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Characterization and validation of olive FAD and SAD gene families: expression analysis in different tissues and during fruit development

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Abstract:	Background : Stearoyl-ACP desaturases (SADs) and fatty acid desaturases (FADs) play a critical role in plant lipid metabolism and also affect oil fatty acid composition introducing double bonds into the hydrocarbon chains to produce unsaturated fatty acids. In the present study, the genomic sequences of three SAD and three FAD candidate genes were characterized in olive and their expression was evaluated in different plant tissues. Methods and Results : OeSAD genes corresponded to olive SAD1 and SAD2 and to a newly identified Oe SAD4 , sharing the conserved protein structure with other plant species. On the other hand, the full-length genomic sequences of two microsomal OeFAD genes (FAD2-1 and FAD2-2) and the plastidial FAD6 , were released.

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When the level of expression was tested on different tissues of cv. Leccino, OeSAD1 and OeSAD2 were mainly expressed in the fruits, while OeFAD genes showed the lowest expression in this tissue. The mRNA profiling of all genes was directly studied in fruits of Leccino and Coratina cultivars during fruit development. In both genotypes, the expression level of OeSAD1 and OeSAD2 had the highest value during and after the pit-hardening period, when oil accumulation in fruit mesocarp is intensively increasing. Furthermore, the expression level of both OeFAD2 genes, which were the main candidates for oleic acid desaturation, were almost negligible during fruit ripening.
Conclusions : These results have made possible to define candidate genes of the machinery regulation of fatty acid composition in olive oil, providing information on their sequence, gene structure and chromosomal location.

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RESPONSE TO REVIEWER COMMENTS

COMMENTS TO THE AUTHOR:

Reviewer #1: I observed the manuscript and noticed that the authors have worked hard for improving the paper. They had responded well to almost my every query raised and corrected as it was advised. However the resolution of Fig. 1 is still poor. I advise authors if they could further improved the quality of figure. Overall based on my comments and authors response, I recommend to accept the manuscript.

R. We have improved a lot the quality during last revision and we visualize really well in our PC. Anyhow, we have done what requested and we hope the quality is at high level.

Reviewer #3: Authors have greatly improved the content of the present manuscript after the review, including some important references that they have previously omitted and emphasizing the most important findings of their work. However, in my opinion, there are still some important points that should be addressed before publication:

- Introduction: "Up to now, the complete genome sequences of two olive cultivars, O. europaea cv. Leccino [15] and O. europaea cv. Farga [16], have been reported."

In addition, the olive genome of Olea europaea var. sylvestris have been reported (Unver et al., 2017). This information should be included in the introduction as well, especially when the data obtained by Unver et al. have been used by authors and discussed in the discussion section.

R. The sentence was added as requested

- Materials and methods:

Have the authors checked RNA quality after extraction?

Mesocarp tissue accumulate considerable amount of oil and polyphenols that interfere with the RNA extraction, so that, in many cases the RNA quality is very poor. Therefore, it is very important to check RNA quality before cDNA synthesis.

R. The use of NANODROP already reported is now better explained in order to describe how the quality and purity of RNA was controlled. Moreover, we added an information about the agarose gel, which is always run in our lab to check the integrity of RNA.

- Discussion: "Thus, OeSAD2 and OeFAD2-2 are the most important contributors for the determination of the peculiar and precious content of fatty acids in olive fruit mesocarp, which has the greatest quantity of oleic acid among all plant species."

Bearing in mind the recently published manuscript by Hernandez et al. (2020), and the data reported by authors in the present manuscript, this statement is not completely true. It should be written:

Thus, OeSAD2 and OeFAD2-2 are the most important contributors, together with OeFAD2-5 (Hernandez et al., 2020), for the determination of the peculiar and precious content of fatty acids in olive fruit mesocarp, which has the greatest quantity of oleic acid among all plant species."

R. We have introduced the gene and the relative citation in the reported sentence.

- Table 1: As far as I understand, the candidate genes have not been amplified. The primers detailed in this table were used only for QPCR analysis, and they not amplified the complete gene. Is this correct? In this case, it should be written:

Specific primers used for quantitative expression of six candidate transcripts

R. Done.

- Figure 2 and Figure 3 contain the same data, but represented in a different way. Therefore, only one figure should be included in the main text and the other one should be moved to supplementary data.

In addition, If I am not mistaken, the difference between both figures is the calibrator used for the relative expression levels calculation using the 2-ΔΔCT method. However, in none of the cases, the calibrator used for the calculations is indicated.

The calibrator used for the calculations should be indicated to better understand the results presented and the differences between figures 2 and 3. In addition, depending on the calibrator used comparison between genes and/or tissue should be analyzed.

R. In the main text we have left the figure 2 while the figure 3 was placed as supplementary figure S5, as requested.

The calibrator used in each 2-ΔΔCT analysis was related to the sample with lowest level of expression, which correspond to the highest CT values (Righetti et al., 2018; Zhu et al., 2017). This information was reported in the new version of manuscript in the material and methods section.

Bibliography

Righetti, L., Paris, R., Ratti, C., Calassanzio, M., Onofri, C., Calzolari, D., ... & Grassi, G. (2018). Not the one, but the only one: about Cannabis cryptic virus in plants showing 'hemp streak'disease symptoms. European Journal of Plant Pathology, 150(3), 575-588.

Zhu, Y., Shao, J., Zhou, Z., & Davis, R. E. (2017). Comparative transcriptome analysis reveals a preformed defense system in apple root of a resistant genotype of G. 935 in the absence of pathogen. International journal of plant genomics, 2017.

Figure 1

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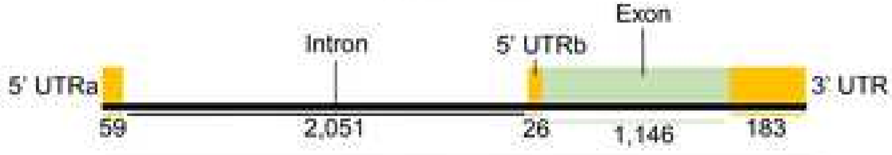
OeFAD2-1

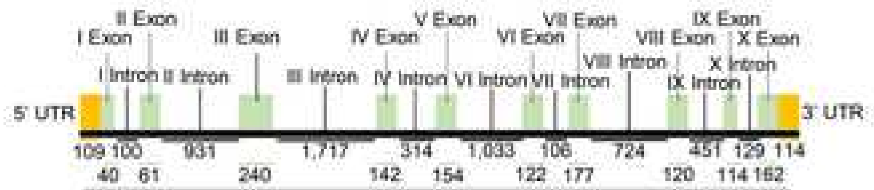


Total length 3,465 bp

OeFAD2-2

Total length 3,687 bp

OeFAD6



Total length 6,930 bp

OeSAD1

Total length 4,063 bp

OeSAD2

I Exon
I Intron
II Exon
II Intron
III Exon
5' UTR
3' UTR
122
108
2,179
505
465
560
209

