two parts forward (5-TTGGCTGAATATATTAGCGGAACTTC-3) and reverse (5- CTCATCATAAAAATGTCCACATCC-3) and Actin gene primer were forward (5-ACCACCTCAGCCGAACGGGA-3) and reverse (5-TGCTGGGAGCCAAGGC AG TG-3).

Chemical analysis

Uses Soxhlet method to determine oil percentage (%) in fresh and dry flesh of 10 olive cultivars.

Results

Figure 1 showed the results of isolated total RNA of the mature leaves (ML) , flesh mesocarp fruit (ME) and seeds (S) of the studied olive cultivars manner filters and then migrated to agar gel 1 %, voltage 100 V for 20 minutes noting the success of the method to isolate RNA from this plant parts.

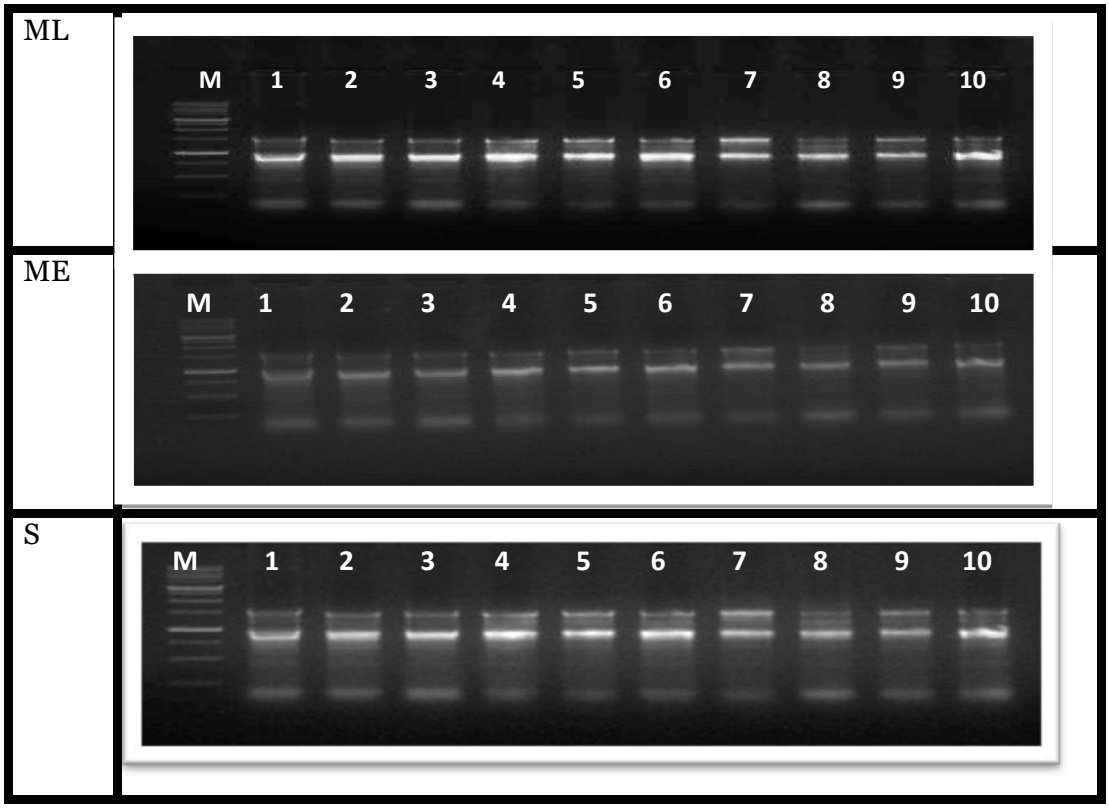
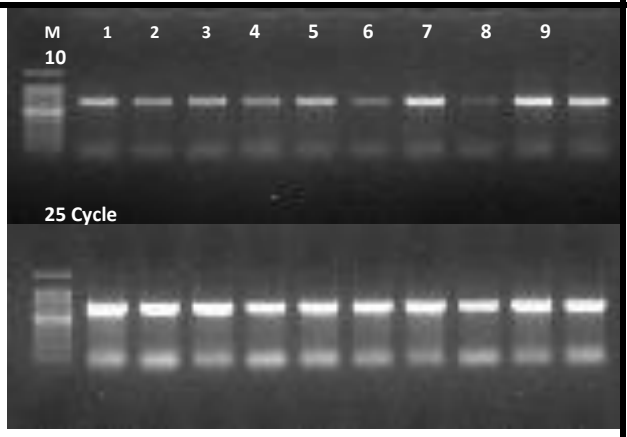
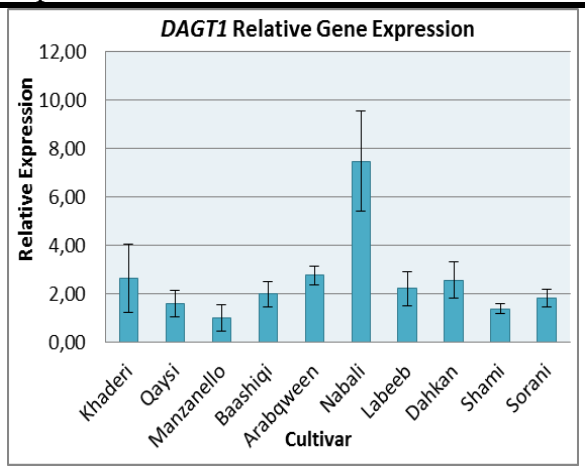
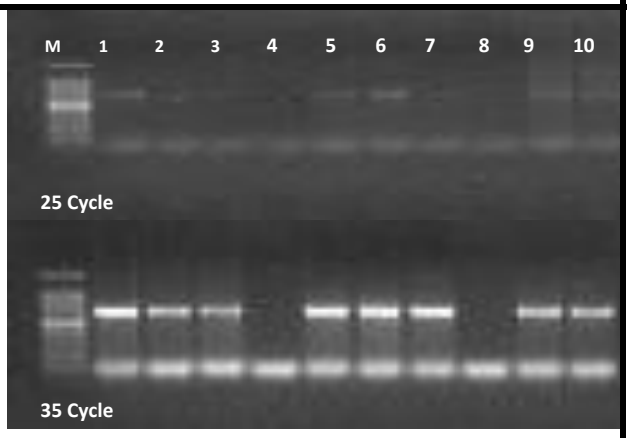
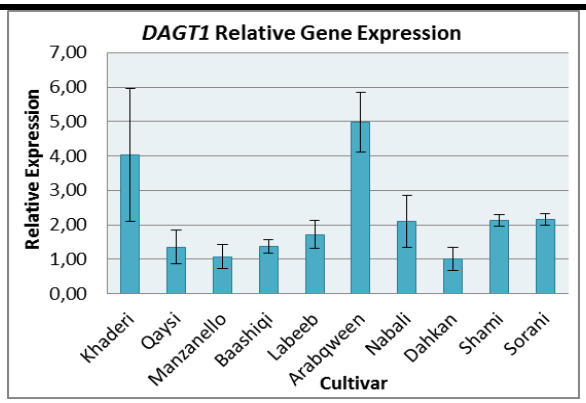