Total length 4,148 bp

OeSAD4

Total length 4,668 bp

Figure 2

Click here to access/download;Figure;Figure_2.jpg

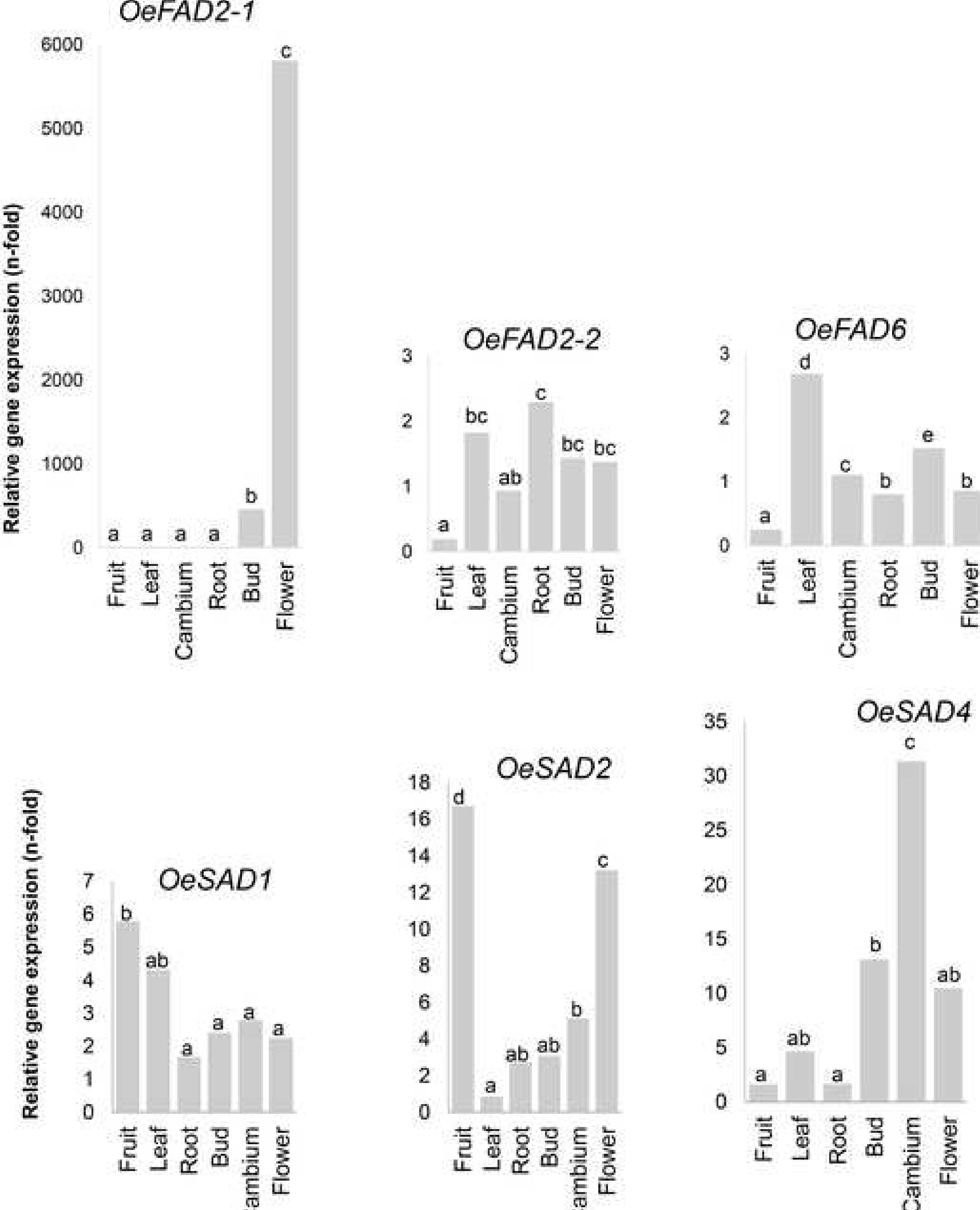


Figure 3

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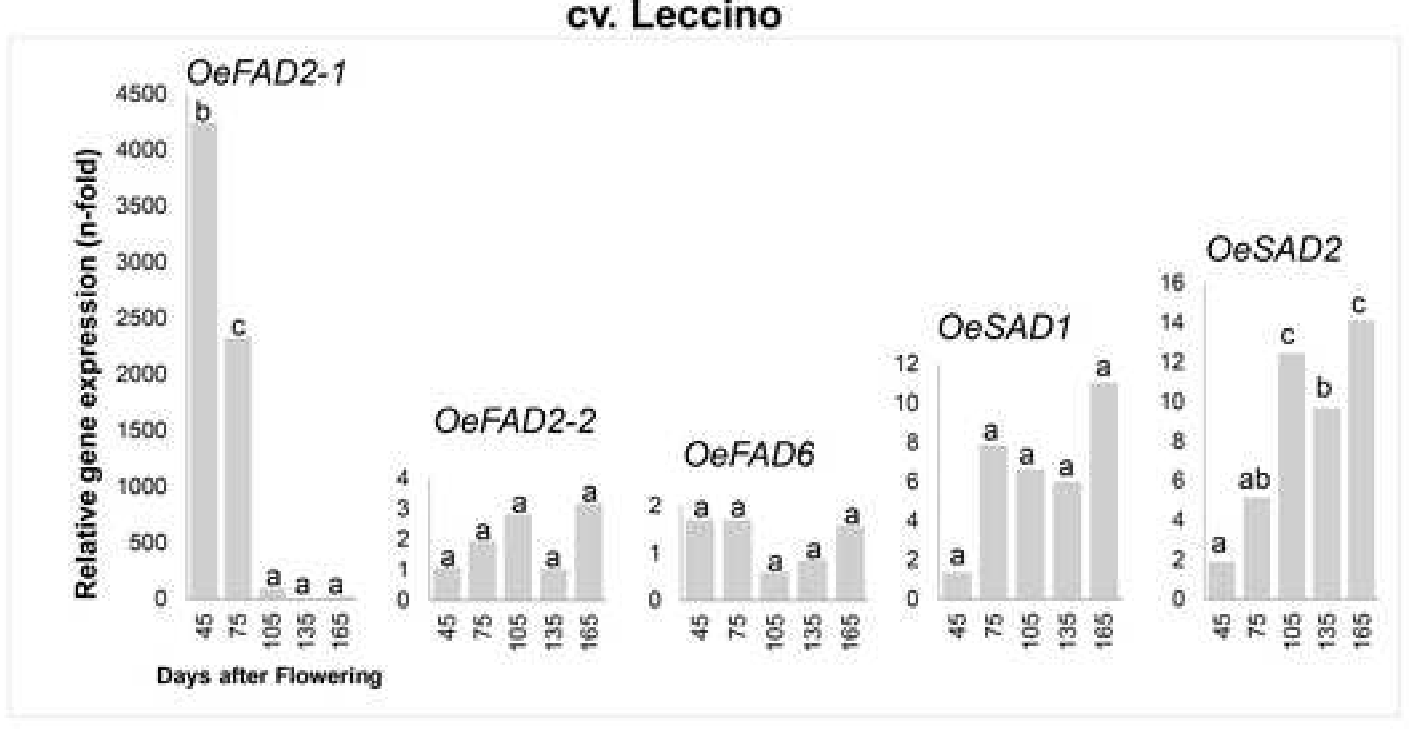

Supplementary Figure 1

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Supplementary Material
ESM_Figure_S1.jpg

Supplementary Figure 2

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Supplementary Figure 3

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Supplementary Figure 4

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Supplementary Figure 5

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ESM_Fig_S5.jpg

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Characterization and validation of olive FAD and SAD gene families: expression analysis in different tissues and during fruit development

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Abstract

Background: Stearoyl-ACP desaturases (SADs) and fatty acid desaturases (FADs) play a critical role in plant lipid metabolism and also affect oil fatty acid composition introducing double bonds into the hydrocarbon chains to produce unsaturated fatty acids. In the present study, the genomic sequences of three *SAD* and three *FAD* candidate genes were characterized in olive and their expression was evaluated in different plant tissues.

Methods and Results: *OeSAD* genes corresponded to olive *SAD1* and *SAD2* and to a newly identified *OeSAD4*, sharing the conserved protein structure with other plant species. On the other hand, the full-length genomic sequences of two microsomal *OeFAD* genes (*FAD2-1* and *FAD2-2*) and the plastidial *FAD6*, were released. When the level of expression was tested on different tissues of cv. Leccino, *OeSAD1* and *OeSAD2* were mainly expressed in the fruits, while *OeFAD* genes showed the lowest expression in this tissue. The mRNA profiling of all genes was directly studied in fruits of Leccino and Coratina cultivars during fruit development. In both genotypes, the expression level of *OeSAD1* and *OeSAD2* had the highest value during and after the pit-hardening period, when oil accumulation in fruit mesocarp is intensively increasing. Furthermore, the expression level of both *OeFAD2* genes, which were the main candidates for oleic acid desaturation, were almost negligible during fruit ripening.

Conclusions: These results have made possible to define candidate genes of the machinery regulation of fatty acid composition in olive oil, providing information on their sequence, gene structure and chromosomal location.

Keywords

Olive (*Olea europaea*, subsp. *europaea*, var. *europaea*), *SAD*, *FAD*, gene expression, RT qPCR, fruit mesocarp.

Key Message

Stearoyl-ACP desaturase, *OeSAD2*, and both fatty acid desaturases, *OeFAD2-1* and *2-2,* are the most important contributors for olive fatty acids content, defining the quantity of oleic acid by their expression.

Introduction

Olive (*Olea europaea* L.) is one of the most important fruit oil crops worldwide [1]. Traditionally grown in the Mediterranean countries, which still represent the main producers, olive oil consumption is steadily increasing worldwide because of its nutritional, organoleptic and therapeutic properties [2, 3]. Olive oil is mainly composed of triacylglycerols (TAGs) and it is mainly producing in the fruit mesocarp and also in the seed in minor quantity (about 5 % of the total oil content) [2]. The monounsaturated oleic acid (C18:1 Δ^{9}) is the most abundant fatty acid in olive oil, accounting for 55-80% of total lipids. The other fatty acids which are highly represented in the olive oil composition are: palmitic (16:0, 10-20%), linoleic (C18:2 $\Delta^{9,12}$, 3-20%), and at a lower quantity, the α–linolenic (C18:3, $\Delta^{9,12,15}$, <1.5%) acids.

Interest in quantity and/or quality of plant oils has encouraged different approaches, such as: conventional breeding, quantitative trait loci (QTL) mapping, metabolomics, transcriptomics and proteomics studies [4]. Regarding olive oil, the limitation of oil content per fruit (only up to 25% of fruit fresh weight) and the variability of oil quality among olive cultivars [5] make necessary a deeper understanding of the oil biosynthesis pathway in this species.

Unlike what happens with oilseeds, little information is available about the regulation of lipid biosynthesis in oil fruits. Olive fruit mesocarp represents a good model to study the regulation of plant lipid metabolism since it behaves as a photosynthetic tissue in the first stages of fruit development, and is involved in the biosynthesis of storage lipids (TAGs) during fruit ripening [2], and it is widely studied [6, 7, 8, 9, 10, 11].

Lately, studies performed in oleaster (*Olea europaea* var. *sylvestris*) have shown that probably the ideal fatty acid composition of olive oil, with high oleic acid and low linoleic acid levels, is due to several genome duplication events. Functional divergence, responsible for the typical proportions of fatty acid in the olive oil, is related to the appearance of different isoforms of SADs, specialized in high oleic acid production, as well as different paralogs of the *FAD2* gene, expressed at very low level in the fruit or repressed during fruit ripening [12]. A similar situation was described in oilseed crops, where several paralogs of *FAD2* gene have emerged probably because of genome duplication events (WGD) [13, 14]. Mutation studies carried out on soybean *FAD2-1* genes have helped to identify new beneficial alleles responsible for high oleic content in this species [14]. Furthermore, in oilseed crops the regulation of *FAD2* genes is important

since it can affect the composition of fatty acids in the oil and it has been reported that oleic and linoleic acid content can be modified genetically by silencing the FAD genes [13].

Up to now, the complete genome sequences of two olive cultivars, *O. europaea* cv. Leccino [15] and *O. europaea* cv. Farga [16], have been described. In addition, the olive genome of *Olea europaea* var. *sylvestris* have been reported [12]. In recent years, significant efforts have been made to identify the main genes responsible for the oleic and linoleic acid biosynthesis in olive oil in some cultivars (i.e., *SAD* and *FAD2* genes) [7, 9, 10, 11]. According to the expression levels of SAD genes, and their correlations with fatty acid concentrations, it seems that *SAD2* is the main gene that contributes to the oleic acid content in the olive fruit. This general finding has been shown in Picual, Arbequina, Hojiblanca, Picudo and Manzanilla cultivars [11]. On the other hand, different authors have suggested that the *FAD2-2* and *FAD2-5* genes could explain the low linoleic content in olive oil of some cultivars [7, 10, 17], and it has been confirmed that the *FAD2-2* gene expression level depends strongly on the olive genotype together with water regime, temperature and light [17].

The expression patterns of distinct *SAD* and *FAD* genes in olive cultivars could be helpful in order to clarify the relative contribution of the different genes to the composition of olive oils. In this paper, two of the most important olive cultivars grown in Central and South Italy, 'Leccino' and 'Coratina', were investigated. Leccino and Coratina cultivars present a well-balanced fatty acid composition, with high levels of oleic acid (71.9% and 74.5% of total lipids, respectively) and low content of linoleic acid (8.2% and 8%, respectively) based on the average of three different environments and two years of study, at the olive collection in Italy, Morocco and Spain [5]. *Olea europaea* cv. Leccino is considered a vigorous olive variety, with a developed trunk and the canopy broad. This cultivar is an early ripening variety and is very appreciated for its high and early productivity; the low resistance of its fruits to harvest is also very advantageous. On the other hand, ‘Coratina’ displays vigorous growth and has a low resistance to cold. It is a medium-late ripening variety. The quality of its oil presents exceptional properties, due mainly to high polyphenol and tocopherol contents. So, both cultivars represent attractive olive varieties to be analyzed [18]. Considering the strong environmental effect on the fatty acid composition together with the recent expansion of olive cultivation outside the Mediterranean Basin, notably in some countries of the southern hemisphere such as

Argentina, Chile, Perú and Australia, the oil chemical parameters of several olive cultivars often do not match those cultivated in the Mediterranean Basin [5, 19]. In this regard, the possible modulation of *SAD* and *FAD* genes due to the environmental factors could provide useful insights on adaptive changes of this crop in relation to oil synthesis and composition. Firstly, it will be necessary to verify, within *SAD* and *FAD* gene families, which genes are more active in the fruit and their expression levels during fruit development and ripening. Thereafter, it will be necessary to clarify the complete sequence and the chromosome localization of the *SAD* and *FAD* genes. A complete structure of the candidate genes could give more opportunity to study the linkage of polymorphisms to the oleic/linoleic content [20]. In this study, we focused on the characterization and validation of genes belonging to the *SAD* and *FAD* families providing, for the first time, the full length of all the studied genes and their location on the chromosomes, studying their expression levels in different tissues and during fruit development.

Materials and Methods

Plant material for RNA extraction

RNA from cv. Leccino tissues like: roots, fruits (mesocarp at 165 days after flowering, DAF), open flowers, young leaves, buds and cambium, was extracted for the expression analysis (Fig S1a). RNA from fruit mesocarp at five stages of development and ripening (referred to as 45, 75, 105, 135 and 165 DAF) was extracted from Leccino and Coratina cultivars (Fig S1b). All tissues were sampled and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction, according to procedures previously detailed [21]. These two cultivars were selected because they were recently studied for fatty acids composition in different olive collections from five countries: Italy, Spain, Morocco, Lebanon and Argentina [5]. In particular, in order to avoid possible environmental effects, the studied materials of cvs. Leccino and Coratina were collected at the same time points and same place. The sampling field was the Italian olive collection of Zagaria, at Enna city, located in the middle of Sicily Island. In this collection the average of oleic acid, calculated on three plants for each variety and two years, was 77% for Leccino and 81.9% for Coratina, while for linoleic acid the average was 4.3% for Leccino and 3.99% for Coratina [5].

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. To eliminate eventual DNA contamination, each sample was treated with DNase I (Ambion) and then tested by amplifying the Glyceraldehyde 3-phosphate dehydrogenase (*OeGAPDH*) as reference gene. The concentration, quality and purity of total RNA was assessed using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by checking the absorptions at 230, 260 and 280nm and the relative ratios A260/A280 for protein and A260/A230 for salts contamination, respectively. Furthermore, an agarose gel (1% standard agarose) was run for all extracted samples in order to monitor the integrity of RNA by controlling the intensity of the double bands and excluding a smearing below them. Single-strand cDNA was synthesized from 500 ng of total RNA using oligo $(dT)_{18}$ and SuperScript III Reverse Transcriptase (Thermo Fisher), as recommended by the supplier company. The amplification ability of cDNA was evaluated by PCR amplification of *OeGAPDH* gene.

Molecular characterization of *SAD* and *FAD* genes involved in fatty acid biosynthesis and desaturation in olive

To carry out the identification and molecular characterization of *FAD2-1, FAD2-2, FAD6, SAD1, SAD2* and *OeSAD4* candidate genes of fatty acid synthesis and desaturation pathways (Fig S2), partial or full-length mRNA sequences previously identified in olive and other related species, were used as queries in a BLAST search of the genome sequences of cv. Leccino (http://oleagenome.org/) [22]. Scaffolds containing sequences related to these genes were identified and aligned with the transcript sequences as predicted by FGENISH software (http://linux1.softberry.com/berry.phtml). Additionally, the BioEdit software (http://www.mbio.ncsu.edu/Bioedit/bioedit) was used to predict the number and length of exons and introns. The alignment of genomic sequences of the cv. Farga [16] with transcript sequences identified in olive fruits [23] enabled the identification of six genes and the definition of their gene structure, the genomic sequence, and the number and length of exons and introns. SAD and FAD amino acid sequences were predicted using the ExPASy translate tool (http://web.expasy.org/translate/). The protein homologs of other oleaginous plant species, including *Elaeis guineensis*, *Cocos nucifera*, *Arachis hypogea*, *Gossypium hirsutum*, *Glycine max*, *Brassica napus-campestris* and *-nigra*, *Helianthus annuus*, *Linum usitatissimum*, *Papaver somniferum*, *Camellia sativa*, *Carthamus tinctorius*, *Sesamum*

indicum, *Ricinus communis*, *Cannabis sativa* and *Zea mays*, were identified by BLASTP in the non-redundant protein sequences database of NCBI (www.ncbi.nlm.nih.gov). The detected sequences were aligned using BioEdit and then two Neighbor joining trees, one for FAD and another for SAD proteins, were built using MEGA7 [24], for all the identified *Olea europaea* genes and those from other species.

Expression analysis by RT-qPCR

The expression analyses of all six genes were performed by quantitative PCR on the reverse transcribed DNA (RT-qPCR) on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA), following manufacturer's instructions. Primers for the RT-qPCR experiments were designed using the program Primer3 version 4.0. Primer efficiency was initially verified by the presence of single PCR product bands after running on agarose gel electrophoresis. Reactions were performed on two biological and three technical replicates for each cDNA sample. Each reaction contained 3 µl of diluted cDNA (1:10), 0.3 µl of each primer (10 pmol/µl) and 5 µl of SYBR Green Master Mix reagent (Life Technologies) in a final volume of 10 µl. The following PCR program was used: 1 cycle at 50ºC for 2 min and 95ºC for 10 min; 40 cycles of 95ºC for 15 s and 60ºC for 1 min; and a final cycle of 95ºC for 15 s, 58ºC for 1 min and 95ºC for 15 s. After each assay, a dissociation kinetic analysis was performed to verify the specificity of the amplification products. Moreover, PCR efficiency was assessed by generating a standard curve for each gene with six dilution points, each one replicated four times. Only primer pairs that produced the expected amplicons and showed similar PCR efficiency were selected (Table 1). *OeGAPDH* and elongation factor (*EF 1α*) genes were used as references for sample normalization.

Statistical analysis

Relative amounts of each transcript were calculated using the $2^{-\Delta\Delta CT}$ method [25] using as calibrator the sample with the lowest expression, which correspond to the highest level of CT. Molecular determinations reported in this study were the average of triplicate measurements from two independent samples. Statistical differences were estimated from ANOVA test at the 5% level ($P \leq 0.05$) of significance for all data. Whenever ANOVA indicated a significant difference, a pair-wise comparison of means by least significant difference (Duncan) was carried out. All statistical analyses were performed using InfoStat software (InfoStat, 2014, http://www.infostat.com.ar).

Results

Molecular characterization of candidate genes related to fatty acid biosynthesis and desaturation: stearoyl-ACP and oleate desaturases

Three oleate desaturase genes (*OeFAD2-1*, *OeFAD2-2* and *OeFAD6*) and three stearoyl-ACP desaturase genes (*OeSAD1*, *OeSAD2* and *OeSAD4*) were characterized and full length gene sequences have been identified in the cv. Leccino (Table 2; Fig 1; Fig S2) and in the published cv. Farga scaffolds.

In both *OeFAD2* genes, the 5' UTR (UTR, Untranslated Region) was divided by a long intron. The analysis of the *OeFAD2-1* gene was carried out based on the mRNA sequences registered in the NCBI database with the codes AY733076 and HQ908422. The alignment of the mRNA sequences with those found in the olive genomes (Farga, wild and Leccino scaffolds) allowed us to generate the full length of Oe*FAD2-1* gene, with only one intron (2,051 bp) and one exon (1,146 bp), for a total gene length of 3,465 bp. The local BLAST search on the published wild olive genome allowed to detect the most identical sequence on chromosome 4 (submitted in NCBI as *FAD2*-like - XM_023019505.1).

The same approach was applied for the *OeFAD2-2* gene. The gene structure resulted identical to that of *OeFAD2-1*, with slight differences in intron length, for a total gene length of 3,687 bp. *OeFAD2-2* gene was located on chromosome 17 of the wild olive genome based on the best hit after blast analysis. Our *OeFAD2-2* corresponds to Farga scaffold Oe6 s00121. The exact sequence, but partial, of the *OeFAD2-1* gene was also found in different scaffolds of Farga and Leccino.

The analysis of plastidial *OeFAD6* gene was performed using three olive coding sequences (cds): AY772187 from cv. Koroneiki, AY733075 from cv. Picual and the partial cds HQ889831 from cv. Canino. The structure of *OeFAD6* was completely different from *OeFAD2* genes. In fact, its length was two times longer than *OeFAD2*, reaching 6,930 bp, and it was composed by ten little exons and nine introns. *OeFAD6* was unplaced in the wild genome, but the full-length gene was located in the Oe6 s07955 scaffold (plus-minus) of Farga genome. By using the recently published genetic map of olive derived from a progeny of Leccino x Dolce Agogia cultivars, it was possible to map this gene at chromosome 11 (Table 2).

The sequence analysis of *SAD* genes allowed the identification of sequences corresponding to three different genes: *OeSAD1*, *OeSAD2* and *OeSAD4*. All *SAD* genes

showed a similar structure, with three exons and two introns, for a total length varying between 4,063 bp and 4,148 bp for *OeSAD1* and *OeSAD2*, respectively, and 4,668 bp for *OeSAD4*, due to its longer 5'UTR region. Furthermore, the longest first exon of *OeSAD4* led to a mature protein nine amino acids longer than *OeSAD1* and *OeSAD2*. For *OeSAD1* gene identification, the complete coding sequence of cv. Picual (KX196198.1) was used to align with the scaffolds Oe6 s02255 and Oe6 s05960 of cv. Farga (the latter in reverse complement) and with the scaffolds of cv. Leccino genome, which corresponded to the *sylvestris* mRNA (XM_023030213.1), named as stearoyl-[acyl-carrier-protein] 9-desaturase (SACPD) and located on chromosome 10 in the oleaster genome.

Using the mRNA coding sequences of cvs. Leccino (U58141), Picual (KX196199), Mari (KP165083) and Shengeh (KP165084), it was possible to identify the *OeSAD2* gene on Leccino scaffolds as well as on the *O. europaea sylvestris* genome. In the wild olive genome, the first part of the gene including 5' UTR, first exon and first intron, was missing. Although, in cv. Leccino the total length of *OeSAD2* gene was equal to *OeSAD1*, the first intron was longer and the second was shorter in *OeSAD2*. It was possible to map this gene on the corresponding chromosome, 23, thanks to the recently published genetic map of Leccino x Dolce Agogia.

Finally, for the identification of the entire gene coding for *OeSAD4*, the cds sequence of cv. Picual (KX196200.1) and the mRNA of the wild genome (XM_023013435.1) allowed us to identify the entire gene in the Oe6 s09854 scaffold of cv. Farga genome and to locate it on chromosome 8 of the wild olive genome.

The Neighbor Joining tree for FAD proteins showed, as expected, two well separated clusters, the first included the microsomal forms and the second with the chloroplast form (Fig S3). In particular, our OeFAD2-2 formed a separated sub-cluster with the delta-12 oleate desaturase already submitted in the NCBI database, where three of them, belonging to the var. *sylvestris*, are called FAD2-like. Moreover, in the same sub-cluster other two FADs belonging to other oleaginous species were present, a FAD2 of *Sesamum indicum* (XP 011075145.1) and not so distant, a so called fatty acid desaturase of *Camellia oleifera* (AFK31315.1). The here studied OeFAD2-1 protein, was placed close to some forms of the *Olea* FAD2 already published and, for what concerns the other species, two forms of sesame and one of oil camellia were placed near to our OeFAD2-1. Regarding the chloroplastic OeFAD6, its placement was close to the chloroplast fatty acid desaturase 6 of previously studied and submitted proteins of *Olea* and, again, the oleaginous species

nearest to our cluster was *Sesamum indicum* and not so distant we found the cotton form of FAD6.

The Neighbor Joining tree for SAD proteins showed a cluster, which included both OeSAD1 and SAD2, even if they were clearly separated in two sub-clusters near to other five proteins of *Olea*'s SADs (Fig S4). The closest oleaginous species were sesame and camellia, and at a narrow distance the chloroplastic stearoyl- acyl-carrier-protein 9-desaturase of *Elaeis guineensis*. The OeSAD4, described for the first time in the present work had a strong similarity with only another stearoyl- acyl-carrier-protein 9-desaturase chloroplastic recently found in the *Olea europaea* var. *sylvestris*. This clade, which is placed a considerable distance with the other two forms, it includes only a *Camellia oleifera* protein (AIN52151.1).

Expression patterns of FAD and SAD genes in different organs of cv. Leccino plants

The relative expression levels (mRNAs) of *FAD* and *SAD* genes are reported in Figure 2.