Figure (1) represents the isolated total RNA of the mature leaves(ML), mesocarp(MS) and seeds(S) of ten olive cultivars on agarose gel (1%) and voltage (100 V) for (20 minutes). M = Marker Leader: 1 = Khaderi, 2 = Qaysi 3 = Manzenillo, 4 = Baashiqi, 5 = Arabqween, 6 = Nabali, 7 = Labeeb, 8 = Dahkan, 9 = Shami and 10 = Sorani.

The semi-quantitative measurement of gene expression of the gene *DGATl* in the mature leaves (ML) of cultivars (Figure 2. ML) refers to the emergence of clear on bands and high intensity in some cultivars and a few intensity in other cultivars at the 25th cycle of the PCR as shine bands cultivars were Labeeb, Shami and Sorani but the cultivars Khaderi, Oaysi, Manzenillo and Arabqween were medium intensity bands, either cultivars less intensity were at cultivars Baashiqi, Nabali and Dahkan, but when 35th cycle that all cultivars were similar and equal approximately. Analysis relative gene expression of the gene *DGATl* in the mature leaves (ML) of cultivars were different then show Nabali variety the highest level gene expression nearly75 fold, but another cultivars were equals in gene expression approximately.

The process of analyzing the cultivars mesocarp (ME) showed clear differences among cultivars. Figure 2.ME refer to supremacy Khaderi, Arabqween, Nabali, Shami and Sorani cultivars which have clear bands in 25th cycle, but in 35th cycle less cultivars intensity were at cultivars Baashiqi and Dahkan if compared with the other cultivars. Notes from the analysis of relative amount of the gene *DGATl* in fruit mesocarp of olive cultivars under study shows that cultivars Khaderi and Arabqween had a higher level of expression of gene after 28 weeks of full bloom and it reached approximately 4 and 5 fold respectively, and either lower the level of gene expression was Dahkan cultiver which amounted to 1 folde approximately.