The expression of *OeFAD2-1* gene resulted extraordinarily high in flowers, low in buds and almost negligible in all other tissues, whereas *OeFAD2-2* gene showed low expression levels in all the tissues, particularly in fruit mesocarp, similarly to *OeFAD6*. However, the expression of *OeFAD6* resulted higher in leaves, compared to *OeFAD2-2*.

Among the *OeSAD* genes, *OeSAD1* expression was low in all organs, but within them, a significantly higher level in the fruit mesocarp was observed. The *OeSAD2* showed the highest expression in fruit mesocarp (two and a half times more than *OeSAD1*), followed by flowers, while a very low expression levels were found for the other organs. *OeSAD4* displayed the maximum expression in the cambium, and minimal in roots and fruit mesocarp.

In order to evaluate which gene of every family had the maximum level of expression in each organ, the expression of the three *FAD* and *SAD* genes was also analyzed separately (Fig. S5).

Regarding *FAD* genes, all the three *FAD* genes had approximately the same level of expression in buds. The expression of *OeFAD2-1* was irrelevant, comparing to the other tissues, in fruits, leaves, roots and cambium, confirming its up-regulation only in flowers; *OeFAD2-2* showed a significantly higher level in leaves, but at meantime it was the most expressed gene in root among the *FAD* family. The plastidial *OeFAD6* showed the maximum level of expression in three out of six tissues, including fruits, leaves and cambium.

Among *SAD* genes, *OeSAD1* expression was highest in leaves and lowest in flowers, while *OeSAD2* was mainly expressed in fruit mesocarp and the minimum expression was in leaves. The *OeSAD2* expression in fruits compared to the other tissues is remarkably higher than for the other two *SAD* genes, ascertaining a clear induction in fruits of *OeSAD2*. *OeSAD4* was the lowest expressed gene in all cases including fruit mesocarp and, for this reason, it was not considered for further analysis related to the evaluation of expression level during the stages of fruit development.

Differential expression of *OeFAD* and *OeSAD* genes in Leccino and Coratina cultivars during fruit development and ripening

Figure 3 shows the relative expression levels of *FAD* and *SAD* genes during fruit ontogeny of the cultivars Leccino and Coratina.

OeSAD1 and *OeSAD2* showed similar patterns of expression in Leccino mesocarp tissues, with increasing expression levels from first to last stage of fruit development, not significant for *OeSAD1*. In cv. Coratina the expression pattern of both genes reached a peak at 135 DAF, followed by a significant decrease at the last stage.

A strong temporal regulation of the oleate desaturase *OeFAD2-1* gene expression was observed in both cultivars, with a sharp decrease from the beginning of fruit development (45 DAF) to the end of ripening (165 DAF), when transcript levels resulted almost negligible. The *OeFAD2-2* gene was poorly expressed in both cultivars with respect to *OeFAD2-1* gene. In cv. Leccino fruits, there were no significant changes along the developmental process, while in cv. Coratina, the maximum level of expression was detected at 45 DAF and then declined dramatically until the last stage, in which it increased slightly, supposedly when the linoleic content reaches the highest levels in the mesocarp. *OeFAD6* level of expression was low in both cultivars and without significant differences among all stages.

Discussion

The peculiarity of olive oil mainly derives from the balanced ratio between mono and polyunsaturated fatty acids and this balance is under a strong genetic and environmental control [5, 17, 19].

High oleic acid oil plants were obtained through the selection of low-active or silenced [26] FAD2 enzyme, in species such as *Brassica napus* [27], sunflower [28], or by induced mutagenesis of *FAD2-2* [29]. *FAD2* mutants have been detected in cottonseed, where the

coding sequence near the end of a mutant allele of *FAD2* appeared prematurely truncated [30]; meanwhile the high oleic acid trait in safflower is controlled by a partially recessive allele *ol* of a defective *FAD2-1* [31]. High oleic lines of rice have been obtained by disrupting the *OsFAD2-1* gene by CRISPR/Cas9-mediated targeted mutagenesis [32] and a recent review article clearly described the involvement of *FAD2* gene in the several oilseed crops [13]. Recent works made on *FAD2-like* genes found thorough deep analysis of EST databases [17] and genome sequences [12] have confirmed the involvement of *FAD2-2* gene or evidenced the participation of a new possible isoform such as *FAD2-5* in the synthesis and balancing of fatty acid composition in the olive mesocarp. Considering that olive is one of the few oil crops naturally producing high oleic acid oil, the involved gene machinery is not fully identified neither are the complete gene sequences or the chromosomes placement of the most important candidates.

In this work, we have provided genomic characterization of three *FAD* and three *SAD* genes putatively involved in this pathway, confirming or establishing their positions in the olive chromosomes. Moreover, their role was ascertained through expression analysis, in different olive organs and at successive fruit developmental stages by analyzing the mesocarp mRNA [33]. Genes under consideration include stearoyl-ACP desaturases (*OeSAD1*, *OeSAD2* and *OeSAD4*) and oleate desaturases (*OeFAD2-1*, *OeFAD2-2* and *OeFAD6*). The studied genes were selected among the others because their complete sequences (UTRs, introns and exons) were confirmed in the sequenced genomes of cultivated [16, 22] and wild (var. *sylvestris*) olives [12]. Moreover, by affirming [12] or identifying [34] their chromosome localization, further opportunities for genetic, genomic and breeding studies can be caught. In this context, thanks to the recently published olive genetic map developed on a controlled cross-progeny from cvs. Leccino and Dolce Agogia [34], two genes, which were not previously assigned to chromosomes, *OeSAD2* and *OeFAD6,* were placed in the 23rd and 11th chromosome respectively.

The *OeFAD2-2* gene corresponds to that of Hernández [6] and Salimonti [20] already published. This latter together with the copy of *OeFAD2-2* found in the cv. Farga genome evidenced the duplication of the unique exon as tandem repeat, features not found in other available olive genomes. In fact, Unver et al. [12] published the same sequence but using *FAD2*-like name (XM_022986507), whereas what they called *FAD2-2* is only a partial replica of *OeFAD2-1* only containing the exon and the 3'UTR part. Moreover, the chromosome's placement was also different between the two reported *FAD* genes. In

other oilseed crops such as flax, up to six *FAD2* gene copies clustered in the same linkage group [35]. The protein sequences of different *Olea* accessions previously submitted in the NCBI database clustered together with ours, showing some amino acid mismatches between different accessions. Moreover, previous published similarity trees showed that *Olea europaea* FADs and SADs clustered apart from most of other oleaginous species [36]. Our analyses demonstrated that all the six studied genes generated proteins whose amino acid sequences were evolutionary close to the *Sesamum indicum* and *Camellia oleifera* ones.

The *OeFAD2-1* and *OeFAD2-2* genes participated in microsomal linoleate synthesis while the *OeFAD6* gene in the plastidial one, and they showed the lowest expression levels in fruits comparing all tissues. These data are in agreement with those observed by Hernández [7]. In fact, his study revealed higher levels of expression of *FAD* genes in seeds, leaves and young drupes, which was correlating to the high content of linoleic acid in these tissues due to the need of plastidial membranes for photosynthesis. In our study, the low expression of both *OeFAD2* genes in fruit mesocarp is in accordance with other oilseed crops where no expression is related to the lack of conversion to linoleic acid from oleic acid [13].

Our *OeFAD2-1* gene could be considered fundamental at the beginning of fruit development in cvs. Leccino and Coratina, as already observed in Picual and Arbequina cultivars by Hernández [7], demonstrating that in all analyzed genotypes *FAD2-1* expression level did not follow the mesocarp linoleic acid synthesis pattern. A link between the up-regulation of *FAD* genes, particularly *FAD2*, and accumulation of linoleic acid has been demonstrated in several oilseed crops [13], such as peanuts [37], maize [38] and oil palm [39]. In the present study, the maximum expression level of *OeFAD2-1* was in flowers and when tested during fruit development the highest expression was found at 45 DAF, with a strong down-regulation from 75 to 165 DAF just after fruit set and throughout fruit ripening, suggesting that this gene is not directly involved in the mesocarp synthesis of linoleic acid which corresponds to what found in the olive oil. If we consider that the suppression of *FAD2* genes in seeds of high-polyunsaturated fatty acid oil crops inhibit an increase in polyunsaturated fatty acids [13, 40], our study confirms the high expression of *OeFAD* was only at the very early stages of fruit development, both in Leccino and Coratina cultivars, when the fruit is still growing and the mesocarp is photosynthetically active.

On the other hand, although its levels of expression are low in the fruit mesocarp of both analyzed cultivars, taking into account that linoleic acid content in the mature fruit is usually low (3-20% of total lipids), *OeFAD2-2* expression profiles were in agreement with the oil biosynthesis process, and therefore the linoleic acid accumulation, in both cultivars which have almost the same quantity of this fatty acid [5]. Our results are in agreement with previous publications in olive, which were performed during fruit developmental stages. In fact, the expression studies made on fruit mesocarp pointed out *FAD2-2* gene as main responsible for the synthesis of linoleic acid at latter fruit developmental stages, while *FAD2-1* was individuated mainly in seeds [7, 10]. A recent publication has confirmed the importance of *FAD2-2* gene both in the linoleic acid of olive seed and mesocarp. At the same time, in a recent publication other three *FAD2* genes were studied for the first time, and it was highlighted the importance of *FAD2-5* gene as a new candidate for linoleate synthesis in the mesocarp while *FAD2-3* and *FAD2-4* were not validated [17]. In the present work, we are facing a similar expression pattern of *OeFAD2-2* in Leccino and Coratina cultivars, which also according to the analyses carried out so far represent the best candidate for the production of linoleic acid content in the olive mesocarp.

Data of *SAD* gene expression obtained in oil crops showed conflicting patterns in oil-accumulating organs. In oil palm [41], the *SAD* gene expression in the mesocarp seems regulated by specific sequence motifs [42]. In flaxseed [43] a decrease in *SAD* gene expression level has been observed during seed development, while the opposite was found in peanut [44]. *OeSAD1* and *OeSAD2* genes showed high expression levels in olive fruit mesocarp. These findings are consistent with those obtained in olive fruits by Parvini [11]. Many evidences suggest that *SAD* genes are mainly regulated in the olive tissues that accumulate oil, and therefore oleic acid, and their level of expression increases simultaneously with the oil synthesis and accumulation [9]. Similar results have also been reported in oil-bearing tissues from other oil crop species, such as oil palm fruit mesocarp [39], cocoa [45], *Jatropha curcas* [46] and barley [47] seeds. Overall, our data reinforce previous observations on the role of *OeSAD1* and *OeSAD2* genes on the synthesis of unsaturated fatty acids in fruit mesocarp [11, 48] and, on the contrary, *OeSAD4* did not show any correlation with oleic acid composition as already observed for *SAD3* by Parvini [11]. In fact, *OeSAD4* showed very low expression in all studied tissues with the lowest level in the mesocarp, where the expression was seven hundred times lower than the

OeSAD2. The expression levels observed for *OeSAD1* and *OeSAD2* genes during fruit development showed low level of expression at the earliest fruit growth stages, followed by a sharp increasing pattern. As indicated by Parvini, *SAD2* gene expression was simultaneous with olive fruit ontogeny [10, 11]. In our work, statistically significant differences between the levels of expression was revealed only for *OeSAD2* in both cultivars, while in cv. Leccino the differences found for *OeSAD1* was not supported statistically. Moreover, the expression pattern of *OeSAD2* is paralleling oleic acid biosynthesis [10, 11, 45, 49] and its expression is significantly high in the mesocarp in comparison with other tissues and with other *SAD* genes, making it the main candidate for the desaturation of stearoyl-ACP to oleyl-ACP, among the genes studied here.