In seed, analysis cultivars gene expression *DGATl* showed clear differences among cultivars then analysis semi-quantitative in the 25thcycle of the PCR program that for cDNA cultivars appeared Baashiqi and Dahkan cultivars weak intensity bands, on the other hand this cycle (25th) showed high intensity of clear bands in Arabqween, Nabali, Labeeb, Shami and Sorani cultivars while another cultivars were has middle intensity (Figure 2.S). In 35th cycle of the PCR program of gene expression to gene *DGAT1* in the seeds of olive cultivars that clear bands for all cultivars especially Khaderi, Manzenillo, Arabqween, Nabali and Labeeb cultivars, except Baashiqi and Dahkan cultivars had weak intensity. Notes from the analysis of relative amount of the *DGAT1* gene in seed (Figuare 2.S) showed Shami, Arabqween and Manzenillo cultivars had high relative gene expression reach (79,51 and 35 fold) respectively. While Khaderi, Qaysi, Sorani and Baashiqi cultivars were less relative gene expression, while Nabali, Labeeb and Dahkan were middle in relative gene expression.

	Semi-quantitative Gene expression	Relative quantitative (realtime PCR) Gene expression
ML		
ME		

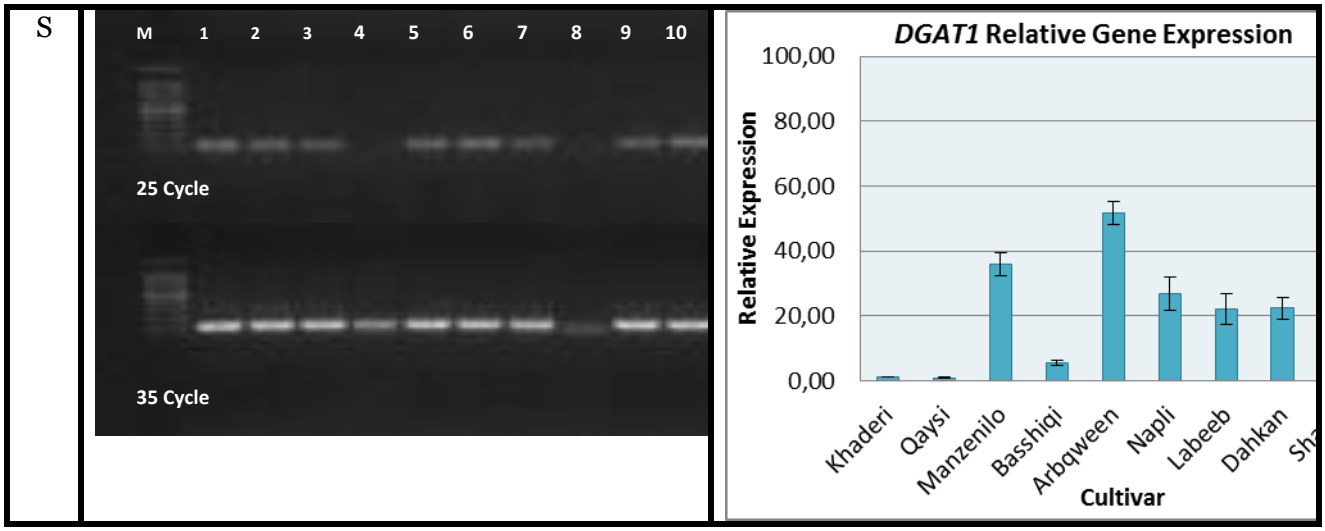


Figure (1) represents the Semi-quantitative measurement and Relative quantitative of *DGATI* Gene expression to the mature leaves(ML), mesocarp(MS) and seeds(S) of ten olive cultivars semi-quantitative were on agarose gel (1%) and voltage (100 V) for (20 minutes). M = Marker Leader: 1 = Khaderi, 2 = Qaysi 3 = Manzenillo, 4 = Baashiqi, 5 = Arabqween, 6 = Nabali, 7 = Labeeb, 8 = Dahkan, 9 = Shami and 10 = Sorani.