Regulation of fatty acid biosynthesis is a major concern in olive because it is directly related to the olive oils composition, palatability and therefore commercial value. As an example some hot regions of the world could not meet current trade standards of the International Olive Oil Council for virgin olive oil. The analyses of gene expression in different olive organs and during fruit development when oil is accumulating, confirmed *OeSAD2* gene as the main candidate gene for oleic acid synthesis and its expression is crucial to guarantee the very high level of this fatty acid in olive oil. At the same time, the low level of *OeFAD2* expression in fruits with consistent profile throughout the ripening stages of fruit development, in accordance with the process of oil accumulation, indicates that oleic acid cannot be actively converted into linoleic acid probably due to the poor *FAD2* activity. Thus, *OeSAD2* and *OeFAD2-2* are the most important contributors, together with OeFAD2-5 [12], for the determination of the peculiar and precious content of fatty acids in olive fruit mesocarp, which has the greatest quantity of oleic acid among all plant species. The gene structure of these candidate genes opens new scenarios directly applicable in the future breeding programs and for oil crop transformation. Furthermore, the chromosome placement of all studied genes could facilitate further studies based on QTL mapping.

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Author Contributions

CC, RM, MTo, DM, LG, MT and LB conceived the study. CC, RM, SM, CG, LR, NC and PP provided the plant material and performed the mRNA synthesis. CC, RM, SM, NC, CG, LR and MTo performed the molecular and bioinformatics analyses. CC, RM, SM, LB, CG, PP, DM, LG, MT and MTo wrote the first draft of the manuscript. CC, RM, SM, LB, CG, PP, DM, LG, MT and MTo contributed to the writing and revised the manuscript. All the authors agreed on the final version of this work.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics declarations

The present research did not involve Human Participants and/or Animals

Figure Legends

Figure 1. Gene structure of the six genes identified in the present study in the Leccino genome. Lengths of UTRs, exons and introns are reported.

Figure 2. Relative expression levels of all the six studied genes (*OeFAD2-1*, *OeFAD2*-2, *OeFAD6*, *OeSAD1*, *OeSAD2* and *OeSAD4*) in different tissues (fruit mesocarp 165 DAF, leaf, root, bud, cambium and flower) of cv. Leccino. Data are the average of two biological replicates and three technical replicates. Multiple comparison test was performed by Duncan test. Different letters correspond to significant level ($p<0.05$) of expression among olive tissues.

Figure 3. Relative expression levels of *OeFAD2-1*, *OeFAD2-2, OeFAD6, OeSAD1* and *OeSAD2*, from drupes of cvs. Leccino and Coratina at distinct stages of fruit development and ripening referred as days after flowering (DAF). Data are the average of two biological replicates and three technical replicates. Multiple comparison test was performed by Duncan test. Different letters correspond to significant level ($p<0.05$) of expression during fruit development.

Electronic Supplementary Material

Figure S1. a) Leccino cultivar tissues images from which RNA extraction was performed: roots, fruits (mesocarp at 165 DAF), flowers, young leaves, buds and cambium. b) Different stages of fruit development and ripening in cv. Leccino, referred as days after flowering (DAF).

Figure S2. Simplified scheme of the olive oil biosynthesis pathway showing the fatty acid formation in the plastid, and the triacylglycerol assembly through the Kennedy pathway in the ER. The key genes coloured in green were studied in the present work.

Figure S3. Distance tree of FAD proteins encoded by the three studied genes after NCBI database blasting. The analysis involved 139 FAD amino acid sequences. All ambiguous positions were removed for each sequence pair. The accession number and the full name for each entry were left as reported in the NCBI database. The FAD protein sequences related to *Olea europaea* were highlighted with different shades of green, full for the sequences found in this work and empty for those previously published. Evolutionary analyses were conducted in MEGA7. The history was inferred using the Neighbor-Joining method. The optimal trees, after a bootstrap analysis running 10,000 times, are shown and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

Figure S4. Distance tree of SAD proteins encoded by the three studied genes after NCBI database blasting. The analysis involved 107 SAD amino acid sequences. All ambiguous positions were removed for each sequence pair. The accession number and the full name for each entry were left as reported in the NCBI database. The protein sequences related to *Olea europaea* were highlighted with different shades of green, full for the sequences found in this work and empty for those previously published. Evolutionary analyses were conducted in MEGA7. The history was inferred using the Neighbor-Joining method. The optimal trees, after a bootstrap analysis running 10,000 times, are shown and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

Figure S5. Relative expression levels of different genes belonging to the *OeFAD* and *OeSAD* families in fruit mesocarp at 165 DAF, flower, leaf, root, bud and cambium of cv. Leccino. Data are the average of two biological replicates and three technical replicates. Multiple comparison test was performed by Duncan test. Different letters correspond to significant level ($p<0.05$) of expression in the same gene family.

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Table 1. Specific primers used for quantitative expression of six candidate transcripts of *Olea europaea SAD* and *FAD*.

Gene	Primers to amplify genomic sequence	Nucleotide sequence of primers (5' - 3')
OeFAD2-1	qRT-*OeFAD2-1* For	TCTGATGTTTGTGTTATCGCCAC
	qRT-*OeFAD2-1* Rev	AATCTCGATCCACAGTCGCC
OeFAD2-2	qRT-*OeFAD2-2* For	TTGGTTAAGGGGAGCGTTGG
	qRT-*OeFAD2-2* Rev	TGTCGCCTCCATTGCATGAT
OeFAD6	qRT-*OeFAD6* For	AAGTTGGAAAGTGGACCCCA
	qRT- *OeFAD6* Rev	GGTTCTCCAATTTGCCTAAAACC
OeSAD1	qRT-*OeSAD1* For	GATGGAGTCCGAGATGAGACG
	qRT-*OeSAD1* Rev	ACCATGCCTATTCTCTTCAGCT
OeSAD2	qRT-*OeSAD2* For	ACACCCTAGATGGAGTTCGAGA
	qRT-*OeSAD2* Rev	ATATTTGTTCAGAAGGTCACCATG
OeSAD4	qRT-*OeSAD4* For	GGAGAACTGGGCTGAGCAAA
	qRT-*OeSAD4* Rev	TGCCCCTGTTTCATCTTGCA

Table 2. Gene name, total sequence length in cv. Leccino, chromosome location on the genome of a wild olive [12], accession numbers (AN) as issued by the NCBI database.

Gene	Total length (bp)	Mapped on chromosome	Accession Number
OeFAD2-1	3,465	4	MN821526
OeFAD2-2	3,687	17	MN821527
OeFAD6	6,930	11	MN821528
OeSAD1	4,063	10	MN821531
OeSAD2	4,148	23	MN821529
OeSAD4	4,668	8	MN821530

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Characterization and validation of olive FAD and SAD gene families: expression analysis in different tissues and during fruit development

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Abstract

Background: Stearoyl-ACP desaturases (SADs) and fatty acid desaturases (FADs) play a critical role in plant lipid metabolism and also affect oil fatty acid composition introducing double bonds into the hydrocarbon chains to produce unsaturated fatty acids. In the present study, the genomic sequences of three *SAD* and three *FAD* candidate genes were characterized in olive and their expression was evaluated in different plant tissues.

Methods and Results: *OeSAD* genes corresponded to olive *SAD1* and *SAD2* and to a newly identified *OeSAD4*, sharing the conserved protein structure with other plant species. On the other hand, the full-length genomic sequences of two microsomal *OeFAD* genes (*FAD2-1* and *FAD2-2*) and the plastidial *FAD6*, were released. When the level of expression was tested on different tissues of cv. Leccino, *OeSAD1* and *OeSAD2* were mainly expressed in the fruits, while *OeFAD* genes showed the lowest expression in this tissue. The mRNA profiling of all genes was directly studied in fruits of Leccino and Coratina cultivars during fruit development. In both genotypes, the expression level of *OeSAD1* and *OeSAD2* had the highest value during and after the pit-hardening period, when oil accumulation in fruit mesocarp is intensively increasing. Furthermore, the expression level of both *OeFAD2* genes, which were the main candidates for oleic acid desaturation, were almost negligible during fruit ripening.

Conclusions: These results have made possible to define candidate genes of the machinery regulation of fatty acid composition in olive oil, providing information on their sequence, gene structure and chromosomal location.

Keywords

Olive (*Olea europaea*, subsp. *europaea*, var. *europaea*), *SAD*, *FAD*, gene expression, RT qPCR, fruit mesocarp.

Key Message

Stearoyl-ACP desaturase, *OeSAD2*, and both fatty acid desaturases, *OeFAD2-1* and *2-2,* are the most important contributors for olive fatty acids content, defining the quantity of oleic acid by their expression.

Introduction

Olive (*Olea europaea* L.) is one of the most important fruit oil crops worldwide [1]. Traditionally grown in the Mediterranean countries, which still represent the main producers, olive oil consumption is steadily increasing worldwide because of its nutritional, organoleptic and therapeutic properties [2, 3]. Olive oil is mainly composed of triacylglycerols (TAGs) and it is mainly producing in the fruit mesocarp and also in the seed in minor quantity (about 5 % of the total oil content) [2]. The monounsaturated oleic acid (C18:1 Δ^9) is the most abundant fatty acid in olive oil, accounting for 55-80% of total lipids. The other fatty acids which are highly represented in the olive oil composition are: palmitic (16:0, 10-20%), linoleic (C18:2 $\Delta^{9,12}$, 3-20%), and at a lower quantity, the α–linolenic (C18:3, $\Delta^{9,12,15}$, <1.5%) acids.

Interest in quantity and/or quality of plant oils has encouraged different approaches, such as: conventional breeding, quantitative trait loci (QTL) mapping, metabolomics, transcriptomics and proteomics studies [4]. Regarding olive oil, the limitation of oil content per fruit (only up to 25% of fruit fresh weight) and the variability of oil quality among olive cultivars [5] make necessary a deeper understanding of the oil biosynthesis pathway in this species.

Unlike what happens with oilseeds, little information is available about the regulation of lipid biosynthesis in oil fruits. Olive fruit mesocarp represents a good model to study the regulation of plant lipid metabolism since it behaves as a photosynthetic tissue in the first stages of fruit development, and is involved in the biosynthesis of storage lipids (TAGs) during fruit ripening [2], and it is widely studied [6, 7, 8, 9, 10, 11].

Lately, studies performed in oleaster (*Olea europaea* var. *sylvestris*) have shown that probably the ideal fatty acid composition of olive oil, with high oleic acid and low linoleic acid levels, is due to several genome duplication events. Functional divergence, responsible for the typical proportions of fatty acid in the olive oil, is related to the appearance of different isoforms of SADs, specialized in high oleic acid production, as well as different paralogs of the *FAD2* gene, expressed at very low level in the fruit or repressed during fruit ripening [12]. A similar situation was described in oilseed crops, where several paralogs of *FAD2* gene have emerged probably because of genome duplication events (WGD) [13, 14]. Mutation studies carried out on soybean *FAD2-1* genes have helped to identify new beneficial alleles responsible for high oleic content in this species [14]. Furthermore, in oilseed crops the regulation of *FAD2* genes is important

since it can affect the composition of fatty acids in the oil and it has been reported that oleic and linoleic acid content can be modified genetically by silencing the FAD genes [13].