Chemical analysis result

Chemical analysis of the fruits of olive cultivars showed highest moisture percentage in Dahkan variety fruits with significant difference reached (71.3 %), while the lowest moisture percentage in Khaderi variety fruit. Arabqween variety fruit contained high percentage of oil in dry weight reached (57.5 %) and Baashiqi fruit had low percentage of oil dry weight reached (15.5 %). Percentage of oil in fresh weight showed which highest significant value in Khaderi variety which was (37.4 %), while the lowest percentage in Iraqi cultivars (Baashiqi and Dahkan) reached (4.9 and 6.6 %) respectively. Table (l).

Table (l) analysis moisture and oil in olive fruits cultivars

Cultivars	Moisture (%)	Oil.dw (%)	Oil.fw (%)
Khaderi	30.4 f	53.7 c	37.4 a
Qaysi	36.4 e	56.3 ab	35.4 b
Manzanello	38.8 de	38.9 e	23.8 e
Baashiqi	68.4 b	15.5 g	4.9 f
Arabqween	41.6 c	57.5 a	33.6 c
Nabali	39.5 d	49.8 d	30.1 d
Labeeb	39.6 d	55.8 b	33.7 c
Dahkan	71.3 a	23.2 f	6.6 f
Shami	37.8 e	49.8 d	31.5 d
Sorani	38.8 de	38.6 e	24.2 e

***Similar letters refer to nonsignificant difference.**
***Dissimilar letters refer to significant difference.**
***This analysis of variance and means were separated by Duncan's multiple range test at the 5% level.**

Discussion

In this study (Figure 1) appear *DGAT1* gene expression in leaves, mesocarp and seeds by two methods (semi- quantitative and relative quantity) were the convergent results and clear, and if these results compared with chemical analysis (Table 1) shows that the best cultivars were Shami, Arabqween, Khaderi, Qaysi and Labeeb. Tables (1) indicates that the cultivars Shami and Khaderi then were contain in fruits desirable qualities of olive oil, low moisture and high oil percentages ratios. While it were Nabali, Manzanello, and Sorani cultivars middle desirable qualities wheres cultivars Baashiqi and Dahkan were undesirable because of low oil quantity and high moisture in fruits. Some cultivars have little intensity in semi- quantitative and little fold in relative quantity but it's have high oil contain in fruits that maybe indicate to that cultivars were complete gene expression and begin to accumulation and save oil in tissue. 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 difference 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 complete covered by oleosins to prevent them from coalescence [37]. The fleshy olive mesocarp possesses much larger (about 30mm diameter) lipid particles TAGs which are devoid of surface oleosins [38, 39]. Accumulation of TAG in olive seeds is relatively fast, if compared with the mesocarp being completed within a relatively short period [40]. Although massive TAG storage in seeds starts at about 11 WAF, coinciding with endocarp lignification, DGATI 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 DGATI in early TAG accumulation in olive drupes, especially in the seed [41]. As the drupe grows further, the rate of oil synthesis in seed tissues accelerates reaching a plateau at about 22 WAF [40]. The pattern of oil deposition in seeds correlates well with DGATI regulation both in embryo and endosperm. The bell-shaped expression pattern of DGATl coincides well with the relative expression of the olive oleosin gene in seed tissues [39]. Similarly, in oilseed species, a transient increase of DGATs activity occurs at the stage of active oil accumulation, but when the lipid content reaches plateau the activity decreases markedly [42, 43]. The present results suggest a prominent role of DGATI in seed olive oil accumulation. 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 [44, 16, 20, 45]. 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 [40]. 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 [46]. DGATI share an overlapping expression pattern after 28 WAF, suggesting that they probably function at those stages. However, following maximal mRNA levels at 22 WAF, DGAT1 transcription declined substantially.

The reference [47] 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 OeDGATl were detected in olive leaves of cultivars, where the regulation of expression was clearly developmentally regulated. Accumulating data, as stated above, suggest that DGATl gene also play roles other than its 'classical' role to synthesize TAGs in the storage organs [23, 41, 48]. The present results point to a differential contribution of each DGAT gene in various organs in a temporal-related manner.

Conclusion

In conclusion DGATI share overlapping but distinct transcription patterns during vegetative 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 DGATI were observed between the leave, seed and mesocarp, with DGATI contributing most of the TAG deposition in seeds, reflecting the large differences in the mode of TAG accumulation among the cultivars fruit compartments. Important differences between the expression profiles of the gene were also apparent during drupe ripening.

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