Up to now, the complete genome sequences of two olive cultivars, *O. europaea* cv. Leccino [15] and *O. europaea* cv. Farga [16], have been reporteddescribed. In addition, the olive genome of *Olea europaea* var. *sylvestris* have been reported [12]. In recent years, significant efforts have been made to identify the main genes responsible for the oleic and linoleic acid biosynthesis in olive oil in some cultivars (i.e., *SAD* and *FAD2* genes) [7, 9, 10, 11]. According to the expression levels of SAD genes, and their correlations with fatty acid concentrations, it seems that *SAD2* is the main gene that contributes to the oleic acid content in the olive fruit. This general finding has been shown in Picual, Arbequina, Hojiblanca, Picudo and Manzanilla cultivars [11]. On the other hand, different authors have suggested that the *FAD2-2* and *FAD2-5* genes could explain the low linoleic content in olive oil of some cultivars [7, 10, 17], and it has been confirmed that the *FAD2-2* gene expression level depends strongly on the olive genotype together with water regime, temperature and light [17].

The expression patterns of distinct *SAD* and *FAD* genes in olive cultivars could be helpful in order to clarify the relative contribution of the different genes to the composition of olive oils. In this paper, two of the most important olive cultivars grown in Central and South Italy, 'Leccino' and 'Coratina', were investigated. Leccino and Coratina cultivars present a well-balanced fatty acid composition, with high levels of oleic acid (71.9% and 74.5% of total lipids, respectively) and low content of linoleic acid (8.2% and 8%, respectively) based on the average of three different environments and two years of study, at the olive collection in Italy, Morocco and Spain [5]. *Olea europaea* cv. Leccino is considered a vigorous olive variety, with a developed trunk and the canopy broad. This cultivar is an early ripening variety and is very appreciated for its high and early productivity; the low resistance of its fruits to harvest is also very advantageous. On the other hand, 'Coratina' displays vigorous growth and has a low resistance to cold. It is a medium-late ripening variety. The quality of its oil presents exceptional properties, due mainly to high polyphenol and tocopherol contents. So, both cultivars represent attractive olive varieties to be analyzed [18]. Considering the strong environmental effect on the fatty acid composition together with the recent expansion of olive cultivation outside the Mediterranean Basin, notably in some countries of the southern hemisphere such as

Argentina, Chile, Perú and Australia, the oil chemical parameters of several olive cultivars often do not match those cultivated in the Mediterranean Basin [5, 19]. In this regard, the possible modulation of *SAD* and *FAD* genes due to the environmental factors could provide useful insights on adaptive changes of this crop in relation to oil synthesis and composition. Firstly, it will be necessary to verify, within *SAD* and *FAD* gene families, which genes are more active in the fruit and their expression levels during fruit development and ripening. Thereafter, it will be necessary to clarify the complete sequence and the chromosome localization of the *SAD* and *FAD* genes. A complete structure of the candidate genes could give more opportunity to study the linkage of polymorphisms to the oleic/linoleic content [20]. In this study, we focused on the characterization and validation of genes belonging to the *SAD* and *FAD* families providing, for the first time, the full length of all the studied genes and their location on the chromosomes, studying their expression levels in different tissues and during fruit development.

Materials and Methods

Plant material for RNA extraction

RNA from cv. Leccino tissues like: roots, fruits (mesocarp at 165 days after flowering, DAF), open flowers, young leaves, buds and cambium, was extracted for the expression analysis (Fig S1a). RNA from fruit mesocarp at five stages of development and ripening (referred to as 45, 75, 105, 135 and 165 DAF) was extracted from Leccino and Coratina cultivars (Fig S1b). All tissues were sampled and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction, according to procedures previously detailed [21]. These two cultivars were selected because they were recently studied for fatty acids composition in different olive collections from five countries: Italy, Spain, Morocco, Lebanon and Argentina [5]. In particular, in order to avoid possible environmental effects, the studied materials of cvs. Leccino and Coratina were collected at the same time points and same place. The sampling field was the Italian olive collection of Zagaria, at Enna city, located in the middle of Sicily Island. In this collection the average of oleic acid, calculated on three plants for each variety and two years, was 77% for Leccino and 81.9% for Coratina, while for linoleic acid the average was 4.3% for Leccino and 3.99% for Coratina [5].

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. To eliminate eventual DNA contamination, each sample was treated with DNase I (Ambion) and then tested by amplifying the Glyceraldehyde 3-phosphate dehydrogenase (*OeGAPDH*) as reference gene. The concentration, quality and purity of total RNA was assessed using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), by checking the absorptions at 230, 260 and 280nm and the relative ratios A260/A280 for protein and A260/A230 for salts contamination, respectively. Furthermore, an agarose gel (1% standard agarose) was run for all extracted samples in order to monitor the integrity of RNA by controlling the intensity of the double bands and excluding a smearing below them. Single-strand cDNA was synthesized from 500 ng of total RNA using oligo $(dT)_{18}$ and SuperScript III Reverse Transcriptase (Thermo Fisher), as recommended by the supplier company. The amplification ability of cDNA was evaluated by PCR amplification of *OeGAPDH* gene.

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Molecular characterization of *SAD* and *FAD* genes involved in fatty acid biosynthesis and desaturation in olive

To carry out the identification and molecular characterization of *FAD2-1, FAD2-2, FAD6, SAD1, SAD2* and *OeSAD4* candidate genes of fatty acid synthesis and desaturation pathways (Fig S2), partial or full-length mRNA sequences previously identified in olive and other related species, were used as queries in a BLAST search of the genome sequences of cv. Leccino (http://oleagenome.org/) [22]. Scaffolds containing sequences related to these genes were identified and aligned with the transcript sequences as predicted by FGENISH software (http://linux1.softberry.com/berry.phtml). Additionally, the BioEdit software (http://www.mbio.ncsu.edu/Bioedit/bioedit) was used to predict the number and length of exons and introns. The alignment of genomic sequences of the cv. Farga [16] with transcript sequences identified in olive fruits [23] enabled the identification of six genes and the definition of their gene structure, the genomic sequence, and the number and length of exons and introns. SAD and FAD amino acid sequences were predicted using the ExPASy translate tool (http://web.expasy.org/translate/). The protein homologs of other oleaginous plant species, including *Elaeis guineensis*, *Cocos nucifera*, *Arachis hypogea*, *Gossypium hirsutum*, *Glycine max*, *Brassica napus-campestris* and *-nigra*, *Helianthus annuus*, *Linum usitatissimum*, *Papaver somniferum*, *Camellia sativa*, *Carthamus tinctorius*, *Sesamum*

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indicum, *Ricinus communis*, *Cannabis sativa* and *Zea mays*, were identified by BLASTP in the non-redundant protein sequences database of NCBI (www.ncbi.nlm.nih.gov). The detected sequences were aligned using BioEdit and then two Neighbor joining trees, one for FAD and another for SAD proteins, were built using MEGA7 [24], for all the identified *Olea europaea* genes and those from other species.

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Expression analysis by RT-qPCR

The expression analyses of all six genes were performed by quantitative PCR on the reverse transcribed DNA (RT-qPCR) on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA), following manufacturer's instructions. Primers for the RT-qPCR experiments were designed using the program Primer3 version 4.0. Primer efficiency was initially verified by the presence of single PCR product bands after running on agarose gel electrophoresis. Reactions were performed on two biological and three technical replicates for each cDNA sample. Each reaction contained 3 µl of diluted cDNA (1:10), 0.3 µl of each primer (10 pmol/µl) and 5 µl of SYBR Green Master Mix reagent (Life Technologies) in a final volume of 10 µl. The following PCR program was used: 1 cycle at 50ºC for 2 min and 95ºC for 10 min; 40 cycles of 95ºC for 15 s and 60ºC for 1 min; and a final cycle of 95ºC for 15 s, 58ºC for 1 min and 95ºC for 15 s. After each assay, a dissociation kinetic analysis was performed to verify the specificity of the amplification products. Moreover, PCR efficiency was assessed by generating a standard curve for each gene with six dilution points, each one replicated four times. Only primer pairs that produced the expected amplicons and showed similar PCR efficiency were selected (Table 1). *OeGAPDH* and elongation factor (*EF 1α*) genes were used as references for sample normalization.

Statistical analysis

Relative amounts of each transcript were calculated using the $2^{-\Delta\Delta CT}$ method [25] using as calibrator the sample with the lowest expression, which correspond to the highest level of CT. Molecular determinations reported in this study were the average of triplicate measurements from two independent samples. Statistical differences were estimated from ANOVA test at the 5% level ($P \leq 0.05$) of significance for all data. Whenever ANOVA indicated a significant difference, a pair-wise comparison of means by least significant difference (Duncan) was carried out. All statistical analyses were performed using InfoStat software (InfoStat, 2014, http://www.infostat.com.ar).

Results

Molecular characterization of candidate genes related to fatty acid biosynthesis and desaturation: stearoyl-ACP and oleate desaturases

Three oleate desaturase genes (*OeFAD2-1*, *OeFAD2-2* and *OeFAD6*) and three stearoyl-ACP desaturase genes (*OeSAD1*, *OeSAD2* and *OeSAD4*) were characterized and full length gene sequences have been identified in the cv. Leccino (Table 2; Fig 1; Fig S2) and in the published cv. Farga scaffolds.

In both *OeFAD2* genes, the 5' UTR (UTR, Untranslated Region) was divided by a long intron. The analysis of the *OeFAD2-1* gene was carried out based on the mRNA sequences registered in the NCBI database with the codes AY733076 and HQ908422. The alignment of the mRNA sequences with those found in the olive genomes (Farga, wild and Leccino scaffolds) allowed us to generate the full length of Oe*FAD2-1* gene, with only one intron (2,051 bp) and one exon (1,146 bp), for a total gene length of 3,465 bp. The local BLAST search on the published wild olive genome allowed to detect the most identical sequence on chromosome 4 (submitted in NCBI as *FAD2*-like - XM_023019505.1).

The same approach was applied for the *OeFAD2-2* gene. The gene structure resulted identical to that of *OeFAD2-1*, with slight differences in intron length, for a total gene length of 3,687 bp. *OeFAD2-2* gene was located on chromosome 17 of the wild olive genome based on the best hit after blast analysis. Our *OeFAD2-2* corresponds to Farga scaffold Oe6 s00121. The exact sequence, but partial, of the *OeFAD2-1* gene was also found in different scaffolds of Farga and Leccino.

The analysis of plastidial *OeFAD6* gene was performed using three olive coding sequences (cds): AY772187 from cv. Koroneiki, AY733075 from cv. Picual and the partial cds HQ889831 from cv. Canino. The structure of *OeFAD6* was completely different from *OeFAD2* genes. In fact, its length was two times longer than *OeFAD2*, reaching 6,930 bp, and it was composed by ten little exons and nine introns. *OeFAD6* was unplaced in the wild genome, but the full-length gene was located in the Oe6 s07955 scaffold (plus-minus) of Farga genome. By using the recently published genetic map of olive derived from a progeny of Leccino x Dolce Agogia cultivars, it was possible to map this gene at chromosome 11 (Table 2).

The sequence analysis of *SAD* genes allowed the identification of sequences corresponding to three different genes: *OeSAD1*, *OeSAD2* and *OeSAD4*. All *SAD* genes

showed a similar structure, with three exons and two introns, for a total length varying between 4,063 bp and 4,148 bp for *OeSAD1* and *OeSAD2*, respectively, and 4,668 bp for *OeSAD4*, due to its longer 5'UTR region. Furthermore, the longest first exon of *OeSAD4* led to a mature protein nine amino acids longer than *OeSAD1* and *OeSAD2*. For *OeSAD1* gene identification, the complete coding sequence of cv. Picual (KX196198.1) was used to align with the scaffolds Oe6 s02255 and Oe6 s05960 of cv. Farga (the latter in reverse complement) and with the scaffolds of cv. Leccino genome, which corresponded to the *sylvestris* mRNA (XM_023030213.1), named as stearoyl-[acyl-carrier-protein] 9-desaturase (SACPD) and located on chromosome 10 in the oleaster genome.

Using the mRNA coding sequences of cvs. Leccino (U58141), Picual (KX196199), Mari (KP165083) and Shengeh (KP165084), it was possible to identify the *OeSAD2* gene on Leccino scaffolds as well as on the *O. europaea sylvestris* genome. In the wild olive genome, the first part of the gene including 5' UTR, first exon and first intron, was missing. Although, in cv. Leccino the total length of *OeSAD2* gene was equal to *OeSAD1*, the first intron was longer and the second was shorter in *OeSAD2*. It was possible to map this gene on the corresponding chromosome, 23, thanks to the recently published genetic map of Leccino x Dolce Agogia.

Finally, for the identification of the entire gene coding for *OeSAD4*, the cds sequence of cv. Picual (KX196200.1) and the mRNA of the wild genome (XM_023013435.1) allowed us to identify the entire gene in the Oe6 s09854 scaffold of cv. Farga genome and to locate it on chromosome 8 of the wild olive genome.

The Neighbor Joining tree for FAD proteins showed, as expected, two well separated clusters, the first included the microsomal forms and the second with the chloroplast form (Fig S3). In particular, our OeFAD2-2 formed a separated sub-cluster with the delta-12 oleate desaturase already submitted in the NCBI database, where three of them, belonging to the var. *sylvestris*, are called FAD2-like. Moreover, in the same sub-cluster other two FADs belonging to other oleaginous species were present, a FAD2 of *Sesamum indicum* (XP 011075145.1) and not so distant, a so called fatty acid desaturase of *Camellia oleifera* (AFK31315.1). The here studied OeFAD2-1 protein, was placed close to some forms of the *Olea* FAD2 already published and, for what concerns the other species, two forms of sesame and one of oil camellia were placed near to our OeFAD2-1. Regarding the chloroplastic OeFAD6, its placement was close to the chloroplast fatty acid desaturase 6 of previously studied and submitted proteins of *Olea* and, again, the oleaginous species

nearest to our cluster was *Sesamum indicum* and not so distant we found the cotton form of FAD6.

The Neighbor Joining tree for SAD proteins showed a cluster, which included both OeSAD1 and SAD2, even if they were clearly separated in two sub-clusters near to other five proteins of *Olea*'s SADs (Fig S4). The closest oleaginous species were sesame and camellia, and at a narrow distance the chloroplastic stearoyl- acyl-carrier-protein 9-desaturase of *Elaeis guineensis*. The OeSAD4, described for the first time in the present work had a strong similarity with only another stearoyl- acyl-carrier-protein 9-desaturase chloroplastic recently found in the *Olea europaea* var. *sylvestris*. This clade, which is placed a considerable distance with the other two forms, it includes only a *Camellia oleifera* protein (AIN52151.1).

Expression patterns of FAD and SAD genes in different organs of cv. Leccino plants

The relative expression levels (mRNAs) of *FAD* and *SAD* genes are reported in Figure 2.

The expression of *OeFAD2-1* gene resulted extraordinarily high in flowers, low in buds and almost negligible in all other tissues, whereas *OeFAD2-2* gene showed low expression levels in all the tissues, particularly in fruit mesocarp, similarly to *OeFAD6*. However, the expression of *OeFAD6* resulted higher in leaves, compared to *OeFAD2-2*.

Among the *OeSAD* genes, *OeSAD1* expression was low in all organs, but within them, a significantly higher level in the fruit mesocarp was observed. The *OeSAD2* showed the highest expression in fruit mesocarp (two and a half times more than *OeSAD1*), followed by flowers, while a very low expression levels were found for the other organs. *OeSAD4* displayed the maximum expression in the cambium, and minimal in roots and fruit mesocarp.

In order to evaluate which gene of every family had the maximum level of expression in each organ, the expression of the three *FAD* and *SAD* genes was also analyzed separately (Fig. S5).

Regarding *FAD* genes, all the three *FAD* genes had approximately the same level of expression in buds. The expression of *OeFAD2-1* was irrelevant, comparing to the other tissues, in fruits, leaves, roots and cambium, confirming its up-regulation only in flowers; *OeFAD2-2* showed a significantly higher level in leaves, but at meantime it was the most expressed gene in root among the *FAD* family. The plastidial *OeFAD6* showed the maximum level of expression in three out of six tissues, including fruits, leaves and cambium.

Among *SAD* genes, *OeSAD1* expression was highest in leaves and lowest in flowers, while *OeSAD2* was mainly expressed in fruit mesocarp and the minimum expression was in leaves. The *OeSAD2* expression in fruits compared to the other tissues is remarkably higher than for the other two *SAD* genes, ascertaining a clear induction in fruits of *OeSAD2*. *OeSAD4* was the lowest expressed gene in all cases including fruit mesocarp and, for this reason, it was not considered for further analysis related to the evaluation of expression level during the stages of fruit development.

Differential expression of *OeFAD* and *OeSAD* genes in Leccino and Coratina cultivars during fruit development and ripening

Figure 34 shows the relative expression levels of *FAD* and *SAD* genes during fruit ontogeny of the cultivars Leccino and Coratina.

OeSAD1 and *OeSAD2* showed similar patterns of expression in Leccino mesocarp tissues, with increasing expression levels from first to last stage of fruit development, not significant for *OeSAD1*. In cv. Coratina the expression pattern of both genes reached a peak at 135 DAF, followed by a significant decrease at the last stage.

A strong temporal regulation of the oleate desaturase *OeFAD2-1* gene expression was observed in both cultivars, with a sharp decrease from the beginning of fruit development (45 DAF) to the end of ripening (165 DAF), when transcript levels resulted almost negligible. The *OeFAD2-2* gene was poorly expressed in both cultivars with respect to *OeFAD2-1* gene. In cv. Leccino fruits, there were no significant changes along the developmental process, while in cv. Coratina, the maximum level of expression was detected at 45 DAF and then declined dramatically until the last stage, in which it increased slightly, supposedly when the linoleic content reaches the highest levels in the mesocarp. *OeFAD6* level of expression was low in both cultivars and without significant differences among all stages.

Discussion

The peculiarity of olive oil mainly derives from the balanced ratio between mono and polyunsaturated fatty acids and this balance is under a strong genetic and environmental control [5, 17, 19].

High oleic acid oil plants were obtained through the selection of low-active or silenced [26] FAD2 enzyme, in species such as *Brassica napus* [27], sunflower [28], or by induced mutagenesis of *FAD2-2* [29]. *FAD2* mutants have been detected in cottonseed, where the

coding sequence near the end of a mutant allele of *FAD2* appeared prematurely truncated [30]; meanwhile the high oleic acid trait in safflower is controlled by a partially recessive allele *ol* of a defective *FAD2-1* [31]. High oleic lines of rice have been obtained by disrupting the *OsFAD2-1* gene by CRISPR/Cas9-mediated targeted mutagenesis [32] and a recent review article clearly described the involvement of *FAD2* gene in the several oilseed crops [13]. Recent works made on *FAD2-like* genes found thorough deep analysis of EST databases [17] and genome sequences [12] have confirmed the involvement of *FAD2-2* gene or evidenced the participation of a new possible isoform such as *FAD2-5* in the synthesis and balancing of fatty acid composition in the olive mesocarp. Considering that olive is one of the few oil crops naturally producing high oleic acid oil, the involved gene machinery is not fully identified neither are the complete gene sequences or the chromosomes placement of the most important candidates.

In this work, we have provided genomic characterization of three *FAD* and three *SAD* genes putatively involved in this pathway, confirming or establishing their positions in the olive chromosomes. Moreover, their role was ascertained through expression analysis, in different olive organs and at successive fruit developmental stages by analyzing the mesocarp mRNA [33]. Genes under consideration include stearoyl-ACP desaturases (*OeSAD1*, *OeSAD2* and *OeSAD4*) and oleate desaturases (*OeFAD2-1*, *OeFAD2-2* and *OeFAD6*). The studied genes were selected among the others because their complete sequences (UTRs, introns and exons) were confirmed in the sequenced genomes of cultivated [16, 22] and wild (var. *sylvestris*) olives [12]. Moreover, by affirming [12] or identifying [34] their chromosome localization, further opportunities for genetic, genomic and breeding studies can be caught. In this context, thanks to the recently published olive genetic map developed on a controlled cross-progeny from cvs. Leccino and Dolce Agogia [34], two genes, which were not previously assigned to chromosomes, *OeSAD2* and *OeFAD6,* were placed in the 23rd and 11th chromosome respectively.

The *OeFAD2-2* gene corresponds to that of Hernández [6] and Salimonti [20] already published. This latter together with the copy of *OeFAD2-2* found in the cv. Farga genome evidenced the duplication of the unique exon as tandem repeat, features not found in other available olive genomes. In fact, Unver et al. [12] published the same sequence but using *FAD2*-like name (XM_022986507), whereas what they called *FAD2-2* is only a partial replica of *OeFAD2-1* only containing the exon and the 3'UTR part. Moreover, the chromosome's placement was also different between the two reported *FAD* genes. In

other oilseed crops such as flax, up to six *FAD2* gene copies clustered in the same linkage group [35]. The protein sequences of different *Olea* accessions previously submitted in the NCBI database clustered together with ours, showing some amino acid mismatches between different accessions. Moreover, previous published similarity trees showed that *Olea europaea* FADs and SADs clustered apart from most of other oleaginous species [36]. Our analyses demonstrated that all the six studied genes generated proteins whose amino acid sequences were evolutionary close to the *Sesamum indicum* and *Camellia oleifera* ones.

The *OeFAD2-1* and *OeFAD2-2* genes participated in microsomal linoleate synthesis while the *OeFAD6* gene in the plastidial one, and they showed the lowest expression levels in fruits comparing all tissues. These data are in agreement with those observed by Hernández [7]. In fact, his study revealed higher levels of expression of *FAD* genes in seeds, leaves and young drupes, which was correlating to the high content of linoleic acid in these tissues due to the need of plastidial membranes for photosynthesis. In our study, the low expression of both *OeFAD2* genes in fruit mesocarp is in accordance with other oilseed crops where no expression is related to the lack of conversion to linoleic acid from oleic acid [13].

Our *OeFAD2-1* gene could be considered fundamental at the beginning of fruit development in cvs. Leccino and Coratina, as already observed in Picual and Arbequina cultivars by Hernández [7], demonstrating that in all analyzed genotypes *FAD2-1* expression level did not follow the mesocarp linoleic acid synthesis pattern. A link between the up-regulation of *FAD* genes, particularly *FAD2*, and accumulation of linoleic acid has been demonstrated in several oilseed crops [13], such as peanuts [37], maize [38] and oil palm [39]. In the present study, the maximum expression level of *OeFAD2-1* was in flowers and when tested during fruit development the highest expression was found at 45 DAF, with a strong down-regulation from 75 to 165 DAF just after fruit set and throughout fruit ripening, suggesting that this gene is not directly involved in the mesocarp synthesis of linoleic acid which corresponds to what found in the olive oil. If we consider that the suppression of *FAD2* genes in seeds of high-polyunsaturated fatty acid oil crops inhibit an increase in polyunsaturated fatty acids [13, 40], our study confirms the high expression of *OeFAD* was only at the very early stages of fruit development, both in Leccino and Coratina cultivars, when the fruit is still growing and the mesocarp is photosynthetically active.

On the other hand, although its levels of expression are low in the fruit mesocarp of both analyzed cultivars, taking into account that linoleic acid content in the mature fruit is usually low (3-20% of total lipids), *OeFAD2-2* expression profiles were in agreement with the oil biosynthesis process, and therefore the linoleic acid accumulation, in both cultivars which have almost the same quantity of this fatty acid [5]. Our results are in agreement with previous publications in olive, which were performed during fruit developmental stages. In fact, the expression studies made on fruit mesocarp pointed out *FAD2-2* gene as main responsible for the synthesis of linoleic acid at latter fruit developmental stages, while *FAD2-1* was individuated mainly in seeds [7, 10]. A recent publication has confirmed the importance of *FAD2-2* gene both in the linoleic acid of olive seed and mesocarp. At the same time, in a recent publication other three *FAD2* genes were studied for the first time, and it was highlighted the importance of *FAD2-5* gene as a new candidate for linoleate synthesis in the mesocarp while *FAD2-3* and *FAD2-4* were not validated [17]. In the present work, we are facing a similar expression pattern of *OeFAD2-2* in Leccino and Coratina cultivars, which also according to the analyses carried out so far represent the best candidate for the production of linoleic acid content in the olive mesocarp.

Data of *SAD* gene expression obtained in oil crops showed conflicting patterns in oil-accumulating organs. In oil palm [41], the *SAD* gene expression in the mesocarp seems regulated by specific sequence motifs [42]. In flaxseed [43] a decrease in *SAD* gene expression level has been observed during seed development, while the opposite was found in peanut [44]. *OeSAD1* and *OeSAD2* genes showed high expression levels in olive fruit mesocarp. These findings are consistent with those obtained in olive fruits by Parvini [11]. Many evidences suggest that *SAD* genes are mainly regulated in the olive tissues that accumulate oil, and therefore oleic acid, and their level of expression increases simultaneously with the oil synthesis and accumulation [9]. Similar results have also been reported in oil-bearing tissues from other oil crop species, such as oil palm fruit mesocarp [39], cocoa [45], *Jatropha curcas* [46] and barley [47] seeds. Overall, our data reinforce previous observations on the role of *OeSAD1* and *OeSAD2* genes on the synthesis of unsaturated fatty acids in fruit mesocarp [11, 48] and, on the contrary, *OeSAD4* did not show any correlation with oleic acid composition as already observed for *SAD3* by Parvini [11]. In fact, *OeSAD4* showed very low expression in all studied tissues with the lowest level in the mesocarp, where the expression was seven hundred times lower than the

OeSAD2. The expression levels observed for *OeSAD1* and *OeSAD2* genes during fruit development showed low level of expression at the earliest fruit growth stages, followed by a sharp increasing pattern. As indicated by Parvini, *SAD2* gene expression was simultaneous with olive fruit ontogeny [10, 11]. In our work, statistically significant differences between the levels of expression was revealed only for *OeSAD2* in both cultivars, while in cv. Leccino the differences found for *OeSAD1* was not supported statistically. Moreover, the expression pattern of *OeSAD2* is paralleling oleic acid biosynthesis [10, 11, 45, 49] and its expression is significantly high in the mesocarp in comparison with other tissues and with other *SAD* genes, making it the main candidate for the desaturation of stearoyl-ACP to oleyl-ACP, among the genes studied here.

Regulation of fatty acid biosynthesis is a major concern in olive because it is directly related to the olive oils composition, palatability and therefore commercial value. As an example some hot regions of the world could not meet current trade standards of the International Olive Oil Council for virgin olive oil. The analyses of gene expression in different olive organs and during fruit development when oil is accumulating, confirmed *OeSAD2* gene as the main candidate gene for oleic acid synthesis and its expression is crucial to guarantee the very high level of this fatty acid in olive oil. At the same time, the low level of *OeFAD2* expression in fruits with consistent profile throughout the ripening stages of fruit development, in accordance with the process of oil accumulation, indicates that oleic acid cannot be actively converted into linoleic acid probably due to the poor *FAD2* activity. Thus, *OeSAD2* and *OeFAD2-2* are the most important contributors, together with OeFAD2-5 [12], for the determination of the peculiar and precious content of fatty acids in olive fruit mesocarp, which has the greatest quantity of oleic acid among all plant species. The gene structure of these candidate genes opens new scenarios directly applicable in the future breeding programs and for oil crop transformation. Furthermore, the chromosome placement of all studied genes could facilitate further studies based on QTL mapping.

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Author Contributions

CC, RM, MTo, DM, LG, MT and LB conceived the study. CC, RM, SM, CG, LR, NC and PP provided the plant material and performed the mRNA synthesis. CC, RM, SM, NC, CG, LR and MTo performed the molecular and bioinformatics analyses. CC, RM, SM, LB, CG, PP, DM, LG, MT and MTo wrote the first draft of the manuscript. CC, RM, SM, LB, CG, PP, DM, LG, MT and MTo contributed to the writing and revised the manuscript. All the authors agreed on the final version of this work.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics declarations

The present research did not involve Human Participants and/or Animals

Figure Legends

Figure 1. Gene structure of the six genes identified in the present study in the Leccino genome. Lengths of UTRs, exons and introns are reported.

Figure 2. Relative expression levels of all the six studied genes (*OeFAD2-1*, *OeFAD2*-2, *OeFAD6*, *OeSAD1*, *OeSAD2* and *OeSAD4*) in different tissues (fruit mesocarp 165 DAF, leaf, root, bud, cambium and flower) of cv. Leccino. Data are the average of two biological replicates and three technical replicates. Multiple comparison test was performed by Duncan test. Different letters correspond to significant level ($p<0.05$) of expression among olive tissues.

~~**Figure 3.** Relative expression levels of different genes belonging to the *OeFAD* and *OeSAD* families in fruit mesocarp at 165 DAF, flower, leaf, root, bud and cambium of cv. Leccino. Data are the average of two biological replicates and three technical replicates. Multiple comparison test was performed by Duncan test. Different letters correspond to significant level ($p<0.05$) of expression in the same gene family.~~

Figure ~~4~~3. Relative expression levels of *OeFAD2-1*, *OeFAD2-2, OeFAD6, OeSAD1* and *OeSAD2*, from drupes of cvs. Leccino and Coratina at distinct stages of fruit development and ripening referred as days after flowering (DAF). Data are the average of two biological replicates and three technical replicates. Multiple comparison test was performed by Duncan test. Different letters correspond to significant level ($p<0.05$) of expression during fruit development.

Electronic Supplementary Material

Figure S1. a) Leccino cultivar tissues images from which RNA extraction was performed: roots, fruits (mesocarp at 165 DAF), flowers, young leaves, buds and cambium. b) Different stages of fruit development and ripening in cv. Leccino, referred as days after flowering (DAF).

Figure S2. Simplified scheme of the olive oil biosynthesis pathway showing the fatty acid formation in the plastid, and the triacylglycerol assembly through the Kennedy pathway in the ER. The key genes coloured in green were studied in the present work.

Figure S3. Distance tree of FAD proteins encoded by the three studied genes after NCBI database blasting. The analysis involved 139 FAD amino acid sequences. All ambiguous positions were removed for each sequence pair. The accession number and the full name for each entry were left as reported in the NCBI database. The FAD protein sequences related to *Olea europaea* were highlighted with different shades of green, full for the sequences found in this work and empty for those previously published. Evolutionary analyses were conducted in MEGA7. The history was inferred using the Neighbor-Joining method. The optimal trees, after a bootstrap analysis running 10,000 times, are shown and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

Figure S4. Distance tree of SAD proteins encoded by the three studied genes after NCBI database blasting. The analysis involved 107 SAD amino acid sequences. All ambiguous positions were removed for each sequence pair. The accession number and the full name for each entry were left as reported in the NCBI database. The protein sequences related to *Olea europaea* were highlighted with different shades of green, full for the sequences found in this work and empty for those previously published. Evolutionary analyses were conducted in MEGA7. The history was inferred using the Neighbor-Joining method. The optimal trees, after a bootstrap analysis running 10,000 times, are shown and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

Figure 3S5. Relative expression levels of different genes belonging to the *OeFAD* and *OeSAD* families in fruit mesocarp at 165 DAF, flower, leaf, root, bud and cambium of cv. Leccino. Data are the average of two biological replicates and three technical replicates. Multiple comparison test was performed by Duncan test. Different letters correspond to significant level ($p<0.05$) of expression in the same gene family.

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Table 1. Specific primers used for quantitative expression of six candidate transcripts~~Specific primers used to amplify genes from~~ of *Olea europaea* *SAD* and *FAD* ~~families and for quantitative expression of six candidate transcripts~~.

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Gene	Primers to amplify genomic sequence	Nucleotide sequence of primers (5' - 3')
OeFAD2-1	qRT-*OeFAD2-1* For qRT-*OeFAD2-1* Rev	TCTGATGTTTGTGTTATCGCCAC AATCTCGATCCACAGTCGCC
OeFAD2-2	qRT-*OeFAD2-2* For qRT-*OeFAD2-2* Rev	TTGGTTAAGGGGAGCGTTGG TGTCGCCTCCATTGCATGAT
OeFAD6	qRT-*OeFAD6* For qRT- *OeFAD6* Rev	AAGTTGGAAAGTGGACCCCA GGTTCTCCAATTTGCCTAAAACC
OeSAD1	qRT-*OeSAD1* For qRT-*OeSAD1* Rev	GATGGAGTCCGAGATGAGACG ACCATGCCTATTCTCTTCAGCT
OeSAD2	qRT-*OeSAD2* For qRT-*OeSAD2* Rev	ACACCCTAGATGGAGTTCGAGA ATATTTGTTCAGAAGGTCACCATG
OeSAD4	qRT-*OeSAD4* For qRT-*OeSAD4* Rev	GGAGAACTGGGCTGAGCAAA TGCCCCTGTTTCATCTTGCA

Table 2. Gene name, total sequence length in cv. Leccino, chromosome location on the genome of a wild olive [12], accession numbers (AN) as issued by the NCBI database.

Gene	Total length (bp)	Mapped on chromosome	Accession Number
OeFAD2-1	3,465	4	MN821526
OeFAD2-2	3,687	17	MN821527
OeFAD6	6,930	11	MN821528
OeSAD1	4,063	10	MN821531
OeSAD2	4,148	23	MN821529
OeSAD4	4,668	8	MN821530